พฤติกรรมการตอบสนองของชันโรง *Tetragonula pagdeni* และมอดยาสูบ *Lasioderma serricorne* ต่อสารให้กลิ่น

นางสาววชิราภรณ์ ฟูนัน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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RESPONSE BEHAVIOR OF STINGLESS BEE *Tetragonula pagdeni* AND TOBACCO BEETLE *Lasioderma serricorne* TO ODORANTS

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วชิราภรณ์ ฟูนัน : พฤติกรรมการตอบสนองของชันโรง *Tetragonula pagdeni* และมอดยาสูบ *Lasioderma serricorne* ต่อสารให้กลิ่น (RESPONSE BEHAVIOR OF STINGLESS BEE *Tetragonula pagdeni* AND TOBACCO BEETLE *Lasioderma serricorne* TO ODORANTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.วรินทร ชวศิริ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.ดร.สุรีรัตน์ เดี่ยววาณิชย์, 106 หน้า.

ปัจจุบัน สารล่อแมลงแสดงบทบาทสำคัญทางการเกษตรเนื่องจากช่วยเพิ่มผลผลิตของพืชและช่วยลดความเสียหายของผลิตภัณฑ์ในโรงเก็บ ฟีโรโมนและสารระเหยจากพืชเป็นสารให้กลิ่นสำหรับล่อแมลงที่มีประสิทธิภาพที่ใช้ได้ทั้งในการจัดการก่อนการเก็บเกี่ยวและหลังการเก็บเกี่ยว การใช้ฟีโรโมนนำทางของชันโรงในแปลงพืชก่อนการเก็บเกี่ยวอาจมีประสิทธิภาพต่อการผสมเกสรของชันโรงในแปลง ในขณะที่สารระเหยจากพืชอาจช่วยดึงดูดแมลงศัตรูในโรงเก็บมาที่กับดักเมื่อใช้ร่วมกับฟีโรโมนเพศของแมลง การศึกษานี้มีจุดมุ่งหมายเพื่อวิเคราะห์สารประกอบกลิ่นนำทางจากต่อมลาเบียลของชันโรง *Tetragonula pagdeni* ที่พบทั่วไปในประเทศไทยเพื่อใช้ในแปลงก่อนการเก็บเกี่ยว และเพื่อหาสารระเหยจากพืชที่มีบทบาทเป็นสารล่อไคโรโมนต่อ *Lasioderma serricorne* แมลงศัตรูที่สำคัญ เพื่อใช้สำหรับผลิตภัณฑ์หลังการเก็บเกี่ยว สำหรับการศึกษาทางกระบวนการก่อนการเก็บเกี่ยว มีสารประกอบไฮโดรคาร์บอน 6 ชนิดอยู่ในสารสกัดจากต่อมลาเบียลของ *T. pagdeni* ไม่สามารถจำแนกสารที่วิเคราะห์ได้ 3 ชนิด ปรากฏอยู่ในสารสกัดกระดาษกรองที่มีกลิ่นเปื้อนอยู่ซึ่งชันโรงได้แต้มไว้ หนึ่งในนั้นแสดงความเป็นสารล่อนำทางต่อชันโรงชนิดนี้ กลิ่นนำทางที่เป็นไปได้นี้คือสารประกอบในพีคที่ 2 ซึ่งอาจเป็นสารประกอบไฮโดรคาร์บอนไม่อิ่มตัวสายสั้น การพิสูจน์สูตรโครงสร้างของสารนี้เพิ่มเติมเป็นสิ่งจำเป็นเพื่อยืนยันฤทธิ์ทางพฤติกรรมของสารนี้ สำหรับการศึกษาทางกระบวนการหลังการเก็บเกี่ยว ชาใบหม่อนให้ฤทธิ์การดึงดูดต่อ *L. serricorne* ที่สูงที่สุดใน 30 พืชทดสอบ ได้วิเคราะห์สารประกอบระเหยได้ 13 ชนิดในใบชาด้วยวิธี headspace-SPME-GC-MS พบว่า Phytol β-ionone และ methyl palmitate เป็นองค์ประกอบสำคัญ β-ionone แสดงฤทธิ์การดึงดูดที่สูงที่สุดด้วยค่าดัชนีการตอบสนอง 60% ที่ขนาด 0.001 มก. ในขณะที่ phytol ซึ่งเป็นองค์ประกอบหลัก ให้ฤทธิ์การดึงดูดที่ต่ำ อย่างไรก็ตาม β-ionone ไม่มีฤทธิ์ที่แตกต่างจากเหยื่อล่อไคโรโมนอย่างมีนัยสำคัญ การใช้ร่วมกันของ phytol และ β-ionone ไม่แสดงความเสริมฤทธิ์กัน งานวิจัยนี้เป็นรายงานแรกในประเทศไทยเกี่ยวกับการใช้สารให้กลิ่นจากชาใบหม่อนต่อ *L. serricorne* และสารประกอบให้กลิ่นจากต่อมลาเบียลในฐานะฟีโรโมนนำทางสำหรับดึงดูด *T. pagdeni* ไปยังแหล่งอาหาร สิ่งเหล่านี้อาจเป็นเครื่องมือทางเลือกที่มีความหวังสำหรับการจัดการก่อนและหลังการเก็บเกี่ยวที่เหมาะสมอย่างน้อยที่สุดในประเทศไทย

ลายมือชื่อนิสิต.....

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2555.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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WACHIRAPORN PHOONAN : RESPONSE BEHAVIOR OF STINGLESS BEE *Tetragonula pagdeni* AND TOBACCO BEETLE *Lasioderma serricorne* TO ODORANTS. ADVISOR: ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., CO-ADVISOR: ASST. PROF. SUREERAT DEOWANISH, Dr.Agr., 106 pp.

Insect attractants presently play an important role in agriculture for increasing crop yields and decreasing loss of stored products. Pheromones and plant-derived volatiles are the effective odorants for attracting insect in both pre- and post-harvest managements. The use of trail pheromone of stingless bee in pre-harvest crop may affect on their pollination in crop field, while plant-derived volatiles may help to attract storage insect pest into trap when combined with their sex pheromone. This study aims to analyze the trail-scent compounds from labial glands of *Tetragonula pagdeni*, a common stingless bee of Thailand, for pre-harvest use, and to find plant-derived volatiles that act as kairomone attractant to *Lasioderma serricorne*, an important insect pest, in use for post-harvest products. For pre-harvest study, six hydrocarbons were detected in labial gland extract of *T. pagdeni*. Among these, three unidentified compounds appeared in scent-marked filter paper extract which the stingless bee rub on it, and one of them displayed the trail-following attractant to this species. This possible trail scent was the compound in peak no.**2** that might be the unsaturated hydrocarbon with short chain compound. Further characterization of this compound could be necessary to confirm its behavioral activity. For post-harvest study, mulberry leaf tea had the highest attractive activity to *L. serricorne* among 30 plants tested. Thirteen volatile compounds containing in this tea were analyzed by headspace-SPME-GC-MS method. Phytol, β-ionone and methyl palmitate were the main volatile constituents. β-Ionone exhibited the highest attractive activity to *L. serricorne*, with 60% response index at 0.001 mg dose, while phytol, the major component, showed low attractive activity. However β-ionone did not show significantly different to commercial kairomone lure. The combination of phytol and β-ionone did not show the synergistic activity. This research is the first report in Thailand about the use of odorants from mulberry leaf tea to attract *L. serricorne,* and odorous compound from labial gland as trail pheromone for attracting *T. pagdeni* to food source. These might be the promising alternative tools for pre- and post-harvest managements that are suitable at least in Thailand.

Student's Signature……...

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CONTENTS

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Page

Page

Page

LIST OF ABBREVIATIONS

amu	atomic mass unit
EI	electron impact
eV	electron volt (s)
°C	degree Celsius
cm	centimeter (s)
g	gram (s)
GC	gas chromatography
GC-FID	gas chromatography-flame ionization detector
GC-MS	gas chromatography – mass spectroscopy
h	hour (s)
HS-SPME	headspace solid-phase microextraction
I.D.	internal diameter
km	kilometer (s)
L	liter (s)
L:D	light:dark
m	meter (s)
mg	milligram (s)
min	minute (s)
mL	milliliter (s)
mm	millimeter (s)
mmole	millimole (s)
n	number
nov.	novel (nova)
R.H.	relative humidity
SE	standard error
w/w	weight by weight
µg	microgram (s)
µL	microlitre (s)
µm	micrometer (s)
var.	variety

CHAPTER I

INTRODUCTIONS

Thailand is an agricultural country. Approximately 21 million ha or 40.9% of the total area is used for agricultural production. About 49.8% of the agricultural land is used for growing rice, 21.5% for field crops, 21.2% for fruit or horticultural crops and 7.5% for others (OAE, 2008). Thailand is almost self-sufficient in food production. Agricultural production does not only support for domestic consumption but also play significant role in export earnings.

Agricultural goods are among the country's primary exports. Over the past decade, Thailand's agricultural sector has shifted from labor- to machine-intensive farming practices. Pressures to sustain high crop yields have led to heavy usage of pesticides. Annually, Thailand imports several thousand metric tons of herbicides, fungicides, and insecticides. Residues, especially organochlorine and organophosphate compounds, have been contaminated in soil, water, and agricultural products throughout the country (Thapinta and Hudak, 2003). Nowadays, agriculture in Thailand has tended to be organic farm. Thus, biological strategies have become a significantly promising alternative for Thai farmers *via* IPM (integrated pest management) which is a strategy to increase crop yield, by combining several elements into an integrated system.

At present, one of biological strategies, insect attractant, plays an important role in protection of stored products from insect infestation and using for increasing crop production. Attractants are a kind of odorants including pheromones, natural food lures, oviposition lures, and poison baits. Odorants are the low molecular weight volatile compounds that humans and animals perceive as odorous *via* the olfactory system (Touhara and Vosshall, 2009; Suwannapong and Benblow, 2011). Pheromones are substances released by individual in a species that elicit behavioral or physical changes to another individual in same species. Pheromone can provide a means of monitoring and controlling insects which is non-toxic to animals and plants, and specific for the target pest. They are fully compatible with other methods of pest control and thus ideal for components of IPM programs (Cork and Hall, 1998).

Moreover the natural plant volatiles can play a major role in response behavior of both male and female insects as direct attractants and oviposition stimulants for females and also acting as synergists to pheromones (Landolt and Phillips, 1997).

Many previous studies reported about the use of odorants both in crop field and post-harvest product store. The examples such as the use of bee pheromones in pollination and colony management to attract bees to a crop or to stimulate foraging (Pettis *et al.*, 1999), or use sex pheromone combining with food attractant of some stored-product insect for population survey or monitoring in mass trapping (Tumlinson *et al.*, 1969; Trematerra, 1997).

In this study, application of odorants in both pre-harvest crops and post-harvest products had been explored. Pre-harvest study focused on the use of pheromone from stingless bees to attract them for crop pollination, and in post-harvest study, improvement of mass trapping to potent attraction for some stored-product insects was investigated.

1.1 Pheromone Attractants for Crop Pollination of Stingless Bees

Stingless bees (Apidae: Meliponini) play an important ecological role as pollinators of many wild plant species and seem to be good candidates for future alternatives in commercial pollination, but evidence for their importance and effectiveness as crop pollinators is lacking for most plant species (Slaa *et al.*, 2006).

Unlike honey bees, stingless bees have many advantages: they are generally less harmful to humans and domestic animals; they are able to forage effectively in greenhouses; propagation of their colonies contributes to preserve biodiversity by conserving species populations; the colonies are rarely able to abscond, as the old queen is flightless (Inoue *et al.*, 1984) and they are resistant to diseases and parasites of honey bees (Delfinado-Baker *et al.*, 1989). Nine species of stingless bees were confirmed to be the effective and important pollinators of annatto, avocados, camu-camu, carambola, chayote, citrus, coconut, cupuacu, mango, mapati, strawberries, lynches macadamia, and watermelons (Heard, 1999).

Pollination has been a fast growing field since the 1960s (Kevan, 1983). Pollinators such as bees, birds and bats affect 35% of the world's crop production. In the continents of Latin America, Africa and Asia, an average of 40% of the land area

of crops is planted to crops with some dependence on animal pollinators. Well-pollinated crops can be of noticeably better quality, and markets are sensitive to quality considerations: in Canada, good pollination in apple orchards resulted in about one extra seed per apple, which produced larger and better formed apples. These improved apples were estimated to provide marginal returns of about 5–6%, or about Can. US$250/ha, compared to orchards with insufficient pollination (FAO, 2008).

Pheromones have just begun to play a role in bee management and pollination. As the understanding and identification of bee pheromones increases, the further utilization of pheromones in the management of bees will be possible (Pattis *et al.*, 1999). Roubik (1989) provides an excellent overview to the wide array of chemicals produced by various bee species that may play a role in foraging behavior. Numerous species produce chemical signals for aggregation and mating and these signals could be used to manipulate these species for pollination. Some progress in using this pheromone sprays on crops has been made but short-lived (Winston and Slessor, 1993).

Pheromone from labial gland or trail pheromone, a pheromone of stingless bees, is used for recruitment, for marking pathways to resources, and for indicating resource richness. Foraging worker of *Tetragonula* will release more trail pheromone near a food source, thus indicating proximity to the food source for other foragers (Zablotny, 2003). There are some previous reports of using pheromone from labial gland of stingless bees for crop pollination (Jarau *et al.*, 2004, 2005, 2006).

In Thailand, 32 species of *Trigona* have been recorded (Schwarz, 1939; Sakagami *et al.*, 1985; Michener and Boongird, 2004; Klakasikorn *et al.*, 2005). Some species of these stingless bees have been cultured for crop pollination, especially in orchards. *Tetragonula pagdeni* (Schwarz, 1939) is a species which is predominant and commonly distributes in Thailand (Sakagami, 1978; Sakagami and Khoo, 1987). This species is an interesting stingless bee from Thai agriculturists for using as pollinator in their orchards aims to increase crop yield.

In this research, the active compounds in pheromone from labial gland of a stingless bee, *Tetragonula pagdeni* (Schwarz, 1939) will be analyzed aiming to promote the crop pollination of stingless bee in case of the limited resources and several species competing for them. The ability of bees in attraction to a compatible

target crop might be essential for ensure cross-pollination (Malerbo-Souza *et al.*, 2004). However, the analysis of constituents in pheromone from labial gland of this species has not been reported.

1.2 Food Attractant Lures for Controlling Tobacco Beetle

Stored product insects have been a serious problem for all stored products. Their infestation results in loss of quality and quantity of products and economic loss will follow. Tobacco beetle, *Lasioderma serricorne* F. (Coleoptera: Anobiidae) is an important pest which can infested a wide range of stored products including: ground grain or seed, whole seeds, grains, rice, pulses, beans, pasta, spices, dried fruit, dried vegetables, yeast, dried insects, dried fish, fishmeal, meat meal, leather, wax, and dried and processed plant material such as tobacco (Fraenkel and Blewitt, 1943; Howe, 1957; Ambadkar and Khan, 1989). In Thailand, besides tobacco products, dried herbs and spices, and tea products (especially mulberry leaf tea) have been reported about seriously infesting by *L. serricorne* (Phimphasali and Kaewruang, 2007). This insect is often encountered as pests in manufacturing, retail and domestic situations. Larvae are the stage that causes the highest damage of foodstuffs. Mature larvae and adults can readily chew through packaging materials to infest the stored products (Rees, 2004).

Manipulation of this insect usually use an early practical monitoring method cooperated with insecticidal strategy. Pheromones are the most common uses as attractant lures in traps to detect the presence of pests and to monitor the activities of pest populations (Phillips, 1997). Anhydroserricornin (2,6-diethyl-3,5-dimethyl-3,4-dihydro-2H-pyran), and serricornin (4,6-dimethyl-7-hydroxynonan-3-one) are the sex pheromones of *L. serricorne* which are produced by adult females, and show a strong attractive response to adult males (Burkholder, 1970; Coffelt and Burkholder, 1972; Levinson *et al.*, 1981; Levinson and Levinson, 1986). Commercial compounds of these pheromones have been synthesized and used as lure in different types of traps for monitoring the presence of *L. serricorne* (Chuman *et al.*, 1980; Chuman *et al.*, 1985; Faustini, 1985; Papadopoulou and Buchelos, 2002; Campbell *et al.*, 2002; Arbogast *et al.*, 2003).

Moreover, the food odors are the effective attractant lures for monitoring stored product insect. Due to most of the stored product insects are phytophagous insects. They generally use volatile semiochemicals from host plants to search for their food and oviposition sites. Plant odors may act as direct attractants for both sex of insects or they may synergistically enhance the effectiveness of insect pheromone traps (Phillips *et al.*, 1993; Cox, 2004). Food volatile attractants for several beetle species that infest broken grain have been identified from cereal grains and their products (Nara *et al.*, 1981; Mikolajczak *et al.*, 1984; Pierce *et al.*, 1990).

In Thailand, the commercial attractant traps have been imported in large quantities particularly traps for tobacco beetle, *Lasioderma serricorne*. Kairomone lures or food attractant traps have had the problem in less effective in practical uses in different countries (Mahroof and Phillips, 2007). Searching for the potential kairomone lures for this insect have been continued. The study on the effective traps for this insect in Thailand is less. Improvement the effective kairomone lures for management of the insect causing many problems in stores, may be more specific for insect species in Thailand and may reduce cost.

1.3 Objectives

1. To analyze the main constituents of pheromone from the labial gland of stingless bee, *Tetragonula pagdeni*.

2. To analyze the active components in food-host materials causing the attractive behavior in tobacco beetle, *Lasioderma serricorne*.

CHAPTER II

RESPONSE BEHAVIOR OF STINGLESS BEE *Tetragonula pagdeni* TO ODORANTS

2.1 Literature Reviews

2.1.1 Taxonomy and Distribution of Stingless Bees

Stingless bees are a group of small- to medium- sized bees which belong to the subfamily Meliponinae (highly eusocial stingless bees) of family Apidae and closely related with honey bees, carpenter bees, orchid bees and bumblebees (Rubix, 1989). Stingless bees are the oldest groups among them. The oldest fossil was found in New Jersey, USA, in Cretaceous amber. It was a worker of a stingless bee, *Trigona prisca* (Velthuis *et al*., 1997). Three main characters of stingless bees are different from other bees: reduction and weakness of the wing venation, the presence of penicillum, and reduction of the sting. According to the last character, they will defend themselves by biting (Wille, 1983).

This highly eusocial bees had their origin in Africa and have dispersed to other tropical and many subtropical regions of the world (Figure 2.1), such as Australia, Southeast Asia, and tropical America (Michener, 1974, 1990, 2000; Sakagami, 1982). Stingless bees are a large and diverse taxonomic group comprising some 60 genera and more than 500 species recorded, but many of which are poorly known (Klakasikorn *et al.,* 2005; Rasmussen and Cameron, 2010). Of these, they are mostly found in America, following by Southeast Asia, Africa, and Australia, respectively (Velthuis *et al*., 1997). Among them, *Melipona* and *Trigona* are the most important genera. They can produce honey, so sometime called “stingless honey bees”. About 50 species of *Melipona* group is restricted to the neotropics, and has more complex communication systems (Nieh and Roubik, 1995).

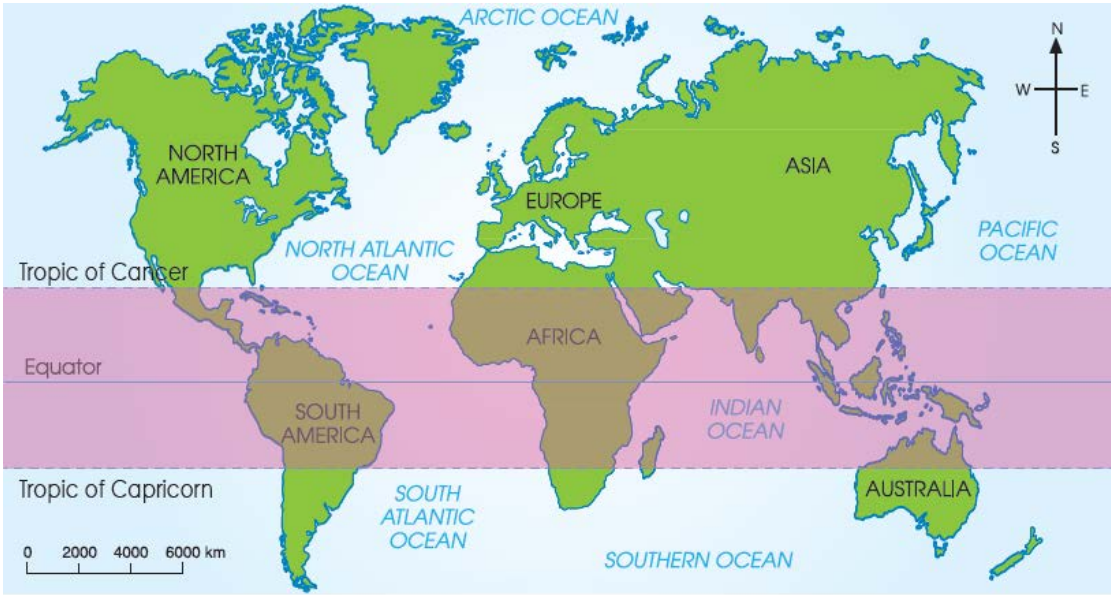


Figure 2.1 Stingless bee distribution in the tropical and subtropical zones (in pink area) (Kwapong *et al.*, 2010).

Trigona is the largest and most widely distributed genus (Heard, 1999). They are found extensively in tropical regions. It extends from Mexico to Argentina, India, Sri Lanka to Taiwan, the Solomon Islands, South Indonesia, and New Guinea, but no member of the genus occurs in Africa. *Trigona* have approximately 150 species presently with 11 subgenera. Many of these former subgenera including *Tetragonula* have been elevated as genus by Moure (1961) *cf.* Sakagami (1975) (Sakagami, 1978; Michener, 2000). In Southeast Asia found 39 species of stingless bees in genera of *Trigona* and *Hypotrigona* (Dejtisakdi, 2005). In Thailand, there are 34 species in 2 genera (*Trigona* and *Hypotrigona*) (Pobsuk, 2006). They are 32 species in *Trigona,* such as *T. thoracica*, *T. fimbriata*, *T. aliceae*, *T. ferrea*, *T. nitidiventalis*, *T. terminata*, *T. ventalis*, *T. canifrons*, *T. apicalis*, *T. melanoleuca*, *T. peninsularis*, *T. binghami*, *T. atripes*, *T. collina*, *T. fuscibasis*, *T. iridipennis*, *T. fuscobalteata*, *T. pagdeni*, *T. geissleri*, *T. melina*, *T. pagdeniformis*, *T. sarawakensis*, *T. hirashimai*, *T. latigenalis*, *T. laeviceps*, *T. sirindhornae* and *T. minor* (Michener and Boongird, 2004; Klakasikorn *et al.,* 2005), and another 2 species in *Hypotrigona* including *H. (Pariotrigona) pendleburyi* and *H. (Lisotrigona) scintillans* (Saibun, 1996).

The former subgenus ***Tetragonula*** (now as a genus), is the largest group of Indopacific stingless bees, involving some most common and widespread forms (Sakagami and Inoue, 1985). Several species in this subgenus including *Trigona (Tetragonula) pagdeni*, *T. melina*, *T. minor*, and *T. fuscobalteata*, is taxonomically problematic for the absence of reliable diagnostic characters different from other groups, *Geniotrigona*, *Heterotrigona*, *Homotrigona*, *Lepidotrigona*, *Lophotrigona*, *Tetrigona*, and *Tetragonilla*, that clearly classified based on their morphological and nest architecture characters (Sakagami, 1978; Sakagami and Inoue, 1985; Dollin *et al.,* 1997).

Due to the virtual absence of reliable structural characters in *Tetragonula* workers, thus the classification must base on size, proportion, coloration and pilosity, which makes difficulty and sometime impossible in partial specimens. Males are more easily distinguished by structural characters, but they are poorly in specimens (Sakagami, 1978). Sakagami (1978) stated that in 1961, Moure defines and descripts the precisely important characters of *Tetragonula* in summarized as followings:

1) Small to very small, body and wing (including tegula) length ~2.5–4.5 mm.
2) Integument polished with fine punctures, not tessellate.
3) Clypeus without stout erect hairs.
4) Bidentate mandible.
5) Malar space much shorter than flagellar width.
6) Flagellomeres very short, shorter than width.
7) Mesoscutellum well projected backward, distinctly exceeding propodeum.
8) Marginal cell nearly closed apically.
9) Bifurcation of media (*M*) (basal vein) and cubitus (*Cu*) nearly at cubitus-anal (*cu-an*).
10) Posterior fringe of hind tibia mostly plumose.
11) Hind tibia below with a moderately broad, raised, and pubescent area.
12) Hind basitarsus narrower than tibia, below basally with a large sericeous area.
13) Propodeum medially smooth and glabrous.
14) Metasoma flat, anterior terga polished.

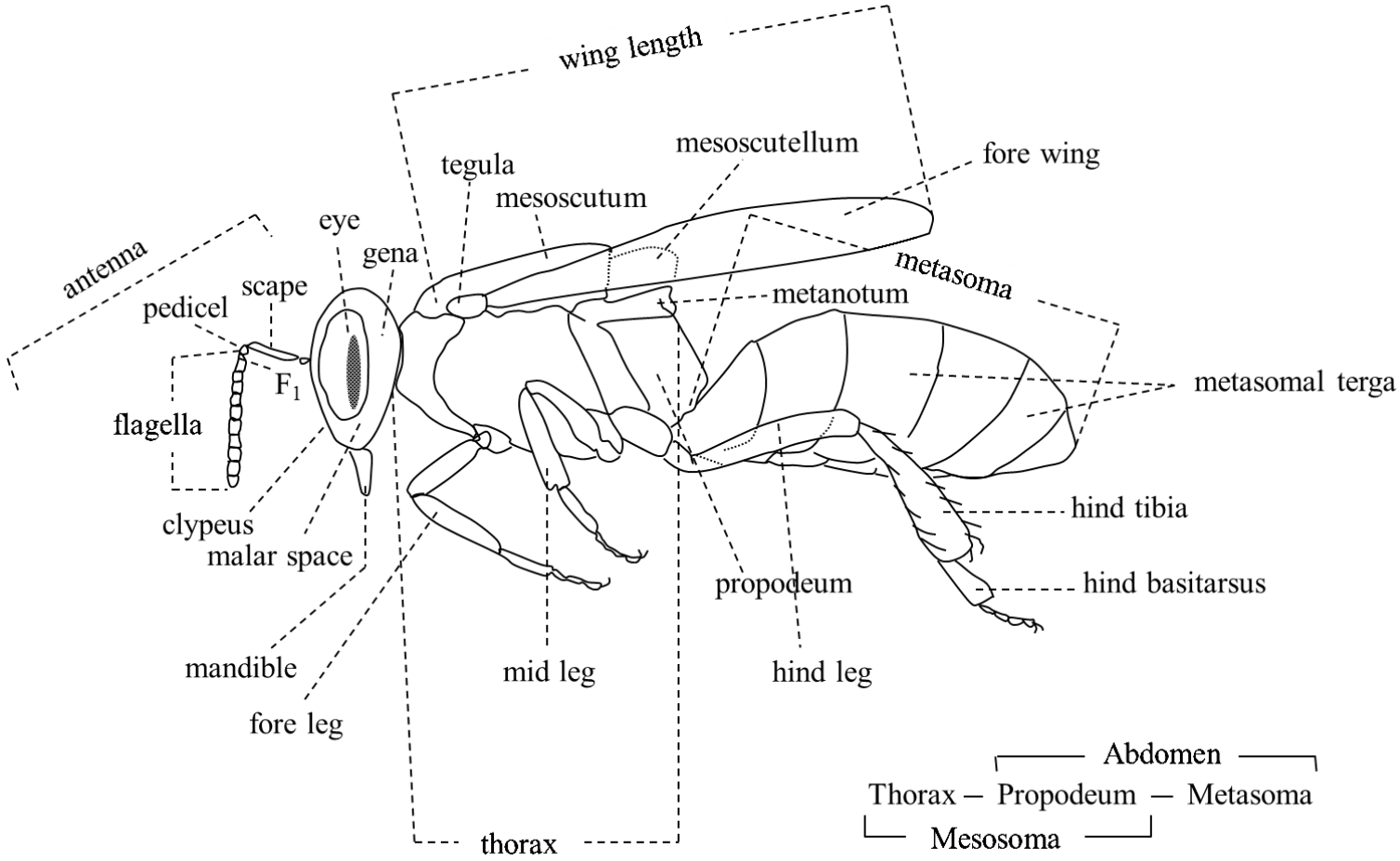


Figure 2.2 Morphological structure of stingless bee (Schwarz, 1939).

2.1.2 General Biology of Stingless Bees

The nest structure: Stingless bees build their nests usually in hollow trunks, tree branches, underground cavities, rock crevices, or even wall cavities. The nests of stingless bees are more elaborate and complex than those of *Apis mellifera* (Sommeijer, 1999). The most common type of nest is found in a tree cavity. The nest is usually made of 5 parts: brood comb, involucrum, store pots, batumen, and an entrance. The comb consists of brood cells, in each of which a single young is reared, and surrounded by a sheath of cerumen, or involucrum. The main building material is cerumen, a mixture of beeswax and plant resin (sometimes called propolis) (Sommeijer, 1999). Other materials used are mud, vertebrate feces, plant fibers, and chewed leaf material. There are many types of nest entrances, with many variations among species (Figure 2.3). The individual species are recognizable from nest entrances and often their particular site (Roubik, 2006).

Some terms of nest structure (Michener, 1961; Wille and Michener, 1973; Sakagami, 1983) were described as followings: **cerumen**: wax and plant resin mixed for pliable construction material; **involucrum**: a single or series of sheaths, made of cerumen, surrounding brood; **batumen**: thick involucrum forming a wall, or resin layer surfacing nest cavity (mud, seeds, wood, and vertebrate feces may be added).

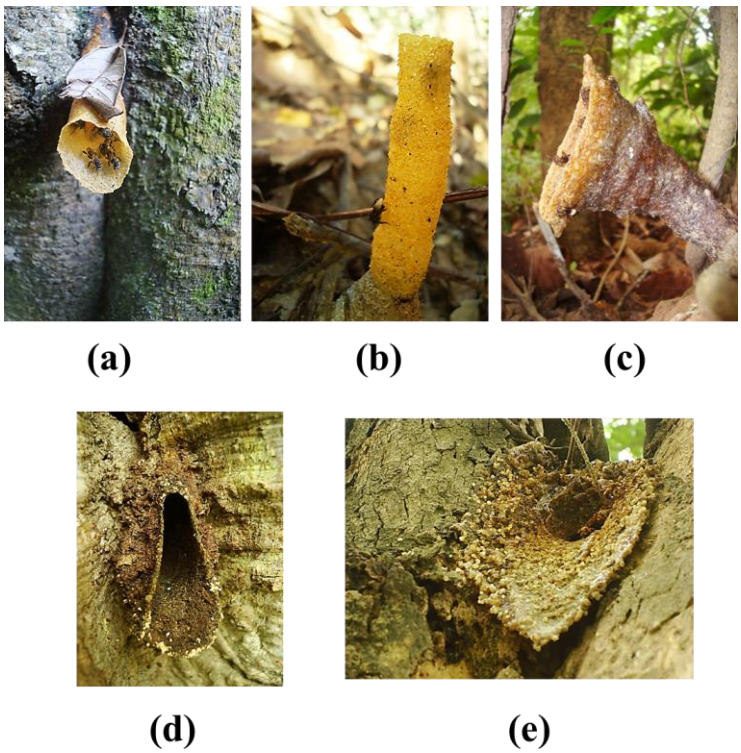


Figure 2.3 Variation of nest entrances of some stingless bee species in Thailand: (a) *Lepidotrigona terminata*; (b) *Tetragonilla collina*; (c) *Tetrigona apicalis*; (d) *Tetragonula pagdeni*; and (e) *Homotrigona fimbriata*.

Inside the nest (Figure 2.4), honey and pollen are stored in separate pots with egg-liked or conical or cylindrical shapes (Roubik, 2006). Brood cells and store pots are mostly arranged in horizontal layers forming combs but in some species they are arranged in clusters. Cluster form is considered the more primitive condition (Velthuis *et al*., 1997). Colony size drastically varies depending on species (Wille and Michener, 1973; Drumond *et al*., 2000; Michener, 2000). Many *Melipona* species have less than 100 till 500 individuals containing in colonies, while colonies of the *Trigona* species may contain more than 100,000 bees. Division of workers of stingless bees can follow the same basic pattern as in honey bees. Very young workers start to work with wax and cerumen. Some of them shift to building and provisioning brood cells and following by cleaning, reception and dehydration of nectar. The old workers around 40 days become to actual foragers (Velthuis *et al*., 1997).

Figure 2.4 Nest structure of *T. pagdeni* (cluster type): (a) honey pots; (b) pollen pots; (c) the younger brood cells; and (d) the older brood cells.

Reproductive system: Stingless bees are perennial colony, there can be two or more queens laying eggs in the same nest. New queens are produced regularly, but most of them are killed and never allowed to produce eggs. Some queens may remain imprisoned in special cells as reserves. The queen lays eggs in a special way. First, a completed cell is half filled with honey and pollen by the workers. Then one or more workers lay an egg in the cell and the queen is encouraged to come near. Then the queen eats the worker egg from the cell and lays her own egg instead, and then proceeds to another cell. One or more workers close the cell by bending the upper collar of the cell against the center. The cell is closed until the adult bee emerges. This is called the mass provisioning system and differs from the situation in honey bees where the honey bee larvae are fed continuously as they develop. Stingless bee queens can provide 10–100 cells with eggs a day, depending on the species. When bee has fully developed, workers tear down the cells for new bee emergence, and the material is reused for building new cells. Fertile eggs from the queens develop into worker bees and queens. Drones (males) come from unfertilized eggs from the queen, or from egg laying workers. It sometimes happens that an egg laying worker bee lays an egg into a cell already containing a queen's egg. The male egg develops into a larva more rapidly than the female egg. The male larva then punctures the queen's egg before it

hatches, and is able to eat all the food in the cell. After 10–15 days, the drones leave their parent colony forever. Where they go is not known (Bradbear, 2009).

Multiplication: Stingless bees multiply themselves by swarming. When a colony has reached a certain size and a usable new nest place is found, some worker bees will start transporting building materials to the new place. More and more bees will fly to the new nest over the next few days, and in the end, a queen from the old nest will transfer to the new nest and begin producing eggs there. Mating between a new queen and drones takes place outside the nest (Bradbear, 2009).

Size and forms: The largest stingless bee is *Melipona flavipennis*, with workers slightly larger than honey bee, *Apis mellifera*. Most stingless bees, however, are much smaller, the smallest being only a few mm in length. Also the form of the bees varies, *Melipona* bees have their robust shape in common with *Apis*, while many species belonging to *Trigonini* are slender, agile bees (Velthuis *et al*., 1997).

The food niches of stingless bees: Stingless bees occur in all kinds of habitats together with many other species of bees. Like all social bees, stingless bees are not specialized visitors of a specific plant species. They need food all year around. Stingless bees have extremely different among the species in their flower preferences, the foraging distances, and the communication systems about food sources. That results in difference of quantity and quality of their honey. Nevertheless each species has its preferences. The tongue length and body size of the bees is very specialized to the flower size. Many species of stingless bees are less than 5 mm in length. They tend to search for food from very small flowers (Velthuis *et al*., 1997).

Importance in crop pollination: Stingless bees (Apidae: Meliponini) play an important ecological role as pollinators of many wild plant species and seem good candidates for future alternatives in commercial pollination, but evidence for their importance and effectiveness as crop pollinators is lacking for most plant species (Slaa *et al.,* 2006). Stingless bees have the following advantages comparable to bees: they are generally less harmful to humans and domesticated animals; they are able to forage effectively in glasshouses; propagation of colonies contributes to preserve biodiversity by conserving populations of species that may otherwise decline owing to human disruption of ecosystems; colonies are rarely able to abscond, as the old queen is flightless (Inoue *et al*., 1984); and they are resistant to the diseases and parasites of

honey bees (Delfinado-Baker *et al*., 1989). They were confirmed to be effective and important pollinators of some plant species, such as annatto, camu-camu, chayote, coconut, cupuacu, carambola, strawberries, watermelons, citrus, avocados, lynches macadamia, mango, and mapati (Heard, 1999).

2.1.3 Cephalic Glandular Organs of Stingless Bees

Much communication in the insect world involves the use of chemicals, which are produced by an individual and that have an impact on the behavior of another individual of the same species. These substances are called pheromones and are produced by specialized glands. The glands are distributed in all body parts (Figure 2.5). The glandular organs in the head linked to the mouth and its appendages, are known as the salivary gland system. **Labial glands** open in the base of the glossa (the tongue). In Apidae, they have a branch in the head and another in the thorax, each with a different morphology and secreted substances. The head branch produces oil-like substances for mouthpart lubrification or trail pheromones in some species (Jarau *et al.,* 2004, 2006; Dambacher 2006; Dambacher *et al.,* 2007; Schorkopf *et al.,* 2007; Stangler *et al*., 2009), while the thoracic part produces digestive enzymes. The mandibular glands open at the base of mandibles. These glands produce mostly pheromonal substances, such as alarm pheromone. The hypopharyngeal gland open into the buccal cavity (the mouth); in the highly social bees they produce food for larvae, digestive enzymes in other bees. The presence and degree of development of these glands varies according to the species, as well as the sex and the caste (Michener, 1974; Velthuis *et al*., 1997; Suwannapong, Chaiwongwattanakul, and Benbow, 2010).

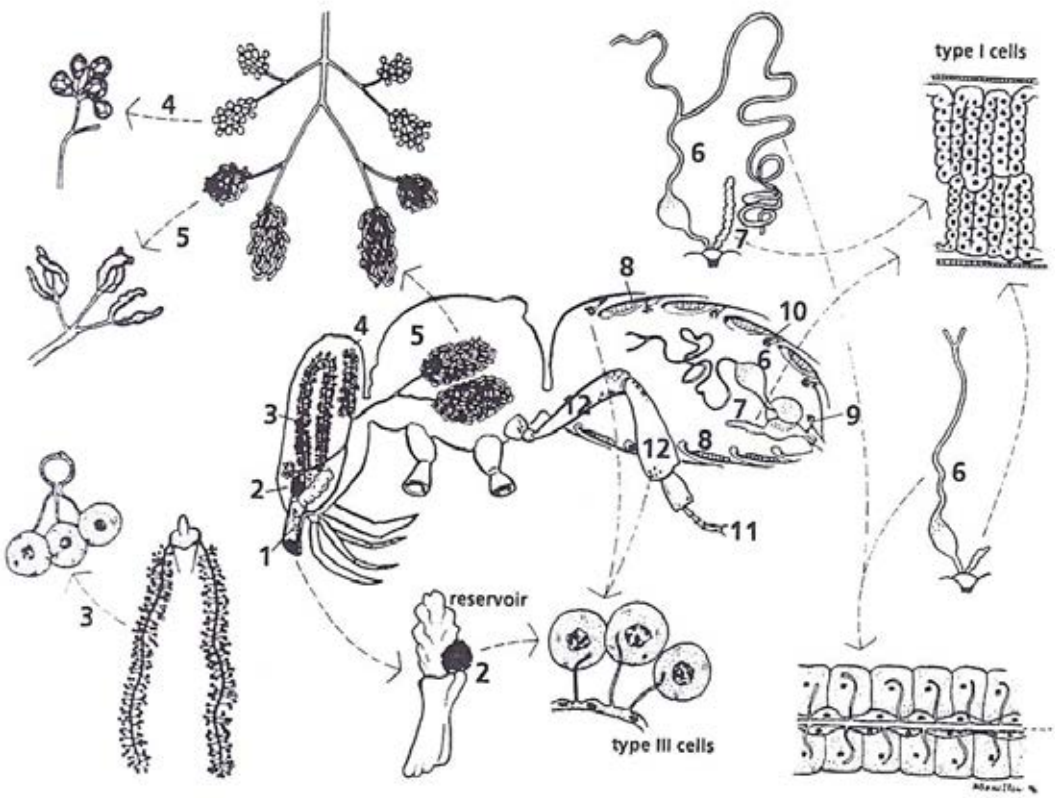


Figure 2.5 The locations and structures of the cephalic glandular organ of an adult female stingless bee (Velthuis *et al*., 1997): 1. intramandibular gland; 2. mandibular gland; 3. hypopharyngeal gland; 4. salivary gland of head; 5. salivary gland of thorax; 6. poison gland; 7. Dufour gland; 8. wax gland (dorsal in stingless bee); 9. Koshevnikow gland; 10. tergal gland; 11. tarsal gland; and 12. various glands in leg segments.

2.1.4 Food Source Communications

Stingless bees recruit their nestmates, mark pathways to resources, and indicate resource richness by the sophisticated communication system. The resources that they recruited are dead animals, pollen, nectar, mud, resin, water, and nests (Nieh, 2004). There are various types of food communication in stingless bees depend on different species (Camargo and Roubik, 1991; Nieh, 2004) which are different from honey bees that using the waggle dance (Dyer, 2002). In some basal species (on the basis of behavioral traits) of stingless bees, such as *Trigona carbonaria,* foragers can recruit nestmates to correct direction, but not correct distance (Nieh *et al*., 2000),

while *Scaptotrigona postica* have a good ability in communicate three-dimensional location (Lindauer and Kerr, 1960). Nevertheless, the *Melipona* genera also have the variation in the ability of different species to communicate three-dimensional food location (Nieh, 2004; Barth *et al.*, 2008).

The possible recruitment behaviors of the successful foragers in their nest may be jostle (bees appearing to purposefully run and bump into nestmates), thorax vibrations, transmitted *via* direct body contact, *via* the substrate or as airborne sound (Lindauer and Kerr, 1960; Hrncir *et al*., 2000; Reichle *et al.*, 2010), zigzag runs (changing direction several times while running) (Kerr, 1994), and spinning (clockwise and counterclockwise body rotations) (Kerr, 1960; Nieh, 1998; Hrncir *et al.*, 2000). In stingless bees, antenna contacts and trophallactic (food exchange) contacts may help to transfer the essential odors and communicate about the relative quality of the food source (Nieh and Roubik, 1998; Hrncir *et al.*, 2000; Hart and Ratnieks, 2002).

Outside the nest, the olfactory communication may range from recruiting foragers complete odor trails that begin in the nearby of the nest and extend to the food source, short odor trails that only extend a short distance away from the food source in the direction of the nest (Nieh *et al.*, 2003, 2004; Jarau, 2009), odor-marking of the food source alone (Nieh, 1998; Hrncir *et al*., 2004) that only reported in many Meliponine species, and aerial odor trail that some species release trail odor into the air aiming recruit their nestmates (Lindauer and Kerr, 1960; Kerr *et al*., 1963; Blum *et al*., 1970; Kerr *et al*., 1981).

However, Nieh (2004) summarized that the stingless bee foragers may use multiple sensory, such as touch, vision, olfaction, and audition, to transfer information about the existence of food resource in location, direction, and in some cases, to communicate its specific 3-dimensional location.

2.1.5 Pheromone from Labial Gland of Stingless Bees

Recent experiments have clearly shown that foragers of *Trigona recursa* (Jarau *et al.*, 2004, 2005, 2006), *T. spinipes* (Schorkopf *et al*., 2007), *T. corvina* (Dambacher, 2006; Dambacher *et al.*, 2007), *Scaptotrigona pectoralis* (Hemmeter,

2008) and *Geotrigona mombuca* (Jarau *et al*., 2009) produce trail pheromones in the labial glands and not in the mandibular glands.

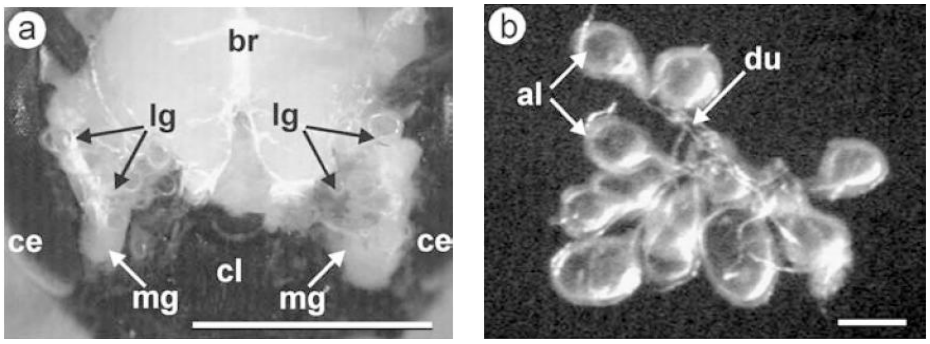


Figure 2.6 The frontally opening head of a forager of *Trigona recursa* (a); a piece of labial gland (b): lg, labial gland; mg, mandibular glands; ce, compound eye; br, brain; cl, clypeus; al, alveoli; du, ducts. Scale bar in (a) = 1 mm, in (b) = 0.1 mm (Jarau *et al*., 2004).

The head of foragers have well-developed in this labial glands (Figure 2.6) containing trail following substances (Jarau *et al.*, 2004). The first trail pheromone identified from stingless bee was hexyl decanoate, a low molecular weight wax-type ester, being dominant in the labial gland extract of *Trigona recursa* and proved to be behaviorally active (Jarau *et al.,* 2006). In 2007, Schorkopf and co-workers concluded that octyl octanoate pheromone in *Trigona spinipes*, a widely distributed species of South America, was a single compound pheromone which induces full trail following behavior. In 2009, Stangler *et al*. postulated that the labial glands of *Geotrigona mombuca* contained a series of terpene- and wax-type esters, with farnesyl butanoate as a major constituent. Recently, Jarau and teams (2010) defined trail pheromone of *Trigona corvina* as a blend of wax-type and terpene that have the relative proportions of the single components different in the pheromones of foragers from 3 different colonies. This trail pheromone comprised of esters of 2 different biogenetic origins proving variability of the system. Pheromone specificity may serve to avoid confusions between the trails deposited by foragers of different nests and, thus, to decrease competition at food sources.

2.1.6 *Tetragonula pagdeni* (Schwarz, 1939)

Their classification can be show as followings (Michener, 2000):

Kingdom Animalia (animals)
Class Insecta
Order Hymenoptera
Superfamily Apoidea
Family Apidae
Subfamily Meliponinae
Genus *Tetragonula*
Species *Tetragonula pagdeni*

Due to *Tetragonula pagdeni* (Schwarz, 1939) has taxonomically problematic identification from other sympatric species (e.g. *T. fuscobalteata*) (Sakagami, 1978). In Thailand, there were previous studies which *T. pagdeni* was misidentified into *T. laeviceps* (Thummajitsakul *et al*., 2011). The differences between morphometric characters of *T. laeviceps* and *T. pagdeni* workers, such as *T. laeviceps* has a head width of ~1.890 mm, a wing length of ~1.311 mm, and a hind tibia length of ~1.797 mm, whereas *T. pagdeni* were ~1.742, ~1.100, and ~1.555 mm, respectively. More important characters are *T. pagdeni* has whitish plumose frontal hairs and well-banded of mesoscutal hairs but not appear such characters in *T. laeviceps* (Sakagami, 1978). Additionally, the difference of their male genitalia also uses for identification (Sakagami, 1978) as well as the recently AFLP technique used to generate a candidate species-specific marker of *T. pagdeni* (Thummajitsakul *et al*., 2011).

Morphologically characters of *T. pagdeni* by Schwarz (1939) are fully descripted as:

(1) At least head has dark brown to black excluding clypeus and mesosoma.
(2) Gena narrower, Gena width/eye width (GW/EW) less than 1.0, ocelloccipital distance about 1/2 of ocellar diameter.
(3) Larger, body length (BL) more than 3.2 mm, head width (HW) 1.6 mm or more, in subtle case (*minor* nov.) mesoscutal hairs darker.
(4) Antenna below testaceous to ferruginous, only exceptionally dark brown.

(5) Mesoscutal hairs more or less well banded, at least G3 detectable.

(6) Small species, BL less than 4.1 mm, wing length (WL_1) less than 4.5 mm, HW less than 1.8 mm.

(7) Larger, BL mostly more than 3.4 mm, WL_1 more than 3.7 mm, HW more than 1.6 mm. Mesoscutal hairs sparser, with fewer admixture of dark bristles.

(8) Wing length/head width (WL_2/HW) 0.60", 0.70, hind tibia length/head width (HTL/HW) 0.85 ~ 1.00, eye length/median ocellus diameter (EL/MOD) 0.90–1.00.

(9) Metasoma, legs and anterior corbicular fringe paler. Frontal hairs are mainly whitish and distinctly plumose (Thailand and Indochina).

T. pagdeni is a predominant and commonly distributed species in most of geographic locations in Thailand, the Malaysian peninsula, and Indochina zone (Sakagami, 1978; Sakagami and Khoo, 1987; Thummajitsakul *et al.*, 2008). Thummajitsakul and others (2011) stated that *T. pagdeni* have sympatric distribution with *T. laeviceps* only in Phitsanulok province and they also found that *T. laeviceps* was only found in 3 provinces of northern of Thailand (Phrae, Phitsanulok, and Pichit provinces). *T. pagdeni* has high levels of genetic variation among individuals in all populations (Thummajitsakul *et al.*, 2008) and among populations of Thailand (Thummajitsakul *et al.*, 2011).

This species is an interesting stingless bee among various species from Thai agriculturists for using as pollinator in their orchards aims to increase crop yield. Additionally, they can produce the medicinal honey and other hive products, such as propolis. Their pollination leads to fertilization of plant sex cells and then provides fruit and seed production. The frequency of their visiting to the flowers and efficiency in pollination (ability of bee in depositing the collected pollen grains onto the stigma of flower) results in high quality and quantity yields of fruits and seeds (Kwapong *et al*., 2010). Besides in agricultural field, stingless bees also are a good pollinator in forest and can be employed for pollination in greenhouse crops in both temperate and tropical regions.

2.2 Materials and Methods

2.2.1 Insect Culture

Stingless bees, *Tetragonula pagdeni*, used in this experiment were isolated from the cultures of Chantaburi province since 1996, and continually cultured at Center of Excellence in Entomology Bee Biology, Biodiversity of Insects and Mites, Department of Biology, Faculty of Science, Chulalongkorn University. The wooden box hive of stingless bee was 1 m high from the ground. The identification of this stingless bee species was performed by professional technician of Center of Excellence in Entomology Bee Biology, Biodiversity of Insects and Mites based on the identified procedures of Schwarz (1939), Sakagami (1978), and Sakagami *et al.* (1985).

Figure 2.7 *Tetragonula pagdeni* (Schwarz, 1939).

2.2.2 Chemicals

All solvents used in this study were analytical grades. Glucose (Glucolin®) and sucrose (Mitrphol®) were commercially available. Pentatriacontane was purchased from Sigma-Aldrich Co. (St. Louis, Missouri).

2.2.3 Training Procedure and Trail Following Bioassay

Due to many previous studies (Jarau *et al.,* 2004, 2006; Dambacher, 2006; Dambacher *et al.,* 2007; Schorkopf *et al.,* 2007; Hemmeter, 2008; Stangler *et al*., 2009) stated that labial glands are the glandular origin of trail pheromone, thus trail following bioassay was introduced to confirm the trail-following behavior of stingless bee in this study. The experiment was conducted in the opening area near Center of Excellence in Entomology Bee Biology, Biodiversity of Insects and Mites. The tested nest of *Tetragonula pagdeni* was cultured in wood hive and located under a tree. This bioassay was applied from the study of Schorkopf *et al*. (2007). Prior to begin the trail following bioassay, the selected foragers were trained followed by the procedure of Schmidt and teams (2006) as descripted next.

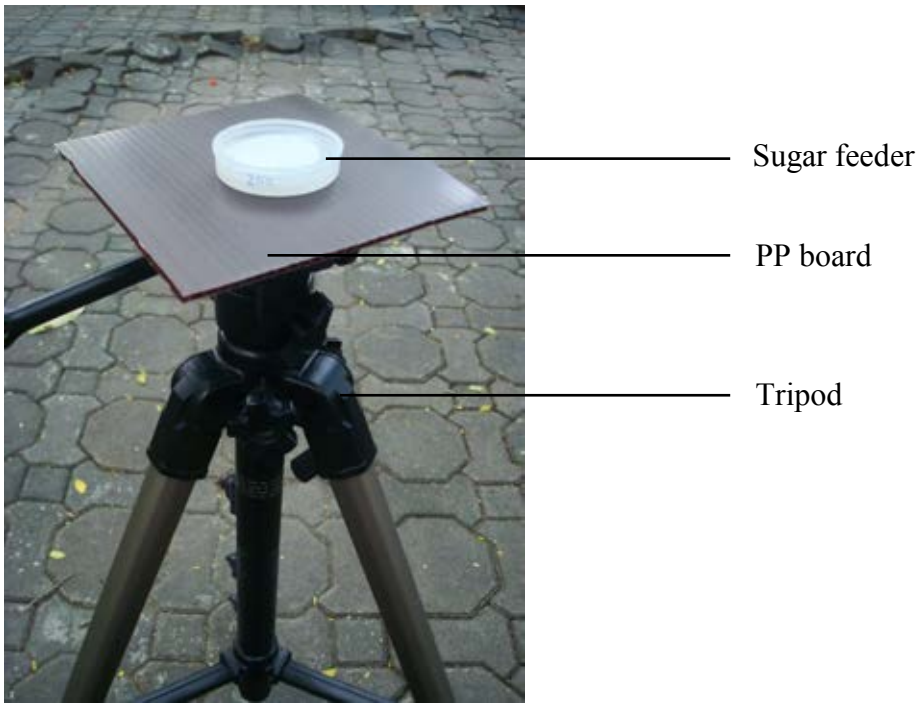


Figure 2.8 Sugar feeder on the PP board supported with tripod

Training program: Training of stingless bee was performed between 09.00 h and 12.00 h. Other nests near the area of the experiments were closed in the morning of experimental days. Ten foragers were trained from their nest entrance to a feeding site at 12 m away from the nest. The artificial unscented sugar solution in feeders was

prepared by using sucrose mixed with 10% of glucose for preventing crystallization (Laos *et al.*, 2007). Each feeder consisted of white plastic dish (5 cm diameter, 1 cm high) containing with cotton wool covering with 4 cm (Ø) filter paper (Whatman No.1, Whatman International Ltd., Maidstone, England), furnished on a Polypropylene board (PP board) with supporting by a 1 m high tripod (Figure 2.8). Cotton wool in feeder was impregnated with sugar solution. Foragers were trained to a training feeder, which contains unscented sugar solution in lower concentrations. At the beginning, let the bees feed sugar solution from syringe at their nest entrance, after that they would follow to the sugar feeder on the tripod in front of the nest. All trained foragers were marked with a water based color (Sakura® poster color) at their thoraces. This sugar feeder was stepwised away from nest by 1 m for 10 min each until the last point (12 m). The trained foragers visited the training feeder did not recruit their nestmates until changing into the new feeder (higher sugar concentration as recruitment feeder; **RF**). At 12 m distance, the old feeder was then put away into an airtight plastic container and, subsequently, the new recruitment feeder was offered to the bees at the same place. When the first recruited newcomer visited the new sugar solution, then the experiment was set up immediately.

The experimental part (Figure 2.9) was conducted after 12.00 h next from the training part. The artificial scent trail (labial gland extract: **T1**) was laid from nest at a branching point (**Bp**) (at 6 m distance from nest) with increasing their concentrations. Another artificial trail (**T2**) was hexane as control and the training trail as natural scent trail (recruitment feeder: **RF**) were used for comparison. Repeating this assay three times was three replications. All the distances of artificial scent trails (**T1** and **T2**), including recruitment feeder (**RF**), were 6 m long from branching point. At the ends of each trail were feeders containing highly profitable 80% (w/w) unscented sugar solutions. The amounts of either labial gland extract (hexane solution) used for the artificially laid scent trails, increased with the distance from the branching point (**Bp**) to feeders (Schorkopf *et al.*, 2007). The order of concentration (bee equivalents dissolved in hexane) was as followings: 0.0 (at the **Bp**, 0 m), 0.05 (1 m), 0.1 (2 m), 0.15 (3 m), 0.2 (4 m), 0.3 (5 m) and 0.9 (at the feeder, 6 m). It is known that several species of stingless bees directly deposited scent mark in the highest at food source and decreased towards the nest (Kerr *et al.* 1963; Johnson, 1987; Nieh *et al.* 2003,

2004). For control trails, the same amounts of solvent were applied. The labial gland extract or solvent were applied on the 2×2 cm filter papers (Whatman No.1) fixed at 1 m interval along the white rope. The experiment was conducted for 40 min, and the artificial scent marks were renewed once after 20 min. Any bee landing on the feeder of **T1** or **T2** was captured by the fabricate mount aspirator. Newcomers visiting at **RF** were also captured, but trained bees were allowed to feed and fly between nest and **RF** for recruiting their nestmates. After the experiment, the numbers of newcomers at treatment feeders were counted and color marked before leaving them. Three replications were performed in different days.

All instruments used in training and bioassay test, including filter paper were soaked with *n*-hexane for 24 h and dried in fume hood before use.

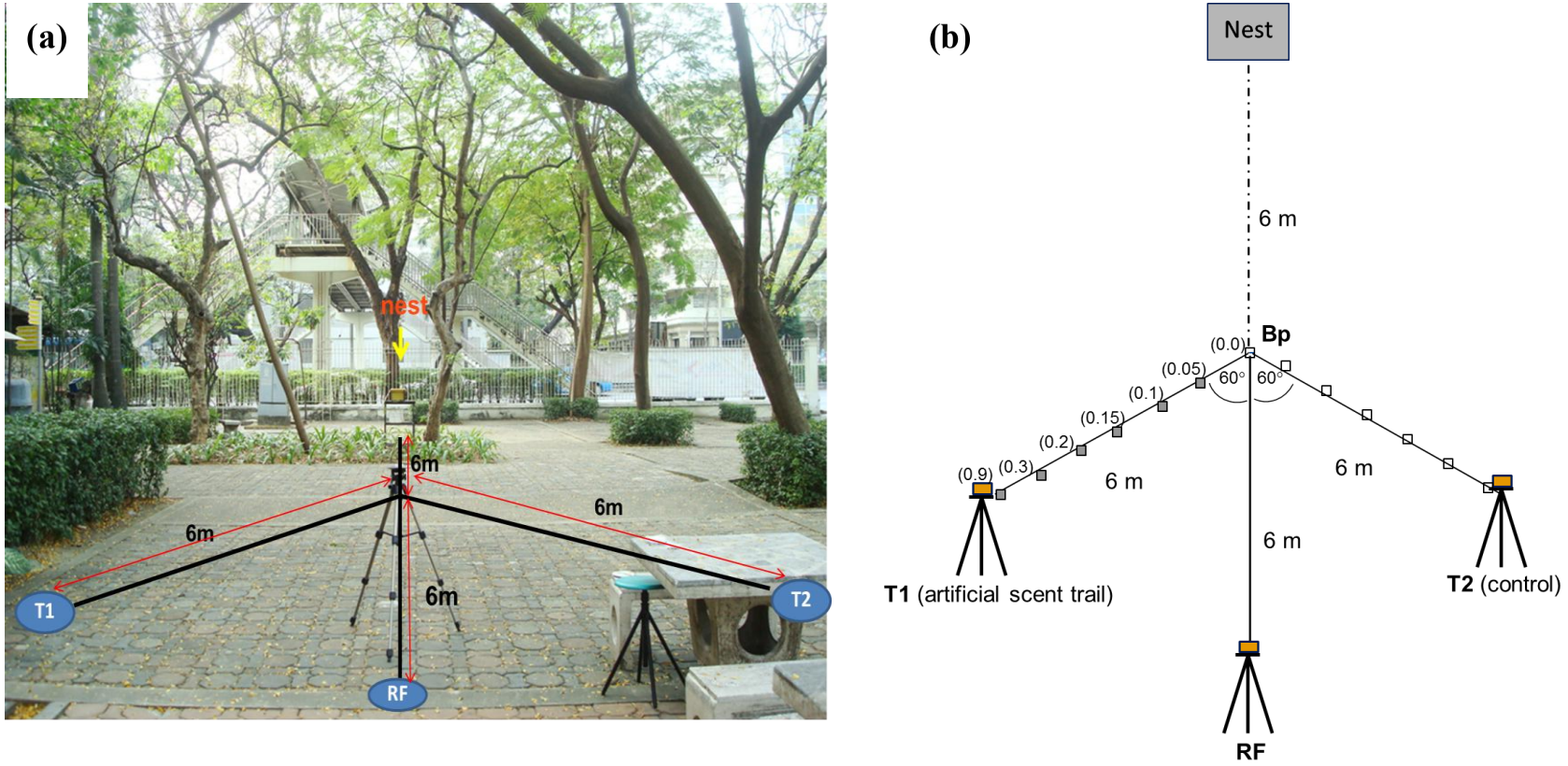


Figure 2.9 Study site (a); pattern of trail following bioassays (b). **T1** and **T2** are treatments with pheromone from labial gland and solvent control, respectively; **Bp** is branching point of artificial trail. The numbers in brackets represent bee equivalent doses. **RF** is recruitment feeder (unscented sugar solution).

2.2.4 Extraction of Labial Glands and Scent-Marked Filter Paper

Labial glands: Foraging workers of *Tetragonula pagdeni* were collected from sugar feeder (80% sugar solution) by sweeping net after training by 40% sugar solution and suddenly sacrificed by freezing at −8°C. The labial glands were carefully dissected from the heads of bees in saline solution under a stereo microscope. The isolated glands were then extracted by homogenizing in *n*-hexane (analytical grade), left at room temperature for 24 h and sonicated for 4 h. After that the labial gland solution were sifted through anhydrous Na_2SO_4 and 0.2 µm nylon filter to eliminate the contaminated water and undesired tissue. The extract was concentrated by rotary evaporator, weighed and redissolved with 100 µL of hexane and kept in −20°C until use.

Scent-marked filter paper: To confirm trail scent produced from labial glands of *T. pagdeni*, a piece of 15×15 cm cleaned filter paper was placed on PP board and sugar feeder was on this paper (Figure 2.10). At recruitment feeder in the final location, many newcomers were recruited from trained bees and laid trail scent by run a short distance while rubbing the proboscis on the filter paper (Jarau *et al.,* 2004). The new cleaned filter paper was put in place of the old one every 20 min for 1 h. These scent-marked filter papers were cut into small pieces and soaked in *n*-hexane immediately. Extraction of these filter papers was left at room temperature for 24 h. The cleaned filter paper with non-scent mark was extracted for comparison. Both extract solutions were sifted through anhydrous Na_2SO_4 and 0.2 µm of nylon filter same in labial gland extraction. After that the extracts were concentrated until dry, redissolved with 100 µL of *n*-hexane and kept in −20°C until use. Furthermore, head of a foraging worker was also performed to confirm the other contamination in labial gland extract.

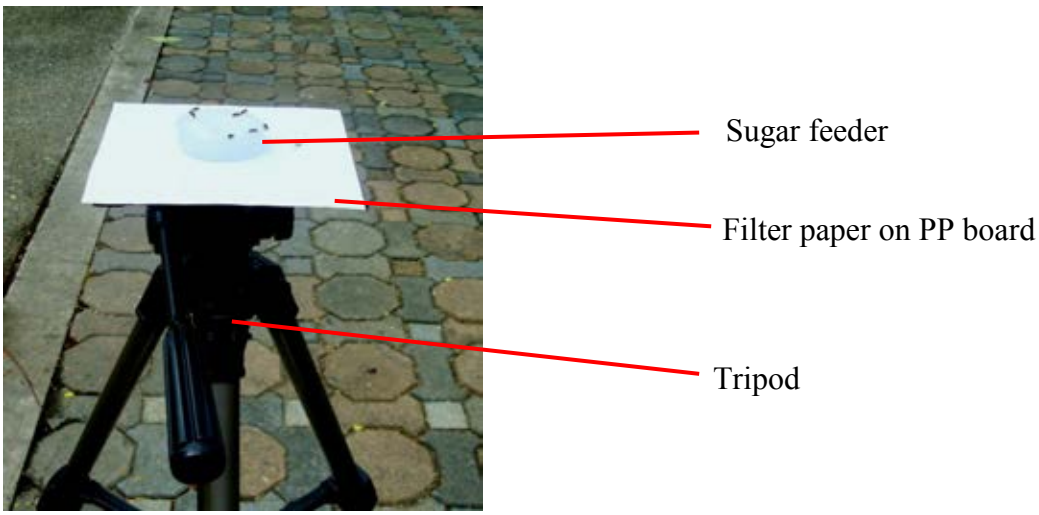


Figure 2.10 Apparatus for scent-marked filter paper analysis.

2.2.5 Preference of *Tetragonula pagdeni* in Sugar Concentration for Training and Bioassay Programs

Two ranges of sugar concentrations were set up for finding the most preferable in sugar concentrations of *T. pagdeni* using in further training and experimental studies. The concentration range of sugar solutions for training program were 20, 30, and 40% (w/w), while such for experiment bioassay were 60, 70, and 80% (w/w). Sugar solutions for training program were only used in the position of training trail, and such for bioassay program was used at the final position of recruitment feeder. Different concentrations were performed in different days to let the experienced workers forget the former sugar concentration. Responses and numbers of visiting workers were recorded.

2.2.6 Chemical Analysis

The quantitative analysis of labial gland extract was performed by GC (Varian®, CP-3800 Chromatograph) equipped with a CP-sil 8 capillary column (30 m × 0.25 mm) and FID, with N_2 as carrier gas (2 mL/min constant linear flow rate). Injection (2 µL) was done in splitless mode with an initial temperature of 60°C, which was kept for 1 min. Subsequently, the temperature was increased by 10°C per minute until the oven reached 280°C and hold for 9 min.

For qualitative investigations, GC-MS was conducted using GC (Agilent Technologies, Burwood, Australia) equipped with a 5973 mass selective detector. A capillary column, HP-5MS, of a dimension 30 m × 0.25 mm I.D. and 0.25 μm film thickness was used. A programmed temperature elution was employed the same as that used for GC-FID analysis. A carrier gas was He. Electron impact (EI) ionization was performed using electron energy of 70 eV and the mass range was 30–400 amu. The components were identified by comparison of their relative retention times and mass spectra with the standards in Wiley7n.1 library data of the GC-MS system.

2.2.7 Statistical Analysis

For normal distribution of data after arcsin square root of percentage value divided by 100 transformation (Lichtenberg *et al.*, 2011), one-way ANOVA was used to test for significant differences in the percentages of stingless bees responding to tested sugar feeders in each group of labial gland extract. Tukey's tests at $P = 0.05$ were applied for the pairwise multiple comparisons. Two treatments were compared in significant difference by *t*-test at $P = 0.05$. Means (±SE) of untransformed data are reported in text and figures.

2.3 Results and Discussions

2.3.1 Labial Gland Dissection

Heads of *Tetragonula pagdeni* foragers were pinned on the dissecting tray. Their antennas were removed, and front cuticle was dissected and removed along the white dash-line (Figure 2.11a). The stingless bees had two well-developed labial glands clearly appearing aside compound eyes (Figure 2.11b). One gland had many alveoli as showed in Figure 2.12. All collected foragers of *T. pagdeni* did not develop labial glands well. This was the result of these foragers having already discharged their scent substance (see the supporting reasons in 2.3.3). Thus the numbers of labial glands for extracting were counted from only well-developed glands.

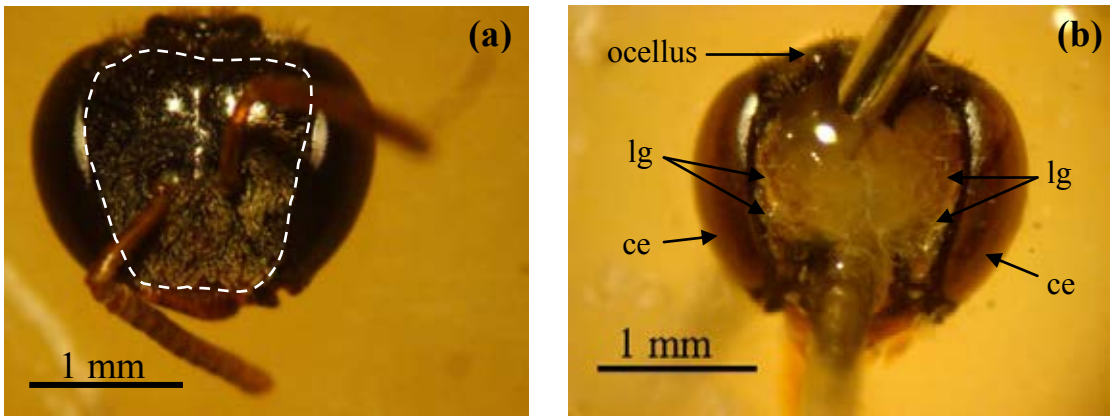


Figure 2.11 (a) Outside of head of foraging workers of *T. pagdeni*, white dash line is the dissected line for cuticle removing; and (b) opening head with labial gland (lg), compound eye (ce), and ocellus.

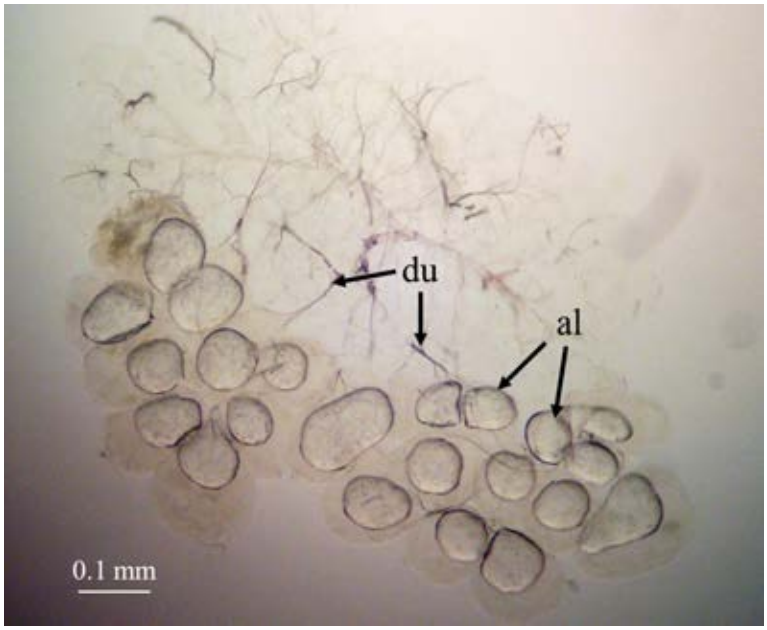


Figure 2.12 Alveoli (al) of a part of labial gland of *T. pagdeni* with ducts (du) under compound microscope (100×).

2.3.2 Preference of *Tetragonula pagdeni* in Sugar Concentration for Training and Bioassay Programs

Numbers of *T. pagdeni* foragers visiting and feeding at different concentrations of sugar solutions were displayed in Table 2.1. Foraging bees showed response behaviors by extending their proboscises into the sugar solution and fed them.

Table 2.1 Responses of *T. pagdeni* workers to the concentrations of sugar solutions

Sugar concentration	**Number of responding workers (Mean ± SE), (*n* = 3)***	
(w/w)	**Training feeder**	**Recruitment feeder**
20%	0.33 ± 0.58^{a}	-
30%	1.67 ± 0.58^{a}	-
40%	7.00 ± 3.00^{b}	-
60%	-	1.33 ± 1.15^{a}
70%	-	3.33 ± 1.15^{a}
80%	-	13.67 ± 1.53^{b}

***** Means within a column followed by the same letter are not significantly different at $P = 0.05$ (Tukey's test).

The result indicated that the appropriate concentration for training feeder is 40% (w/w) sugar solution and such for recruitment feeder in bioassay testing is 80% (w/w) sugar solution. These optimal concentrations of sugar solutions in this study slightly differed from other studies. There was a variation in sugar solution or syrup preferences of stingless bees in each area (Jarau *et al.,* 2004, 2006; Schorkopf *et al*., 2007; Stangler *et al.*, 2009), even though in fact stingless bees used nectars in average about 65% water and then converted this into honey of 30% water (Roubik and Buchmann, 1984; Roubik *et al.,* 1995). Fidalgo and Kleinert (2010) concluded their study that floral species and climate in each area affected to stingless bees in nectar and pollen foraging.

2.3.3 Compositions of Labial Gland Extract

Labial gland extract: Forty-six labial glands of *T. pagdeni* were extracted with hexane, and then concentrated until dry. After solvent evaporation by rotary evaporator, the extract appeared as a clear substance coating on the inside surface of glass vial. The extract was weighed and redissolved in 100 µL of hexane for GC-FID analysis. The yield of the extract was 0.0001 mg per 46 glands. One labial gland contained 2.17 ng, thus 2 glands (a bee) had 4.35 ng. From gas chromatogram (Figure

2.13a), there were 6 peaks from the retention time ranging from 2.65 to 30.79 min with 2 major peaks (peaks no. **2** and **4** at retention time of 3.14 and 21.00 min).

The active components in trail-following scent of labial gland extract were confirmed by extraction of scent-marked filter paper comparing with unscent-marked filter paper, and forager head extracts. Their gas chromatograms were presented in Figure 2.13 (b-d). All three additional gas chromatograms showed that peaks no. **1**, **2**, and **6** might be the trail scent produced by labial glands of *T. pagdeni*. There were no peaks no. **3**, **4**, and **5** appearing in chromatogram of scent-marked filter paper. Peak no. **1*** was confirmed as a contaminant from unscent-marked filter paper. Moreover, peaks of no. **2**, and **6** appeared in bee head extract as well.

Three identical peaks of labial gland and scent-marked filter paper extracts were possible to be the substances triggering the trail-following behavior of *T. pagdeni*. The previous study of Schorkopf *et al.* (2007) proved that *Trigona spinipes* laid saliva on the glass plate for trail communication to food source to their nestmates. Octyl octanoate was identified to be the trail-marked scent found in both glass plate and cephalic labial glands of the salivary system of this species. Therefore one of three constituents of labial gland extract or this bouquet compound might be the trail-marked scents of *T. pagdeni* for recruitment their nestmates to the rich food source. Spectroscopic techniques were essential for further identification and trail-following bioassay was necessary to confirm their activity.

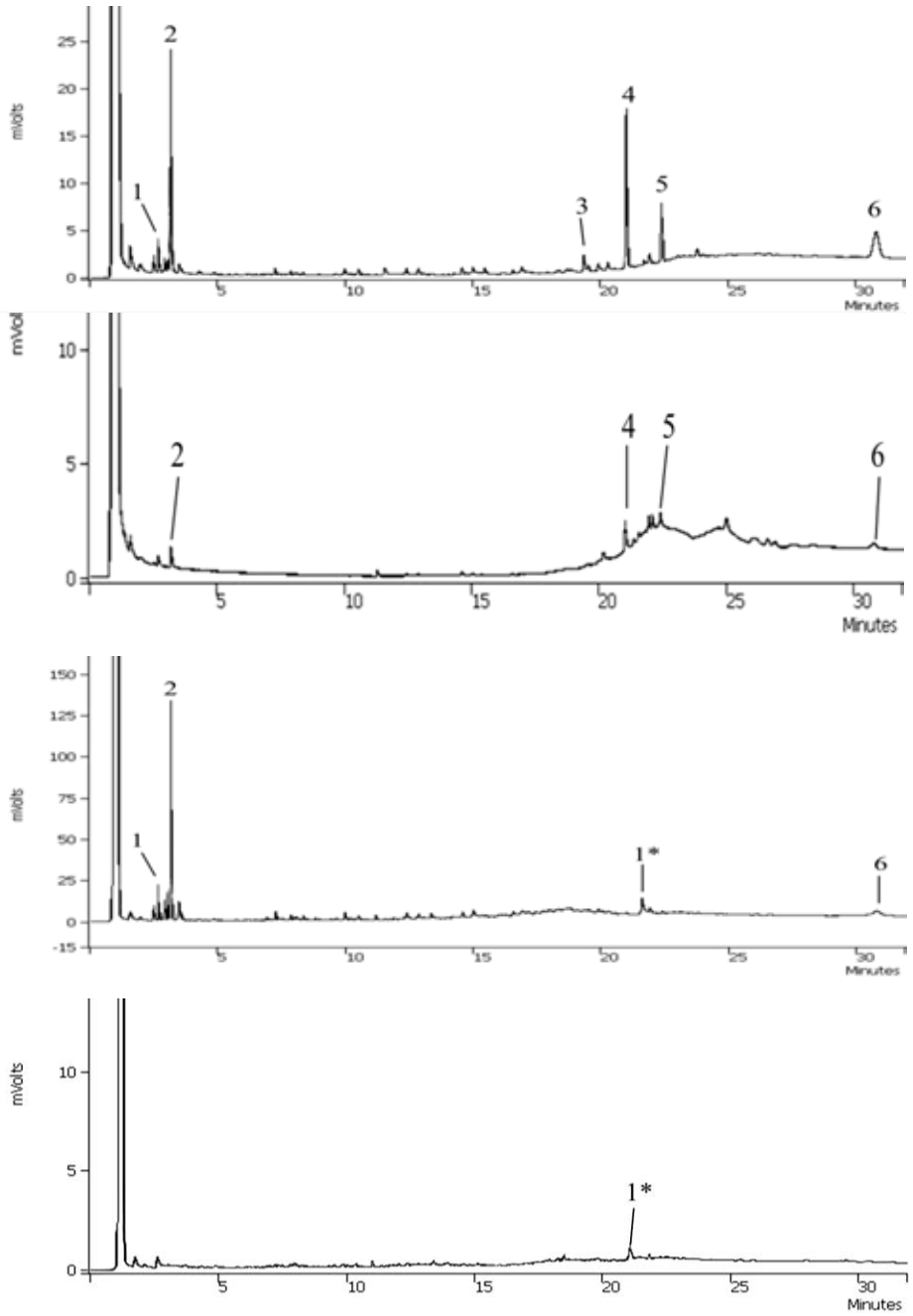


Figure 2.13 Comparison between gas chromatograms of extracts from labial glands (**a**); a bee head (**b**); scent-marked filter paper (**c**); and unscent-marked filter paper (**d**). Peak no. based on gas chromatogram of labial gland extract, unidentical peaks from such in labial gland extract with the asterisk on peak number.

2.3.4 Chemically Composition Identification

GC-MS was used for quality analysis. The constituents of labial gland extract were identified by comparison of mass spectra with such compounds in reference library. Compound names with %composition of each peak were presented in Table 2.2. Chemical structures of identified constituents of labial gland extract were displayed in Figure 2.14.

Table 2.2 Constituents of labial gland extract of *T. pagdeni*

No.	Retention time (min)	Compound name*	Quality (%)	%Composition***	
				LG extract	Paper extract
1	2.65	2,4-Dimethylheptane	10**	5.35	8.62
2	3.14	2,4-Dimethyl-2-pentene	47**	26.30	50.51
3	19.34	Tetracosane	99	3.90	nd
4	21.00	Pentatriacontane	91	31.75	nd
5	22.38	Hexatriacontane	91	12.96	nd
6	30.79	3-Hydroxy-α-(methylamino)methyl-benzenemethanol	38**	19.73	18.96

*Mass spectrum of each identified compound was revealed in Appendix.

**The unidentified compounds had less than 70% in percentage quality (matchability) of their mass spectra with reference compounds in the available mass spectra library (Wiley7n.1).

***LG extract is labial gland extract; paper extract is scent-marked filter paper; nd means not detected.

Most constituents of labial gland extract of *T. pagdeni* were hydrocarbons. Both saturated and unsaturated hydrocarbons were appeared in this extract. Three saturated hydrocarbons with more than 20 carbon atoms were identified with higher %quality (matchability), while other three hydrocarbons could not be identified (lower in %quality).

The identified components were found to be wax-type compounds occurring in labial gland of stingless bee besides trail pheromone comparable to the report of Jarau *et al.* (2004). Pentatriacontane ($C_{35}H_{72}$), a paraffin wax that can found naturally occurring in vanilla beans (Ramaroson-Raonizafinimanana *et al.,* 1997) and were a hydrocarbon component extracted from cuticle of pear psylla, *Cacopsylla pyricola*,

(Homoptera: Psyllidae) (Guédot *et al.*, 2009). Hexatriacontane ($C_{36}H_{74}$), a saturated alkane with 36-carbon atoms, was reported as a constituent in the extract of moth scale (*Heliothis armigera* and *Corcyra cephalonica*) with kairomonal activity (Ananthakrishnan *et al.,* 1991). This compound could be volatile constituents in plants, such as *Prunus mahaleb* (Mastelic *et al.,* 2006), and vanilla bean species (Ramaroson-Raonizafinimanana *et al.,* 1997). Tetracosane ($C_{24}H_{50}$) was found to be an attractive substance from some plants (cacao) or have pheromonal activity to many insect such as flying insect, European house spider, American warble fly, pear psylla, European beewolf, social wasp, milk-white termite, cotton leafroller, common walkingstick, blue milkweed beetle, West Indian sugarcane root borer, Australian cocktail ant, Australian meat ant, Argentine ant, Western drywood termite, banana stemborer and whitemarked tussock moth (Vrkoc and Ubix, 1974; Warthen *et al*., 1981; Brophy *et al*., 1983; Grant *et al*., 1987; Himeno and Honda, 1992; Young and Severson, 1994; Trabalon *et al.*, 1997; Haverty *et al*., 2000; Steinmetz *et al*., 2002; Schmitt *et al*., 2003; LaPointe *et al*., 2004; Guédot *et al*., 2009). In stingless bee, tetracosane was found as a cuticular substance extracted from *Melipona bicolor* (Lepeletier 1836) (Hymenoptera, Meliponini) (Abdalla, 2003).

According to Figure 2.13, the comparison of constituents of labial gland and substances on filter paper extracts revealed that 3 unidentified compounds in labial gland extract might be the possible active components for trail communication of *T. pagdeni.* Previous studies stated that the trail-marked scents from labial glands were the wax- and terpene-type esters (Jarau *et al.,* 2006; Stangler *et al.,* 2009). These possible active compounds tended to be small hydrocarbons with less than 10 carbon atoms or derivatives of wax compounds. It was slightly different from other reports that the trail scent from labial glands was the compounds with carbon between 10–20 atoms. Differences in their origin, geography, genera, and species could influent to the constituents of labial gland of stingless bees. Chemical constituents of labial gland of stingless bees have been extensively studied mostly in South America and less in other regions, particularly in Southeast Asia. This is thus the first report on the compositions of labial gland extract of a native stingless bee, *T. pagdeni* originated in Southeast Asia.

Moreover, it was noticed that the quantity of the peak at retention time of 3.14 min of filter paper extract was higher than that of labial gland extract (Table 2.2). This might be foragers of *T. pagdeni* have already laid trail-marked scent at filter paper before they were captured. That leaded to higher amount of the main compound in filter paper extract than such in labial gland extract.

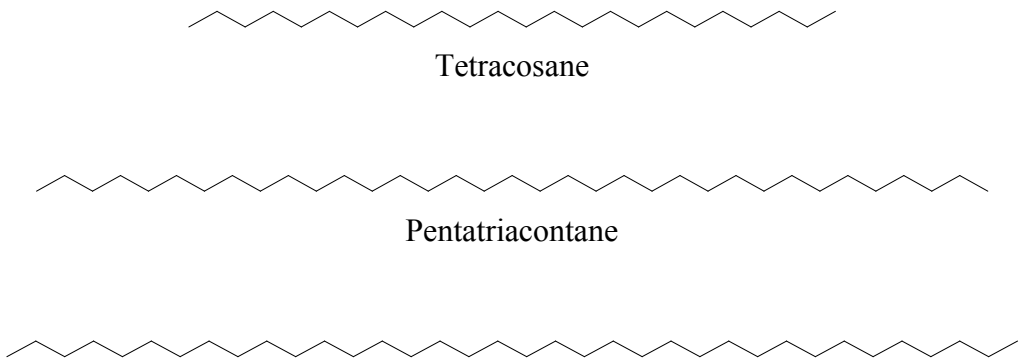
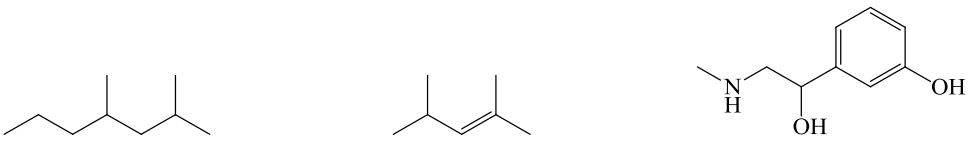


Figure 2.14 Chemical structures of identified constituents of labial gland extract from *T. pagdeni.*



2,4-Dimethylheptane 2,4-Dimethyl-2-pentene 3-Hydroxy-α-(methylamino)methyl-benzenemethanol

Figure 2.15 Chemical structures of unidentified constituents of labial gland extract from *T. pagdeni*.

2.3.5 Trail-Following Response of *Tetragonula pagdeni* to Their Labial Gland Extracts

This study was investigated by trail-following bioassay as described before. Labial gland extract of *T. pagdeni* foragers was prepared into the concentration of 1 bee equivalents (= 2 glands) in 100 µL hexane. Two groups of labial gland extracts with differences in total of %compositions of possible active compounds, especially the compound of peak no. **1** and **2** (higher and lower levels), were determined. Due to the variation in %composition of possible active constituents of labial gland extract in each time, this comparison was conducted. Gas chromatograms of these two groups are displayed in Figure 2.16.

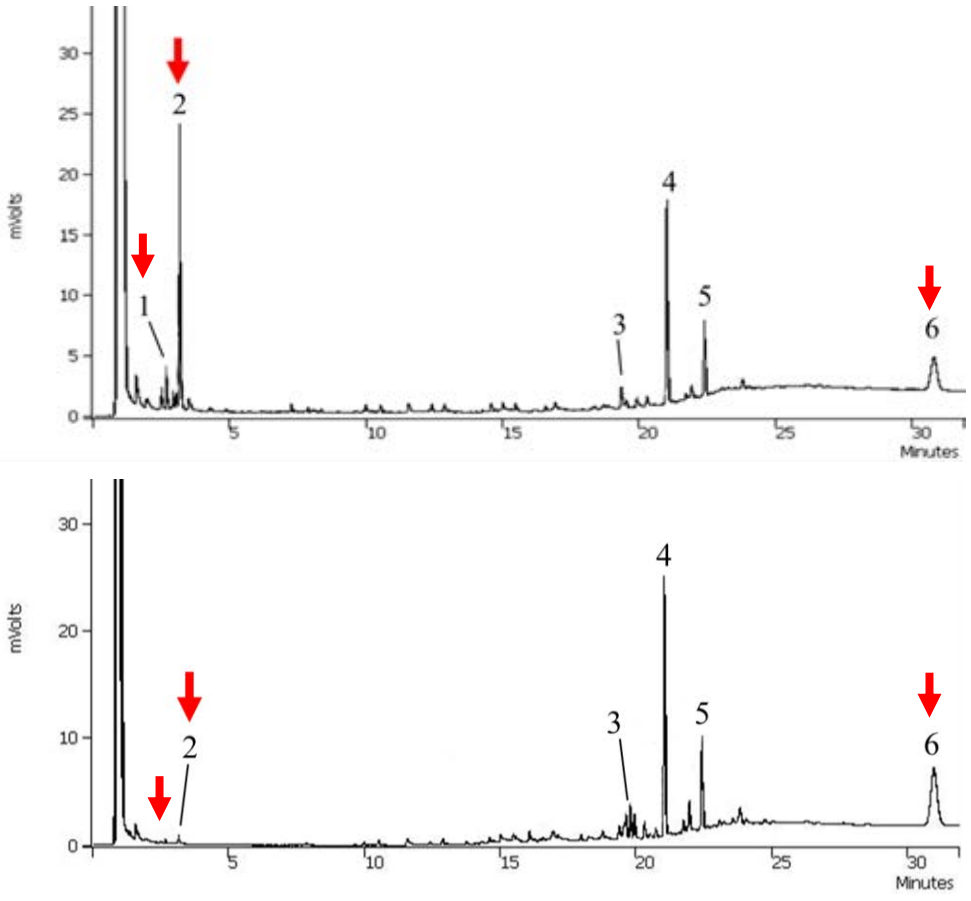


Figure 2.16 Comparison of gas chromatograms of labial gland extracts with higher %composition (a); and lower %composition (b) of compounds of peak no. **1**, **2**, and **6**.

Results in comparison of trail-following behavior of *T. pagdeni* to two groups of labial gland extracts are shown in Table 2.3 and Figure 2.17.

Table 2.3 Trail-following response (%) of *T. pagdeni* to different %compositions of compounds of peak no. **1**, **2**, and **6**

Artificial trail substances*	**%Composition (peak no.)**	**Group***	**%Trail-following response (Mean ± SE, *n* = 3)****
1. Labial gland extract **A** (higher level)	5.35 (**1**) 26.30 (**2**) 19.73 (**6**)	Treatment	12.29 ± 6.52[a]
		Control	9.78 ± 2.82[a]
		RF	77.93 ± 3.96[b]
2. Labial gland extract **B** (lower level)	0.00 (**1**) 1.16 (**2**) 35.62 (**6**)	Treatment	1.23 ± 1.07[a]
		Control	6.00 ± 3.78[a]
		RF	92.76 ± 3.72[b]

* Treatment is artificial scent trails baited with labial gland extracts **A** and **B**; Control is hexane as artificial trail; RF is the natural scent trail

** Means within a column followed by the same letter are not significantly different within group at $P = 0.05$ (Tukey's test).

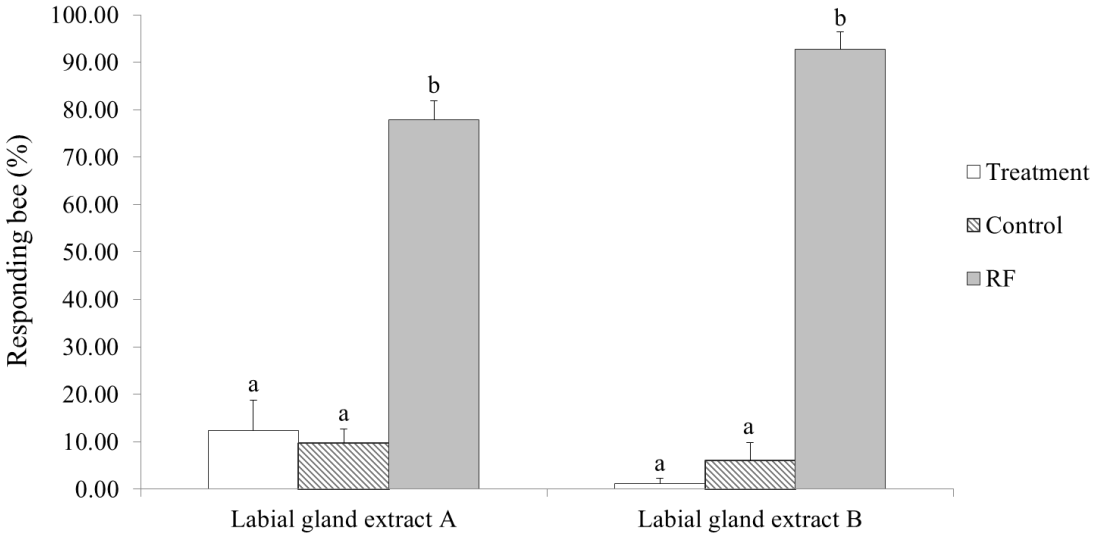


Figure 2.17 Percentages of responding bees in each group of labial gland extracts (the same letter are not significantly different within group at $P = 0.05$, Tukey's test).

During bioassay, each group of the extracts was found that newcomers always followed the natural scent trail (**RF**) in significantly different ($P < 0.05$) from the artificial scent trails (Figure 2.17). This can be explained by a higher concentration of trail scent marked by more trained bees in several times near the food source (Kerr *et al.* 1963; Johnson, 1987; Nieh *et al.* 2003, 2004). This strong mark could attract the newcomers to recruitment feeder rather than to an artificial scent trail which baited the amount only 2 times, not over than one bee equivalent of gland extract per each point. This phenomenon was similar to such of a stingless bee, *Geotrigona mombuca*, addressed by Stangler *et al.* (2009). The recruited bees might receive other cues from trained bees, *i.e.* local enhancement (Slaa *et al.*, 2003), or might directly follow the experienced foragers to recruitment feeders (Stangler *et al*., 2009). Further studies about the scent trail behavior of *T. pagdeni* were essential to explain this situation.

Number of newcomers visiting at sugar feeder of the treatment trail was not significant difference ($P > 0.05$) to the control trails in both groups of labial gland extract. Newcomers could follow the artificial treatment trails more than control trail of labial gland extract **A** group, but less than such labial gland extract **B** group. This was probably derived from some different substances in both labial gland extracts having an effect on stingless bee response including solvent baited in control trail. Figure 2.18 revealed an additional description for this effect.

From Figures 2.17 and 2.18, it was noticed that newcomers can follow the artificial scent trails baited with labial gland extract **A** more than such baited with labial gland extract **B** significantly at $P < 0.05$, while that number in control trails of both groups of labial gland extracts was not significantly different ($P > 0.05$) (Figure 2.18). This was possible that higher quantity of compounds of peaks no. **1** and **2** of labial gland extract **A** could affect on *T. pagdeni* in increasing trail-scent response. Compound of peak no.**6** might affect or did not have any trail-following activity to this stingless bee because it slightly differed in % composition of both extracts. This indicated that compound of peak no. **1** and **2** may be an important scent to trigger the trail following behavior of *T. pagdeni* although synthetic compounds were not confirmed. It was comparable with the previous reports (Jarau *et al.,* 2004, 2006; Dambacher, 2006; Dambacher *et al.,* 2007; Schorkopf *et al.,* 2007; Hemmeter 2008;

Stangler *et al*., 2009) that proved constituents of labial gland extracts of stingless bees have trail-following activity. In addition, by observation, the newcomers or recruited bees following by artificial scent trail baited with labial gland extract **A**, displayed their response behavior by flying around and landing at scent-baited filter papers fixed along the rope. While they did not display such behaviors in solvent trail or treatment trail which was baited with labial gland extract **B**. Their behaviors helped to prove the possible active compounds (peak no. **1** and **2**) as the scent triggering trail-following behavior in *T. pagdeni*. Jarau and co-workers (2004) described these behaviors in scent trail communication of *Trigona recursa*.

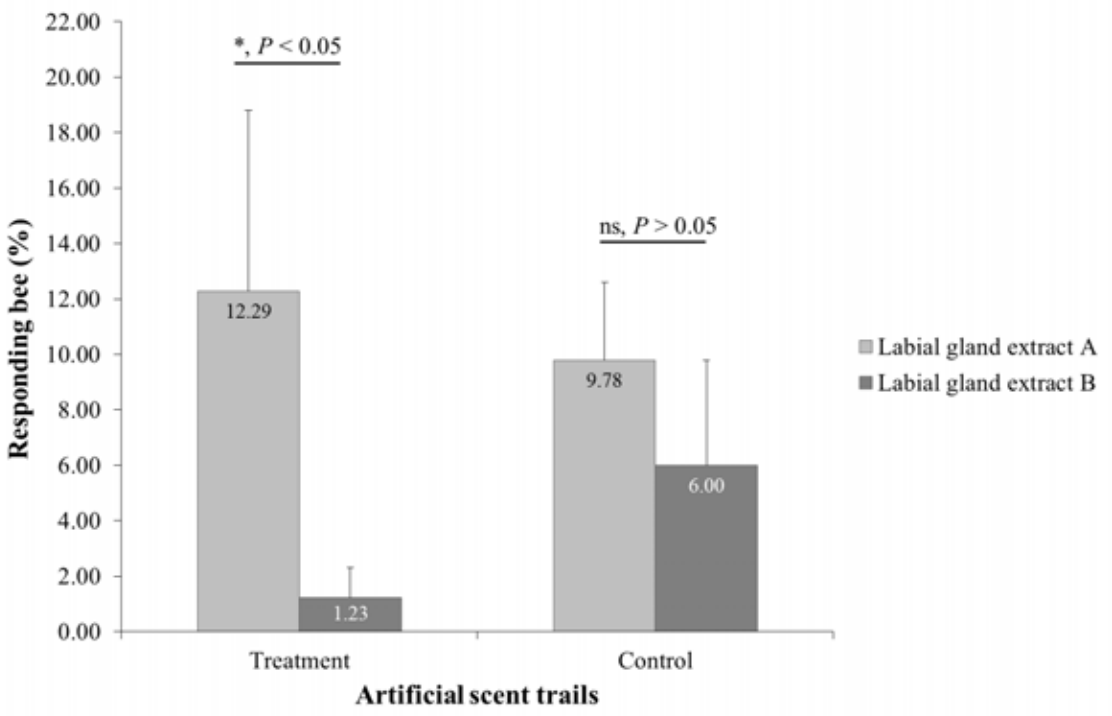


Figure 2.18 Comparison of responding bees (%) among treatment and control of groups of labial gland extracts; (* means significantly different, and ns means not significantly different at $P = 0.05$, *t*-test).

Furthermore, newcomers also followed the control trail to the feeders of both extract groups (Figure 2.18) with no significant difference ($P > 0.05$). It might be from the wind carrying scent from artificial treatment trail to the direction of control trail or the effect of hexane which has a slightly scent response to *T. pagdeni*.

Previous report by Khan and Shahjahan (1998) stated that hexane displayed attractant activity to *Callosobruchus chinensis* (L.) (Coleoptera: Bruchidae).

2.4 Conclusions

In Thailand stingless bees were extensively cultured for crop pollination to increase product yields because of their advantages more than bees. The study about trail-following odors may help supporting foraging behavior of stingless bees. There have not been any reports of trail scents of stingless bees in Thailand. *Tetragonula pagdeni* was selected because they are an abundant species and most culture for crop pollination in Thailand. The study in their trail scents and glandular origin was conducted. The cephalic labial glands of saliva system were proved to be the glandular origin of possible trail scents. Constituents of labial gland extract composed of 6 compounds: 3 compounds were identified as saturated hydrocarbons, whereas the other 3 were unidentified as unsaturated hydrocarbons. The compound with the highest %composition was a straight chain saturated hydrocarbon, pentatriacontane, while the second major component was an unidentified compound with the possible structure as 2,4-methyl-2-pentene (compound of peak no. **2**). The comparison of extracts from scent-marked filter paper and forager head confirmed that the compounds of peaks no. **1**, **2** and **6** might have the trail-following activity to *T. pagdeni*. Bioassay was a tool to confirm their activity. Comparison of labial gland extracts which have different in %composition of the possible active compounds revealed that newcomers were higher following the artificial trail baited with labial gland extract containing higher %composition of compound of peak no. **1** and **2**. This study indicated that there were some substances triggering the trail following the behavior of *T. pagdeni* originated in cephalic labial glands.

CHAPTER III

RESPONSE BEHAVIOR OF TOBACCO BEETLE *Lasioderma serricorne* TO ODORANTS

3.1 Literature Reviews

3.1.1 Biology of Tobacco Beetle

The tobacco beetle (or cigarette beetle), *Lasioderma serricorne* Fabricius (Coleoptera: Anobiidae), is the most ubiquitous of all stored-product insects. It breeds on a wide variety of commodities, including both plant and animal materials (Howe, 1957; LeCato, 1978; Ashworth, 1993). It was found firstly in the tomb of Tutankhamun (Carr *et al.*, 1994) and Rameses II (Steffan, 1982) and present in the Old World over 3000 years ago, but Runner (1919) suggested its origin in America. The first record of this beetle in Paris in 1848 and in America in 1886, was related to tobacco (Runner, 1919; Tenhet and Bare, 1951). *L. serricorne* is now distributed world-wide and has been a serious problem in economy in tropical to temperate regions (U.S.D.A., 1972). The tobacco beetle was described their morphology by Fabricius in North America in 1792 (Powell, 1931).

The **adult** beetles are 2.0–3.7 mm long, light to dark brown color, and humpbacked, with saw-like antennae (serrate) and smooth wing covers (Figure 3.1). They are different from another resembled Anobiidae, drug-store beetle (*Stegobium paniceum*), with clubbed antennae and grooved wing covers. Size of *L. serricorne* depends on the type of food (Jones, 1913), temperature and humidity during development (U.S.D.A., 1972); however, larger beetles tend to be females (Leflcovitch, 1963; Lefkovitch and Currie, 1963; Kohno, 1982). They live in dark or semi-dark places, often in crevices. They avoid the daylight but can attract to the artificial light. They are usually active and fly before the evening, at sunset, and continue their activity throughout the night (Howe, 1957). Adults of this insect can fly in a wide range up to 3 km (Buchelos, 1981). Egg laying (oviposition) is stimulated by the chemical (Fletcher and Long, 1971) and physical (Kohno and Ohnishi, 1986) factors of host food. Sex determination is hardly identified in living adult stage, but

easily identify in deadly adults by the shape of apodeme after treatment with 70% alcohol for 5 min (Papadopoulou and Buchelos, 2002b) (Figure 3.2).

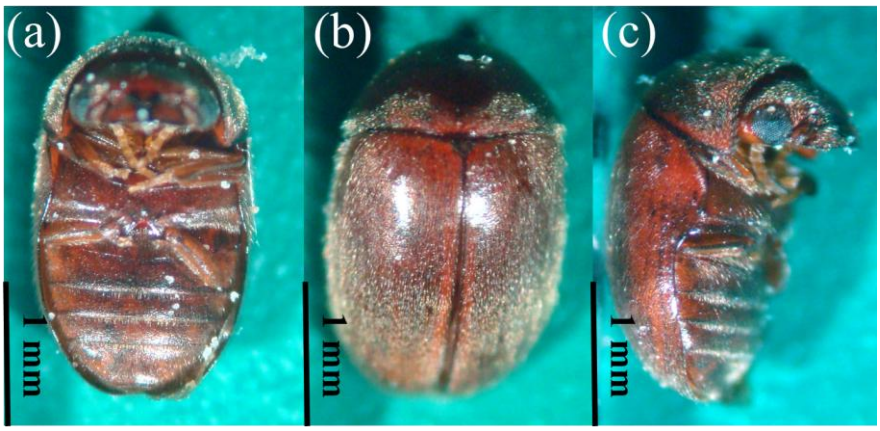


Figure 3.1 Adults of *L. serricorne*: (a) ventral; (b) dorsal; and (c) lateral views.

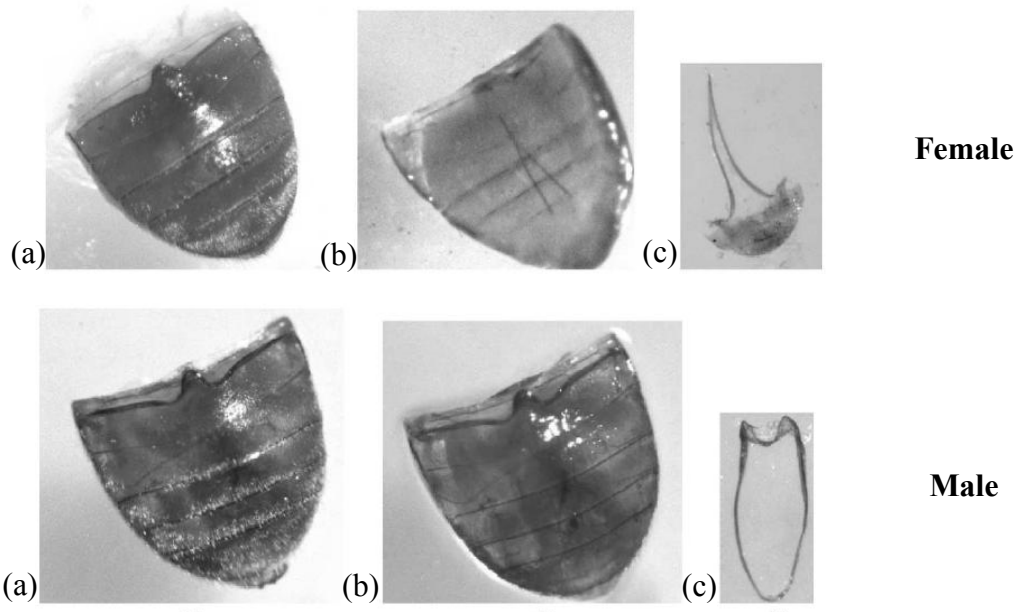


Figure 3.2 Abdomens of female and male adults of *L. serricorne*: (a) before treatment; (b) after treatment; and (c) V- and U-shape apodeme out of abdomen in female and male, respectively (Papadopoulou and Buchelos, 2002b).

Eggs are oviposited onto dried material by females which are one day emerging and have sexually mature development (Cooper and Bengston, 1974). The eggs are pearly white (Retief and Nicholas, 1988), 0.4 to 0.5 mm long and 0.2 mm wide (Jones, 1913; Sivik *et al.*, 1957). They are very fragile waxy shell to protect them from moisture. The eggs are able to hatch in 6 to 8 days (Retief and Nicholas, 1988) and the newly emerged larva will eat the egg shell after that (Pant and Fraenkel, 1950).

Larvae generally complete in four growth stages (instars) and growing from less than 1–4.5 mm long (Niiho, 1984). Larvae are scarabaeiform type with creamy colored or greyish white and covered with fine light brown hairs (Sivik *et al.*, 1957) (Figure 3.3). Newly hatched larvae are extremely active away from light into the small hole and searching for food (Runner, 1919; Bovingdon, 1931). The larvae can eat substrate during their development around 13–16 mg of foodstuff (Howe, 1957; Sivik *et al.*, 1957; Kurup and Parkhe, 1961; Lefkovitch and Currie, 1963; Lefkovitch, 1967). The larva feeding causes most of damage to stored products (Minor, 1979; U.S.D.A., 1972). Activity of *L. serricorne* larvae decreases when the temperature falls below 19.5°C and the insect overwinters in the larval stage (Runner, 1919).

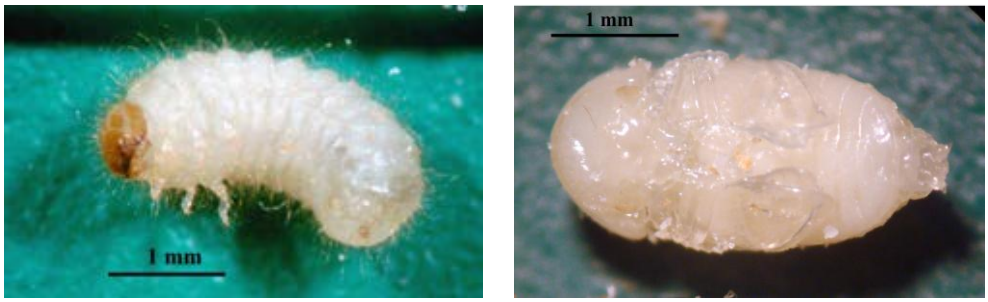


Figure 3.3 Larval (left); and pupal (right) stages of *L. serricorne*.

Pupae (Figure 3.3) develop from fully grown larvae that stop feeding in the pupal cell (or cocoon) within the foodstuff or attached to a surface, such as container surface, by their biological cement. The cell is made of food and waste cemented secreted by their midgut (Howe, 1957; Carr *et al.*, 1994). The pupal period ranges between 4–12 days (Runner, 1919; Howe, 1957; Samuel *et al.*, 1984). Adult emerging from the pupal cell is mainly during the night (Samuel *et al.*, 1984). Sex of the pupae

is determined by genital papillae which are globular and not projecting in the males, and three-segmented and divergent in the females (Halstead, 1963; Ryan, 2001). The sex ratio of the emerging adults is 1:1 (Sivik *et al.*, 1957).

A **life cycle** of this insect takes about 40 days at 30°C (Powell, 1931; Howe, 1957; Savik *et al.*, 1957; Fletcher, 1980; Samuel *et al.*, 1984; Retief and Nicholas, 1988). It may be longer when the larvae overwinter (Meyer, 1980). The duration of the developmental period has been related to temperature and relative humidity (Powell, 1931; Edwards *et al.*, 1980; Niiho, 1984). Populations of this insect can build up very quickly (Howe, 1957) depended on temperature and humidity (Powell, 1931). Optimal conditions for rearing *L. serricorne* are at 28°C and 70% R.H. (Powell, 1931; Howe, 1965; Fletcher and Long, 1976; Fletcher, 1980; Buchelos, 1981) on 20:1, flour: yeast mixtures (Fletcher and Long, 1976; Singh, 1977; Samuel *et al.*, 1984) or ground tobacco (Fletcher, 1980). The adult has a short life, between 2−7 weeks (Howe, 1957), depending on larval food, temperature and humidity again. All stages of *L. serricorne* may survive at temperatures between 2 and 36°C, or below 2°C for short periods (Powell, 1931).

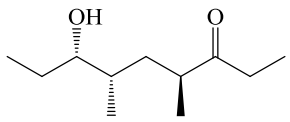
Food preferences: *L. serricorne* infests a wide variety of foodstuffs including: ground grain or seed, whole seeds, grains, rice, pulses and beans, pasta, spices, dried fruit and vegetables, yeast, dried animal, animal feed, leather and wax, and dried and processed plant material, such as tobacco (Fraenkel and Blewitt, 1943; Howe, 1957; Ambadkar and Khan, 1989). Females are attracted to tobacco (Kohno *et al.*, 1983) and will lay more eggs on a better grade of flue-cured (Kurup, 1961). Milne (1963) records flue-cured and some light air-cured tobaccos are the most susceptible to cigarette beetle attack, while dark air-cured and burley tobaccos are only lightly infested. The Turkish and flue-cured tobaccos are a better food for this beetle because they are high in sugar, vitamins, and starch with low in nicotine (Milne, 1963). *L. serricorne* can tolerant to nicotine up to 4% (Yamamoto and Fraenkel, 1960; Milne, 1963). They can metabolize, and detoxify nicotine into cotinine and other alkaloids (Self *et al.*, 1964), but the larvae cannot survive on tobacco containing 8.25% nicotine (Milne, 1963; Jurzitza, 1969).

Food Requirements: *L. serricorne* has yeast-like intracellular symbionts in the gut mycetomes (Jurzitza, 1977). These may be *Symbiotaphrina buchneri* or

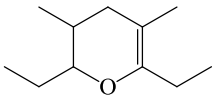
S. kochii (Pant and Fraenkel, 1950). The symbionts are transmitted to the new generation *via* the egg after migration to the oviduct. When the larvae hatch, they eat the egg shell for acquiring the symbionts. The symbionts can synthesize vitamins, amino acids, sterols, and essential nutrients for tobacco beetle (Plant, 1972; Saxena and Gohain, 1976). The esterases produced from the symbionts, such as l-naphthyl acetate esterase, was reported in detoxification a wide range of pesticides, mycotoxins and plant toxins (Dowd, 1989; Shen and Dowd, 1991), and thus resulting in this beetle can tolerant to insecticides (Milne, 1963; Saxena and Gohain 1976; Dowd and Shen, 1990; Shen and Dowd, 1991).

Reproduction: Sex pheromone is the importance attractive substance evolved to bring the two sexes of *L. serricorne* together for reproduction. Female sex pheromones are attractant to male and active over longer distances than those of the male (Rangaswamy, 1985). The pheromone producing gland is in the second abdominal segment of females (Levinson *et al.*, 1983). Pheromone is produced after 10 h of adult emergence, increasing to a maximal level in 4 day age and gradually declining from the 6 days after emergence until death (Coffelt and Burkholder, 1972). The responsiveness of males to the sex pheromone increases from the first to the fourth week of life (Levinson and Levinson, 1987).

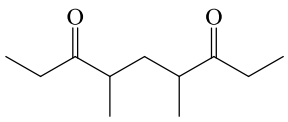
Chemical study of the female sex pheromone of *L. serricorne* (Chuman *et al.*, 1979, 1985) revealed the isolation of seven active components (Figure 3.4), such as (4*S*,6*S*,7*S*)-7-hydroxy-4,6-dimethyl-3-nonanone **(serricornin)** (**I**), 2,6-diethyl-3,4-dihydro-3,5-dimethyl-2H-pyran (**anhydroserricornin**) (**II**), 4,6-dimethyl-3,7-nonanedione (**III**), 4,6-dimethyl-3,7-nonanediol (**IV**), 7-hydroxy-4,6-dimethyl-4-nonen-3-one (**V**), (2*S*,3*R*)-2-ethyl-2,3-dihydro-3,5-dimethyl-6-(1-methyl-2-oxobutyl)-4H-pyran-4-one (**serricorone**) (**VI**), and (2*S*,3*R*)-2-ethyl-2,3-dihydro-6-(2-hydroxy-1-methylbutyl)-3,5-dimethyl-4H-pyran-4-one (**serricorole**) (**VII**).



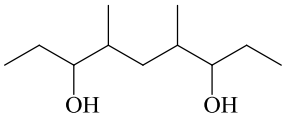
(**I**) Serricornin



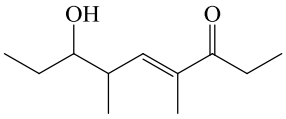
(**II**) Anhydroserricornin



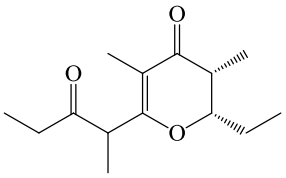
(**III**)



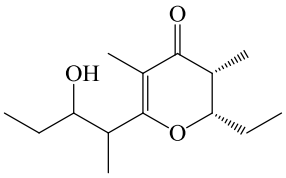
(**IV**)



(**V**)



(**VI**) Serricorone



(**VII**) Serricorole

Figure 3.4 Chemical constituents of *L. serricorne* sex pheromone.

Mating behavior occurs within 3 days of adult emergence. When exposed to sex pheromone from virgin females, males follow to the females, and lower their head and upper body (prothorax), vibrate their antennae, and rapidly walk around the female. Prior to copulation, the male touches the females with his antennae and palpi, grasps her elytra, and inserts the aedeagus into the genital opening. The male descends and remains paired with the female in an end to end position for about an hour. During this stage, the copulatory movements are performed, and sperm are transferred. Males still respond to females after previous mating (Levinson and Levinson, 1987). Multiple mating occurs with over 90% of females mating at least twice and over 90% of males mating at least 6 times (Coffelt and Burkholder, 1972).

The most important parasites of the cigarette beetle are the wasp, *Anisopteromalus calandrae* (How.), and mite, *Moniezella angusta* (Banks) (Bare, 1942).

Control management: The effective pest management program requires integration of various methods. The effective monitoring (pheromone and light traps), good sanitation, best physical control, and judicious applications of insecticides (methophrene or fumigants) methods are a concerted effort. However, the control by prevention is the best strategy to manage *L. serricorne* and other stored-product insects (Carr *et al.*, 1994).

Pheromone trap is a prevention strategy generally uses to monitor and control tobacco beetle (Levinson and Buchelos, 1988; Buchelos and Levinson, 1993). Discovery of female sex pheromone and its synthesis led to the development of pheromone traps. The essential element of the trap consist of a lure permitting controlled release of sex pheromones placed on a sticky board (Levinson and Buchelos, 1988) for catching the beetles and counting (Barak *et al.*, 1991). All commercial pheromone lures are use serricornin as the main component combined with different proportions of minor pheromones, anhydroserricornin (Levinson *et al.*, 1981; Levinson and Levinson, 1986). The advantages of pheromone trap are: easy use, small size, not required electricity to supply, without maintenance, mobile, and specific to insect (Rangaswamy, 1985). A disadvantage is that the sticky surface of trap always becomes coated with dust. Since the sex pheromone trap can attract only male adults of *L. serricorne*, the product losses still risk by infestation of females. There were reports that female adults of *L. serricorne* have attractive response to suitable foods for their oviposition (Kohno *et al.*, 1983; Saeed *et al.*, 2008). There were the combination of host-food extracts and pheromone lure to use for increasing the attractiveness of both sexes in monitoring of *L. serricorne* population (Papadopoulou and Buchelos, 2002; Mahroof and Phillips, 2007, 2008).

3.1.2 Responses of Tobacco Beetle to Plant Volatiles

Due to the fact that stored-product insects mostly are phytophagous insects, they generally utilize the odorous semiochemical cues from host plants during host selection process. Plant volatile odorants may act as direct attractants for insects or they may synergistically enhance the pheromone activity of insects that have contacted the host plant (Phillips *et al.*, 1993). “Semiochemicals” are the chemical substances that deliver behavioral message intraspecific (between individuals of same

species) or interspecific (between members of different species) communications. Semiochemicals are divided into 3 groups: “allomones” the interspecific semiochemicals that benefit to the producers, “kairomones” those that benefit to the receivers and “pheromones” the semiochemicals used for intraspecific communication (Ghosh, 1995).

Many previous reports stated that volatile odorants attracting to several beetle species that infest broken grain have been identified from cereal grains and their products (Nara *et al.*, 1981; Mikolajczak *et al.*, 1984; Pierce *et al.*, 1990). Work on the maize weevil, *Sitophilus zeamais* Motschulsky, a species that attacks sound grain, demonstrated that odors from cracked wheat synergistically enhanced responses to male-produced pheromone (Walgenbach *et al.*, 1989). Trapping technology for the red flour beetle, *Tribolium castaneum* Herbst, utilizes wheat germ oil as a food attractant in combination with a synthetic male-produced pheromone (Barak and Burkholder, 1985). A mixture of phenethyl propanoate and eugenol (4-allyl-2-methoxyphenol) was found to be attractive to both sexes of Japanese beetle (McGovern *et al.*, 1970).

For tobacco beetle, Kohno and co-workers (1983) indicated that female adults of *L. serricorne* were strongly attracted to the lower leaves more than the upper ones of cured tobacco *var*. Turkish. They also found that toasted coffee bean meal was the most attractive material to this insect among 11 items of other host-food, but less than cured tobacco leaves. Finally they suggested that the active components tend to be the volatiles from essential oil of host food, more than from solvent extracts. In 2007, Mahroof and Phillips studied on the orientation of adult tobacco beetle to plant volatiles. They revealed that mated females of *L. serricorne* have relatively higher attracted to volatiles from different *Capsicum* products than that from tobacco. However, they also stated that volatile active components have both polar and non-polar characteristics in each active plant samples. After that in 2008, Mahroof and Phillips proved the volatiles from dried red chili (*Capsicum frutescens* L.) plus serricornin lures could enhance the attractive activity of *L. serricorne* adults.

3.2 Materials and Methods

3.2.1 Insect Rearing

Tobacco beetle, *Lasioderma serricorne*, was obtained from cultures of Tobacco products analysis subdivision, Thailand Tobacco Monopoly and maintained since 2007 in laboratory. The insects were reared in 200 g of 95% coarse-ground whole wheat mixed with 5% dried ground brewer's yeast in 24 oz. glass jars covered with blotting paper. Rearing conditions were at 30 ± 2°C, 60 ± 10% relative humidity (R.H.), and 12: 12 (L: D) h photoperiod. Parent beetles of insect were removed from cultures to new fresh medium one week after inoculation. All new adult progeny were introduced to new medium after a day of emergence. Mated females at age 4–5 days were used for all bioassays because mating and oviposition initiation of adult insect would be ready within three days after emergence (Howe, 1957). Mated females showed higher response to plant volatiles than males (Mahroof and Phillips, 2007). Mated females were isolated by their body sizes descripted by Kohno (1982).

3.2.2 Plant Materials and Chemicals

Thirty different samples of host-plant materials (Table 3.1) could be divided into 5 categories: spices, tobacco, cereal grain, tea, and miscellaneous types. All dried materials were ground by a mechanical grinder (Panasonic MX-795N, 220-240 Volt Blender with Spice Mill), and then sifted using a 20 cm standard flour sifter. The powder of plant materials was dried in hot-air oven at 55°C until their weights were stable, and kept in desiccator until use.

Chemicals used in this experiment, such as phytol was purchased from Merck Schuchardt OHG (Germany) while β-ionone was obtained Fluka Chemies A.G. (Switzerland), and methyl palmitate was from Sigma-Aldrich Co. (St. Louis, Missouri). All solvents were purified by distillation before use, except diethyl ether and acetone which were analytical grade.

A commercial kairomone lure for *L. serricorne*, the STORGARD® oil-based kairomone attractant (Trécé Incorporated, OK) was used as a standard to compare the activity with other plant volatiles.

Table 3.1 Host-plant materials used in the experiment

Categories	Host-plant materials	Scientific name	Sources*
1. Spices	Paprika	*Capsicum annuum* L.	(1)
	Red chili	*Capsicum frutescens* L.	(2)
	Rosemary leaf	*Rosmarinus officinalis* L.	(1)
	Oregano	*Origanum vulgare* L.	(1)
	Cumin	*Cuminum cyminum* L.	(3)
	Pepper	*Piper nigrum* L.	(3)
	Turmeric	*Curcuma longa* L.	(4)
	Curry powder	-	(3)
2. Tobacco	Cured-tobacco leaf *var.* Turkish	*Nicotiana tabaccum*	(5)
	Cured-tobacco leaf *var.* Virginia	*Nicotiana tabaccum*	(5)
	Cured-tobacco leaf *var.* Burley	*Nicotiana tabaccum*	(5)
	Chewing tobacco cut	*Nicotiana tabaccum*	(6)
3. Cereal grain	Wheat flour	*Triticum* spp.	(7)
	Rice flour	*Oryza sativa* L.	(8)
	Glutinous rice flour	*Oryza sativa* var. *glutinosa*	(8)
	Cassava flour	*Manihot esculenta* (L.) Crantz	(9)
	Corn flour	*Zea mays* L.	(10)
	Cracked whole wheat	*Triticum* spp.	(11)
4. Tea	Chinese Oolong tea leaf	*Camellia sinensis*	(12)
	Commercial green tea	*Camellia sinensis*	(13)
	Commercial black tea	*Camellia sinensis*	(14)
	Mulberry leaf tea	*Morus rotunbiloba*	(15)
5. Miscellaneous	Ground cocoa bean	*Theobroma cacao* L.	(16)
	Ground coffee bean	*Coffea Arabica* L.	(17)
	Dark cocoa powder	*Theobroma cacao* L.	(18)
	Light cocoa powder	*Theobroma cacao* L.	(18)
	Lingzhi	*Ganoderma lucidum* (Curtis) P. Karst	(4)
	Brewer's yeast	*Saccharomyces* spp.	(19)

*(1) McGarrett®, Continental Food Co., Ltd., Bangkok; (2) Raitip®, Thai Cereals World Co., Ltd.; (3) Nguan Soon Hand no.1®, Bangkokchili ,.Ltd, Bangkok; (4) Local shop, Yoawarat China town, Bangkok; (5) obtained from Thailand Tobacco Monopoly, Bangkok; (6) Black cat®, Blackcat Industry Co., Ltd., Nakhon Pathom; (7) Kite®, UFM Food Centre Co., Ltd., Bangkok; (8) Erawan®, Cho Heng Rice Vermicelli Factory Co., Ltd., Nakhon Pathom; (9) Pla Mongkorn®, Tong Chan ROP, Bangkok; (10) Kanor®, Friendship Corn Starch Co., Ltd., Samutprakan; (11) UFM Food Centre Co., Ltd., Bangkok; (12) Sam mah no.1®, Three Horse Tea Co., Ltd., Bangkok; (13) Raming®, Raming Tea Co., Ltd., Chiangmai; (14) Liptan®, PT Uniliver Indonesia, Indonesia; (15) manufactured under Thai Tea Suwirun Partnership, Mae Salong Mountain, Chiang Rai; (16) obtained from Chumphon Horticultural Research Center, Sawee, Chumphon; (17) The Coffee Bean®, The Coffee Bean Roasting Co., Ltd., Bangkok; (18) Tulip®, Theobroma B.V. Co., Ltd., Nigeria; and (19) Saf Instant®, S.I.lesaffire, Germany

3.2.3 Preference Pitfall Bioassay

Two-choice pitfall bioassay with passive air was used for testing the response behavior of *L. serricorne* to test materials which was adapted from the previous report of Mahroof and Phillips (2007). The bioassay apparatus (Figure 3.5) consisted of standard 9 cm plastic petri dish plate having two pitfall holes, each 1 cm diameter, in the bottom of plate directly opposite from each other, 6 cm apart and 0.5 cm from the side wall. The 3 mL volume, 1.5 cm bottom diameter, and 3.5 cm height glass vial for holding test materials was connected directly underneath each pitfall holes by adhesive latex (TOA® LA-22S). The top inner edges of vials were coated with 100% pure petroleum jelly (Milott laboratories Co. Ltd., Samut Prakan) to prevent the responding beetles climbing the wall and escape. The bioassay apparatus was prepared prior testing 24 h to let the odor of glue evaporate and the glue set up properly. Adults of *L. serricorne* were isolated from their colony by clearly plastic bag with some ground peanut inside and tightly covered on the edge of rearing jar by elastic band. The adults of insect will climb up into plastic bag to search for the new food source. Ten mated female adults were isolated out based on their size and introduced into the arena of petri dish plate by an inverted small plastic lid and allowed to acclimate in the test condition for 10 min. Prior to release, beetles were starved for 6 h. The assay started when the small lid was removed from the arena and the lid of petri dish was covered tightly with binding paraffin film. The assay was conducted in dark phase at rearing condition for 3 h. After that the assay was finished by switching on the light. The number of beetles found in the treatment vial and the control vial were counted and calculated by a percentage of response index (%RI) in the following formula,

$$\%RI = [(T - C)/Total] \times 100$$

According to formula, T was the number of beetles responding to the treatment vial, C was the number of beetles responding to the control vial and “Total” was the total number of beetles introduced in the arena of petri dish plate in the beginning. A positive RI indicated attraction to the test material whereas a negative RI indicated repellence.

All glass apparatus were cleaned thoroughly between experiments by sonication in detergent for 30 min, followed by distilled water for 1 h, rinsed with double distilled water and again with acetone, then oven-dried at 110°C for 3 h before reuse.

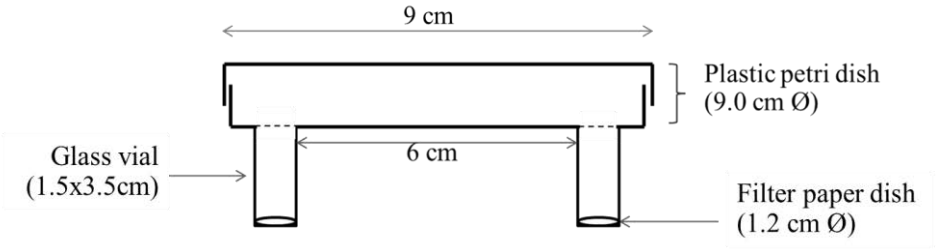


Figure 3.5 The experimental apparatus for two-choice pitfall bioassay.

3.2.4 Screening for Response Behavior of *Lasioderma serricorne* to Host-Plant Materials at 0.2 g Dose

Thirty host-plant materials (Table 3.1) were screened for attractive activity of *L. serricorne* by 2-choice pitfall apparatus that descripted above. Each plant material was weighed for 0.2 g dose for each treatment vial. Another empty vial was used as control. Ten female adults of insect were applied for a replication. Nine replications were carried out (n = 9). The assay was conducted in a dark room for 3 h. From preliminary observation, after beetles released into the arena they moved gradually towards the treatment group and spent 3 h for completely responses. After statistical computing of the number of beetles responding to each host-plant material, the highest group of treatment materials was selected to further determine the response activity at 0.02 g dose by the former assay.

3.2.5 Chemical Analysis

Headspace solid-phase microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS) was used for analysis the volatile constituents of selected host-plant samples. Headspace-SPME-GC-MS is the innovative technique for widely use in identification the volatile organic compounds

(VOCs) (Kusch and Knupp, 2004). The dried plant samples were ground and kept in glass mason jar to use for analysis. Three grams of ground mulberry leaf tea was put in a 22 mL bottle fitted with a PTFE/silicone septum and aluminium cap. The sample bottle was heated at 120ºC for 20 min (Chumpolsri, Suttiarporn and Wongpornchai, 2007). A SPME fiber, 50/30 µm DVB/Carboxen™/PDMS StableFlex™ was mounted in the manual SPME holder and was preconditioned in a GC injection port set at 250ºC for 10 min. By insertion through the septum of the sample bottle, the fiber was then exposed to the sample headspace for 20 min prior to desorption at 250ºC for 10 min of the volatiles into the splitless injection port of the GC-MS. GC-MS analysis was performed using an Agilent Technologies 6890 model gas chromatograph (Agilent Technologies, Burwood, Australia) equipped with a 5973 mass selective detector. A capillary column, HP-5MS, of a dimension 30 m × 0.25 mm I.D. and 0.25 µm film thickness was used. A programmed temperature elution was employed with an initial temperature of 40°C. Then it was ramped to 150 and 250°C at the rate of 10°C/min both, and hold at 250ºC for 10 min. A carrier gas used was He. Electron impact (EI) ionization was performed using electron energy of 70 eV and the mass range was 20–400 amu. The components were identified by comparison of their relative retention times and mass spectra with the standards in Wiley7n.1 library data of the GC-MS system.

3.2.6 Response Behavior of *Lasioderma serricorne* to Plant-Derived Volatiles

Each dose (1.0, 0.1, 0.01, and 0.001 mg) of each commercial compound as volatile constituents in mulberry leaf tea, was diluted in 10 µL of acetone and applied to a piece of 1.2 cm (Ø) filter paper (Whatman No.1, Whatman International Ltd., Maidstone, England) for treatment group. The control group was a piece of such filter paper with 10 µL of acetone. Before the applied filter papers were introduced into the vials, they were left in fume hood for 5 min to allow solvent evaporation. Two-choice pitfall apparatuses were kept in dark at rearing condition for 3 h. The number of beetles in each treatment was calculated comparing with control groups.

3.2.7 Response Behavior of *Lasioderma serricorne* to Synergism of Phytol and β-Ionone

The commercial compounds, phytol and β-ionone, were evaluated the synergistic activity at various ratios of 0.001 mmole. Two-choice pitfall apparatus descripted above was used in this study. Acetone was used as a diluting solvent. After experiment in dark phase, the number of beetle was counted and computed into RI value.

3.2.8 Statistical Analysis

Percentage response index (%RI) (Mahroof and Phillips, 2007) was calculated and transformed to arcsin square root of percentage divided by 100 to convince the assumptions of normality and homogeneity for analysis of variance (ANOVA) using SPSS software version 17th. Duncan's test at $P = 0.05$ were applied for the pairwise multiple comparisons. Means (% ±SE) of untransformed data are reported in text and figures.

3.3 Results and Discussions

3.3.1 Behavioral Response of *Lasioderma serricorne* to Host-Plant Materials at 0.2 g Dose

Thirty different host-plant materials were screened for behavioral response of *L. serricorne* by 2-choice pitfall assay at 0.2 g dose ($n = 9$). The results are shown in Table 3.2 and Figure 3.6.

Table 3.2 Response index (%) of *L. serricorne* to host-plant materials at 0.2 g dose*****

Categories	Host-plant materials	%Response Index (Mean ± SE), (*n* = 9)******
1. Spices	Paprika	48.89 ± 7.82 ^{efgh}
	Red chili	66.67 ± 14.14 ^{bc}
	Rosemary leaf	38.89 ± 10.54 ^{hi}
	Oregano	26.67 ± 7.07 ^{ijk}
	Cumin	38.89 ± 10.54 ^{hi}
	White pepper	47.78 ± 13.94 ^{efgh}
	Dries turmeric	30.00 ± 8.66 ^{ij}
	Cinnamon	-20.00 ± 8.66 ^{l}
	Anise	-15.56 ± 7.26 ^{l}
	Curry powder	37.78 ± 9.72 ^{hi}
2. Tobacco	Cured-tobacco leaf var. Turkish	72.22 ± 18.56 ^{b}
	Cured-tobacco leaf var. Virginia	55.56 ± 11.30 ^{cdefg}
	Cured-tobacco leaf var. Burley	46.67 ± 10.00 ^{efgh}
	Chewing tobacco cut	62.22 ± 10.93 ^{bcd}
3. Cereal grains	Wheat flour	38.89 ± 12.69 ^{hi}
	Rice flour	28.89 ± 15.37 ^{ijk}
	Glutinous rice flour	16.67 ± 7.07 ^{k}
	Cassava flour	22.22 ± 9.72 ^{jk}
	Corn flour	18.89 ± 7.82 ^{jk}
	Cracked whole wheat	38.89 ± 10.54 ^{hi}
4. Teas	Chinese oolong tea leaf	48.89 ± 16.16 ^{efgh}
	Commercial green tea	62.22 ± 15.63 ^{bcd}
	Commercial black tea	57.78 ± 13.94 ^{cdef}
	Mulberry leaf tea	91.11 ± 9.28 ^{a}
5. Miscellaneous	Ground cocoa bean	53.33 ± 8.66 ^{defg}
	Ground coffee bean	58.89 ± 16.16 ^{cde}
	Dark cocoa powder	63.33 ± 19.36 ^{bcd}
	Light cocoa powder	45.56 ± 7.26 ^{fgh}
	Lingzhi	67.78 ± 14.81 ^{bc}
	Brewer's yeast	44.44 ± 8.82 ^{gh}

*****Each datum represents the means of nine replicates, each set up with 10 adult beetles.
****** Means within a column followed by the same letter are not significantly different at $P = 0.05$ (Duncan's test). Mortalities were transformed to arcsine square root before ANOVA. Means (±SE) of untransformed data are reported.

Every host-plant materials were significantly different at $P < 0.05$ ($df = 30, 248$; $F = 34.662$) (Table 3.2). The results showed response index of all host-plant materials were widely range by -20.00–91.11% (Table 3.2). Seven host-plant materials (red chili, cured-tobacco leaf *var.* Turkish, chewing tobacco cut, commercial green tea, mulberry leaf tea, dark cocoa powder, and lingzhi) in four categories (spices, tobacco, teas, and miscellaneous except cereal grains) displayed high attractive response to *L. serricorne* (RI > 60%). Fifteen materials: paprika, rosemary leaf, cumin, white pepper, curry powder, cured-tobacco leaf *var.* Virginia, cured-tobacco leaf *var.* Burley, wheat flour, cracked whole wheat, Chinese oolong tea leaf, commercial black tea, ground cocoa bean, ground coffee bean, light cocoa powder, and brewer's yeast were in the medium group (RI = 31–60%) of response activity to female adults of *L. serricorne*. Oregano, dried turmeric, rice flour, glutinous rice flour, cassava flour, and corn flour were found to have low attractive activity (RI = 0–30%) to this beetle, while two spices; cinnamon and anise, exhibited the repellent activity with negative value (-20.00 ± 8.66 and -15.56 ± 7.26%, respectively).

Among 5 categories, teas had the highest mean at 65.00% RI and tended to be a strong attractants to this tobacco beetle, followed by tobacco and miscellaneous groups of 59.17 and 55.56%, respectively. On the other hand, cereal grains had the lowest mean value of 27.41% RI, followed by spices group (30.00% RI). This might be that cereal grains don't have suitable quality for *L. serricorne* to develop nor have less volatile organic compounds emitted from materials. In addition, materials in spices group tended to have repellent activity to *L. serricorne* because of their essential oil containing comparable with the study by Hori (2003).

Mulberry leaf tea had the highest response index in attractive activity to *L. serricorne* among thirty host-plant materials (91.11 ± 9.28%), followed by cured-tobacco *var.* Turkish (72.22 ± 18.56%) and lingzhi which is not significantly different ($P > 0.05$, $n = 9$) to red chili with RI 67.78 ± 14.81 and 66.67 ± 14.14%, respectively. This is the first report about efficacy of mulberry leaf tea and lingzhi in behavioral response of *L. serricorne*. Cured tobacco *var.* Turkish and red chili showed the high attractive responses to this insect similar to previous reports (Kohno *et al.*, 1983; Mahroof and Phillips, 2007, 2008). Different responding results of this beetle to their host plant might depend on cultivar or variety of plant, culturing practices, harvesting

conditions, and post-harvest processing, which are important to chemical constituents (Mahroof and Phillips, 2007). Differences in quantity and quality of chemical constituents in each plant material may directly influence the attractiveness of *L. serricorne*.

Seven host-plant materials with more than 60% of response index to *L. serricorne* were selected for further study in the lower dose at 0.02 g (Figure 3.6). All effective materials except tobacco products were another host food generally infested by this beetle, especially mulberry leaf tea. Phimphasali and Kaewruang (2007) reported that *L. serricorne* is the most serious stored insect pest of mulberry leaf tea in Thailand resulting in high cost for management.

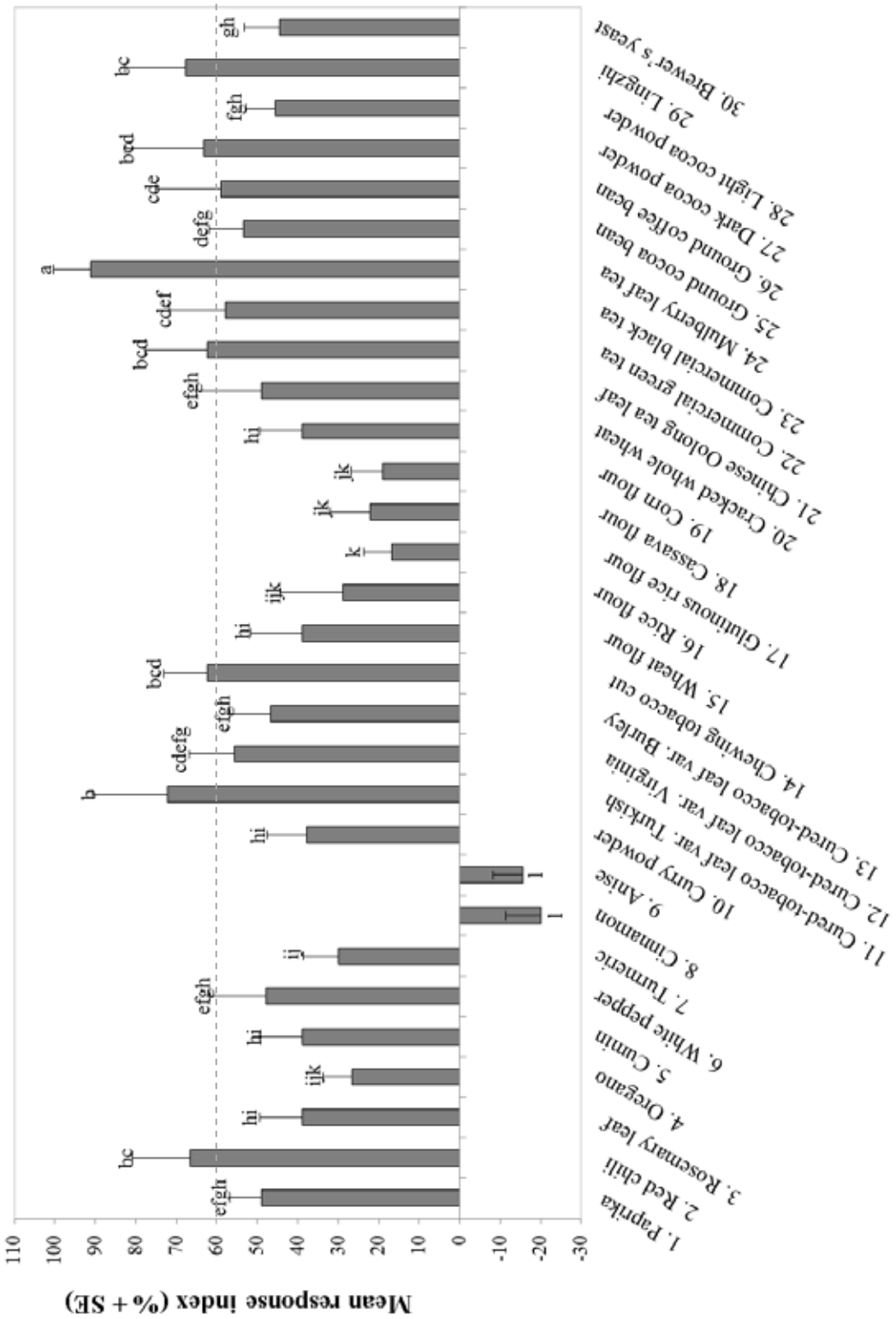


Figure 3.6 Response index (%) of *L. serricorne* towards various host-plant materials at 0.2 g dose.

3.3.2 Behavioral Response of *Lasioderma serricorne* to Selected Host-Plant Materials at 0.02 g Dose

Seven selected plant samples were next tested for the behavioral response of *L. serricorne* at the lower 0.02 g dose. The results show in Table 3.3 and Figure 3.7.

Table 3.3 Response index (%) of *L. serricorne* to selected host-plant materials at 0.02 g dose*

No.	Host-plant materials	%Response Index (Mean ± SE), (*n* = 9)**
1	Red chili	64.44 ± 19.44 [ab]
2	Cured-tobacco leaf *var.* Turkish	66.67 ± 15.00 [a]
3	Chewing tobacco cut	32.22 ± 10.93 [d]
4	Commercial green tea	42.22 ± 15.63 [cd]
5	Mulberry leaf tea	68.89 ± 12.69 [a]
6	Dark cocoa powder	50.00 ± 12.25 [c]
7	Lingzhi	52.22 ± 12.02 [bc]

*Each datum represents the means of nine replicates, each set up with 10 adult beetles.
** Means within a column followed by the same letter are not significantly different at $P = 0.05$ (Duncan's test). Mortalities were transformed to arcsine square root before ANOVA. Means (±SE) of untransformed data are reported.

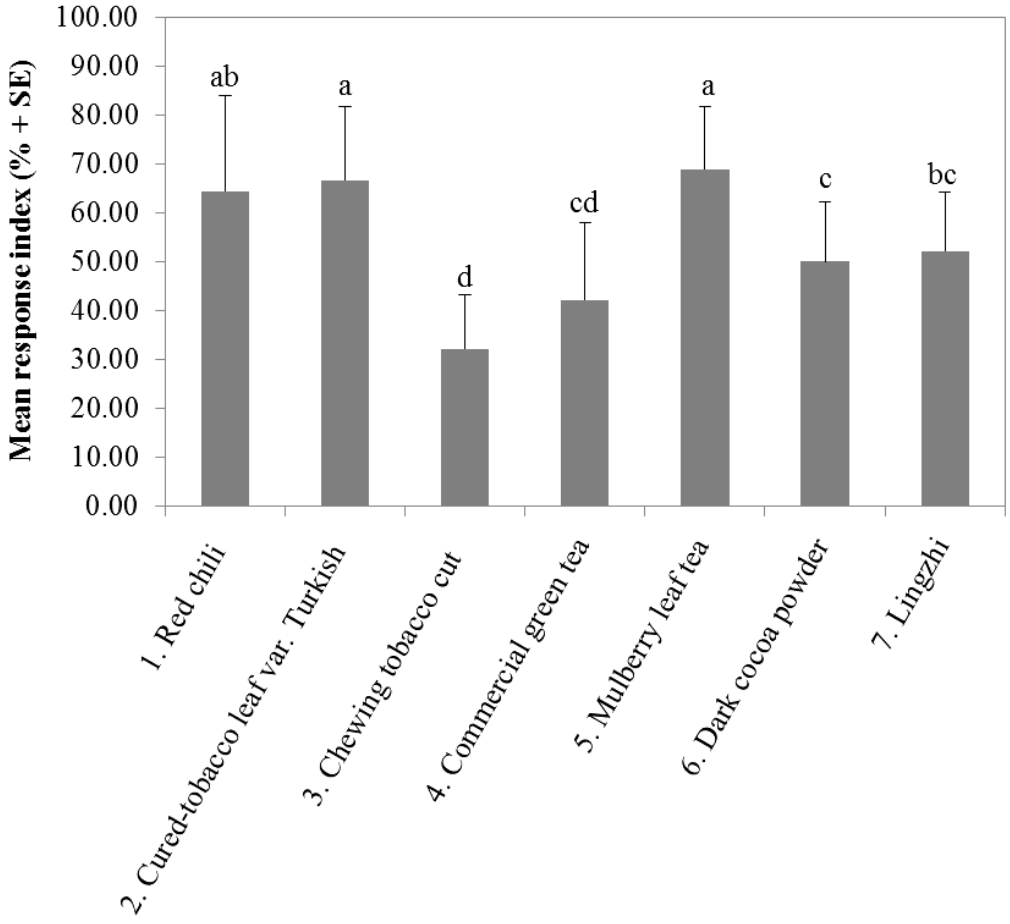


Figure 3.7 Response index (%) of *L. serricorne* towards various host-plant materials at 0.02 g dose.

Every host-plant materials were significantly different at $P < 0.05$ ($df = 11, 96$; $F = 8.623$) (Table 3.3). Among seven selected host-plants, mulberry leaf tea also showed the highest (68.89 ± 12.69%) attractive response to *L. serricorne* at lower dose of 0.02 g that not significantly different ($P > 0.05$) with cured-tobacco leaf *var.* Turkish (66.67 ± 15.00%). Red chili had high activity with RI more than 60% but lower than the two formers. Lingzhi, dark cocoa powder, commercial green tea, and chewing tobacco cut displayed moderately (31−60% RI) attractive response to this insect, as the lowest in chewing tobacco cut (32.22 ± 10.93%). *L. serricorne* had responding differences in each type of tobacco, even though tobacco is major host plant of them. This result was comparable with the study of Mahroof and Phillips (2007).

Due to the fact that mulberry leaf tea exhibited the best attractive activity to tobacco beetle at both higher and lower doses, it was interesting to further identify its volatile constituents that causing potential attractant activity.

3.3.3 Chemical Analysis

The effective mulberry leaf tea was then subjected for determining its volatile constituents by headspace-SPME-GC-MS technique.

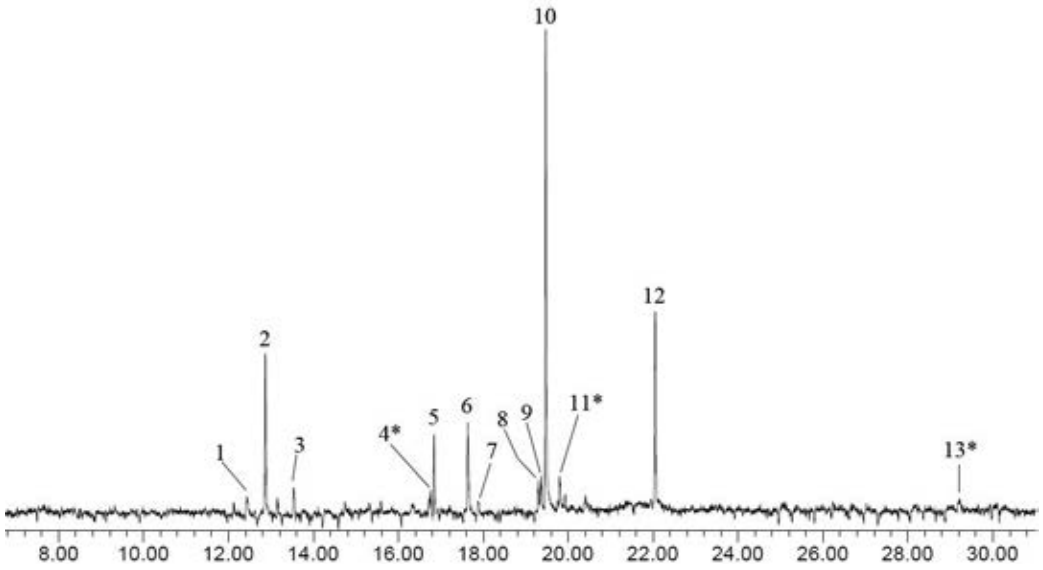


Figure 3.8 Gas chromatogram of volatile compounds emitted from mulberry leaf tea by headspace-SPME-GC-MS technique; * are unidentified compound.

The gas chromatogram indicated that thirteen volatile compounds can be detected (Figure 3.8). Volatile organic compounds were identified using the same criteria of mass spectral matching. The details of each compounds and composition percentages are displayed in Table 3.4.

Table 3.4 The volatile constituents of mulberry leaf tea by headspace-SPME-GC-MS analysis*

no	Compound name**	RT (min)	%Composition
1	α-Ionone	12.12	trace
2	β-Ionone	12.87	13.13
3	Dihydroactinidiolide	13.54	4.62
4	unidentified	16.76	trace
5	6,10,14-Trimethyl-2-pentadecanone	16.84	5.74
6	Methyl palmitate	17.64	10.03
7	Isophytol	17.88	trace
8	Methyl linoleate	19.30	4.45
9	Methyl linolenate	19.37	4.80
10	Phytol	19.47	37.59
11	unidentified	19.79	2.84
12	*Bis*-(2-ethylhexyl) hexanedioate	22.05	16.80
13	unidentified	29.20	trace

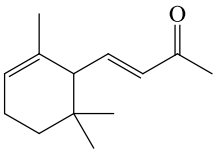
* Mass spectrum of each identified compound was showed in Appendix.

**The unidentified compounds had less than 70% in matching of their mass spectra with reference compounds in the available mass spectra library.

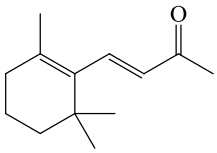
Mass analysis of volatiles emitted from mulberry leaf tea revealed that phytol is a major constituent with the highest composition of 37.59% following by *bis*-(2-ethylhexyl) hexanedioate, β-ionone, and methyl palmitate with composition of 16.80, 13.13, and 10.03%, respectively. Minor components of this tea were 6,10,14-trimethyl-2-pentadecanone, methyl linolenate, dihydroactinidiolide, and methyl linoleate with less than 10% composition. It was also found the trace amount of α-ionone and isophytol emitted from this plant sample. This main constituent could comparable with the only one previous report of Chumpolsri *et al.* (2007). They analyzed the aroma active components of mulberry leaf tea products by SPME-GC-MS technique. Phytol was the major component similar to this study; nonetheless, with difference in minor components.

Phytol, acyclic diterpene alcohol, is generally found in green tea more than oolong (semi-fermented) and black (fermented) teas (van den Brink and Wanders, 2006; Jumtee *et al.*, 2011; Pripdeevech and Machan, 2011). Mulberry leaf tea is a non-fermented tea or green tea, so that phytol was detected from aroma analysis of this mulberry leaf tea. *Bis*-(2-ethylhexyl) hexanedioate is a second major component found in this tea; however, there was no evidence of this compound as natural occurring substance. *Bis*-(2-ethylhexyl) hexanedioate or di-(2-ethylhexyl) adipate, a low volatile liquid, is a plasticizer in the flexible vinyl industry and widely use in flexible poly (vinyl chloride) (PVC), food film, and also important in processing of nitrocellulose and synthetic rubber, in plasticizing polyvinyl butyral, cellulose acetate butyrate, polystyrene and dammar wax and in cosmetics (cellulose-based liquid lipsticks) (IARC, 2000). These were the reasons supporting that this compound might be contaminated to this tea during some processes. β-Ionone was found as a constituent in general tea aroma with trace amount of α-ionone (Cloughley *et al.*, 1982; Shimoda *et al.*, 1995; Chaturvedula and Prakash, 2011; Jumtee *et al.*, 2011) and also found as a major volatile flavor component in flue-cured and burley tobacco (Lee *et al.*, 2010). Methyl palmitate was reported as a derivative of palmitic acid occurring in cut tobacco leave (Huang *et al.*, 2007). 6,10,14-Trimethyl-2-pentadecanone was not found in any host-plant of tobacco beetle, but found mainly in essential oil of *Minuartia meyeri* (Yayli *et al.*, 2006), *Stachys persica* and *S. byzantina* (Khanavi *et al.*, 2004), and *Centaurea sessilis* and *C. armena* (Yayli *et al.*, 2005). Methyl linoleate and methyl linolenate were two volatile compounds commonly found in various parts of plants (Kawasaki *et al.*, 1998; Tellez *et al.*, 2002; Pino *et al.*, 2005; Mastelic *et al.*, 2006). Dihydroactinidiolide is a volatile terpene, which has a sweet, tea-like odor and is used as fragrance. It occurs naturally in both animals and plants (Binder *et al.*, 1990; Buttery *et al.*, 1990; Pino *et al.*, 2005) and additionally found in tobacco (Leffingwell and Alford, 2005; Huang *et al.*, 2007) and also in essential oil from silkworm-mulberry, *Morus rotunbiloba* Koidz (Patharakorn *et al.*, 2010). Isophytol, trace amount in this study, was found to be a volatile compound composing in *Jasminum sambac* (Kaiser, 1988) and *Osmanthus fragrans* tea (Chun *et al.*, 2010).

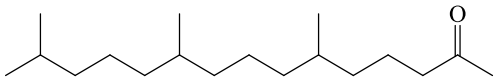
(a) Ketone group



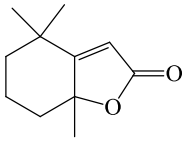
α-Ionone



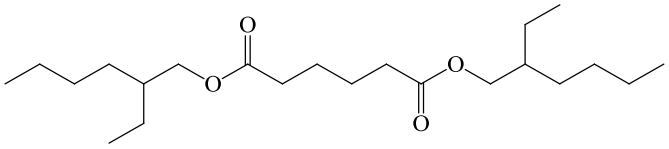
β-Ionone



6,10,14-Trimethyl-2-pentadecanone

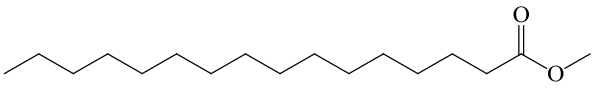


Dihydroactinidiolide

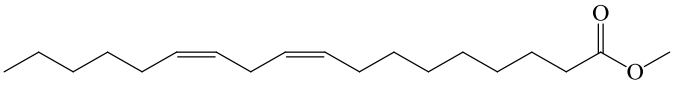


Bis-(2-ethylhexyl) hexanedioate

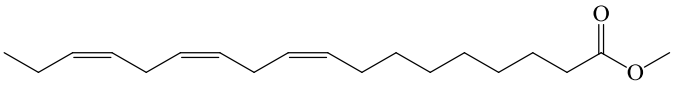
(b) Ester group



Methyl palmitate

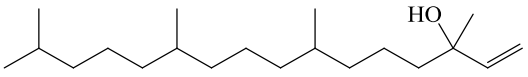


Methyl linoleate

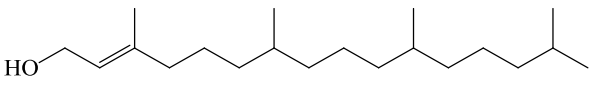


Methyl linolenate

(c) Alcohol group



Isophytol



Phytol

Figure 3.9 Chemical structures of volatile constituents from mulberry leaf tea classified by functional groups.

The compositions in mulberry leaf tea could be classified into three groups including 5 ketones, 3 esters, and 2 alcohols (Figure 3.9). The major group of identified volatile compounds was ketones. It is different to previous report about Thai mulberry leaf tea that hydrocarbons are a major group, following by ketones (Chumpolsri *et al.*, 2007). This might be from several factors, such as stored duration, genotype, plant origin, and tea processes (Pripdeevech and Machan, 2011).

3.3.4 Behavioral Response of *Lasioderma serricorne* to Major Volatile Constituents of Mulberry Leaf Tea

Three major volatile constituents of mulberry leaf tea (> 10.00%composition), such as phytol (37.59%), β-ionone (13.13%), and methyl palmitate (10.03%), were subjected to determine the response activity of *L. serricorne* at various doses. Due to the fact that *bis*-(2-ethylhexyl) hexanedioate was considerably as a contaminant although it revealed high %composition in this tea, thus it was not included in this study.

Table 3.5 Behavioral response of *L. serricorne* to volatile constituents of mulberry leaf tea*****

No.	Substances	%response index (mean ± SE), n = 9 at dose (mg)**			
		1	**0.1**	**0.01**	**0.001**
1	Phytol	61.11 ± 16.16^{a}	63.33 ± 19.36^{a}	45.56 ± 20.68^{a}	30.00 ± 13.23^{b}
2	β-Ionone	57.78 ± 13.94^{a}	64.44 ± 15.90^{a}	60.00 ± 14.14^{a}	60.00 ± 17.32^{a}
3	Methyl palmitate	26.67 ± 10.00^{b}	15.56 ± 13.33^{b}	8.89 ± 14.53^{b}	8.89 ± 12.69^{c}
4	Kairomone lure	60.00 ± 10.00^{a}	56.67 ± 13.23^{a}	50.00 ± 11.18^{a}	47.78 ± 12.02^{a}

*****Each datum represents the means of nine replicates, each set up with 10 adult beetles.

****** Comparison of means within a column of each dose followed by the same letter are not significantly different at P = 0.05 (Duncan's test). Mortalities were transformed to arcsine square root before ANOVA. Means (±SE) of untransformed data are reported.

From Table 3.5, all three main constituents of mulberry leaf tea displayed attractive activity to *L. serricorne,* including commercial kairomone lure. Phytol, β-ionone, and kairomone lure showed no significant difference ($P > 0.05$) of high response index at most of dose values, while methyl palmitate had the lowest response index among tested substances in all doses significantly ($P < 0.05$). At the lowest dose, 0.001 mg, β-ionone exhibited the highest response index (60.00 ± 17.32%) but not significantly different to kairomone lure ($P > 0.05$). It showed response index value more than phytol which is the major component of mulberry leaf tea. This revealed that the high attractive activity of mulberry leaf tea to *L. serricorne* was not derived from a major component. β-ionone was reported as major component in some tobacco products (Lee *et al*., 2010), the major host plant of this beetle, so that might be the result of high preference of *L. serricorne* to this compound which has not been reported as attractant to this insect.

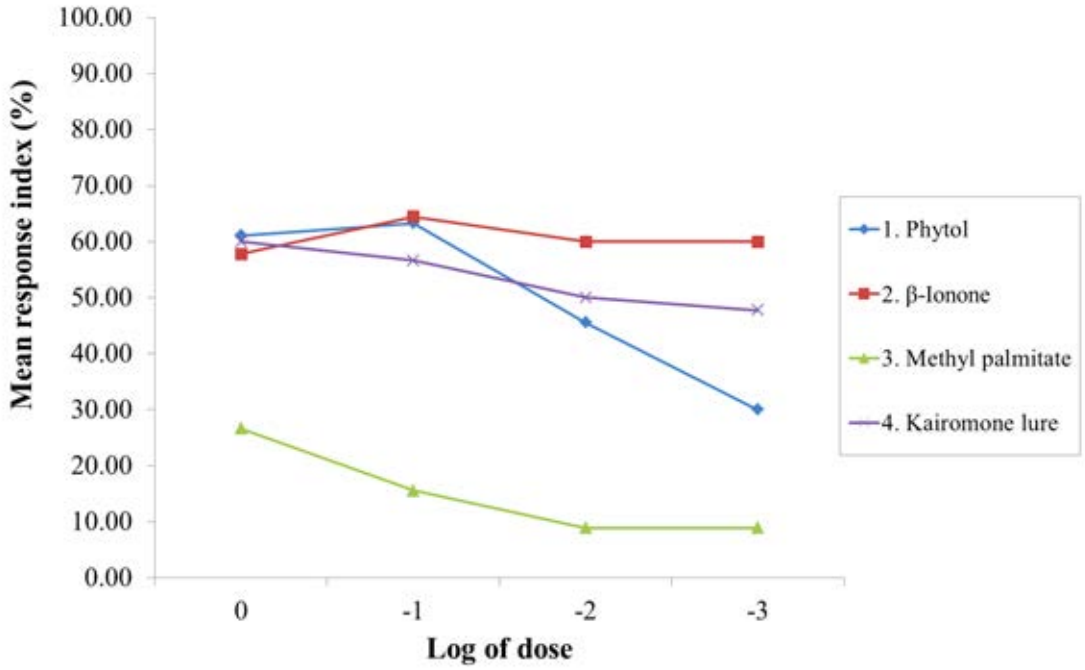


Figure 3.10 Behavioral response of *L. serricorne* to 3 main volatile constituents of mulberry leaf tea and kairomone lure at dose; 1.0, 0.1, 0.01, and 0.001 mg (as log of dose; 0, -1, -2, and -3, respectively).

The dose-responses of *L. serricorne* to main volatile constituents of mulberry leaf tea (Figure 3.10) showed different thresholds. Phytol and β-ionone showed the same dose threshold at 0.1 mg with high attractive response. Kairomone lure had threshold at 1 mg dose similar to methyl palmitate, with higher response activity than methyl palmitate.

The high potential activity of β-ionone in attractive response of *L. serricorne* was more than the commercial kairomone lure which might be a good sign in using these compounds for alternative management against this beetle.

3.3.5 Behavioral Response of *Lasioderma serricorne* to Synergism of Phytol and β-Ionone

Due to the fact that phytol and β-ionone exhibited high level of attractive activity to this beetle, so synergistic of these two compounds were determined.

Table 3.6 Behavioral response of *L. serricorne* to various ratio of mixture between β-ionone : phytol in a dose of 0.001 mmole*

β-ionone : phytol	%response index (mean ± SE), n = 9**
0 : 10	26.67±11.18^{c}
1 : 9	28.89±12.69^{c}
3 : 7	35.56±11.30^{bc}
5 : 5	34.44±20.07^{bc}
7 : 3	41.11±12.69^{bc}
9 : 1	48.89±16.91^{b}
10 : 0	67.78±13.94^{a}

*Each datum represents the means of nine replicates, each set up with 10 adult beetles.
** Means within a column followed by the same letter are not significantly different at P = 0.05 (Duncan's test). Mortalities were transformed to arcsine square root before ANOVA. Means (±SE) of untransformed data are reported.

The result indicated that a mixture of β-ionone and phytol had an effective activity less than individual β-ionone (10:0, β-ionone:phytol). They did not have synergism to each other. Only β-ionone revealed the highest attractive activity (67.78 ± 13.94%) to *L. serricorne* among other mixtures and individual phytol significantly ($P < 0.05$). The attractant activity of mixtures tended to be increase depended on the increasing amount of β-ionone. This study revealed for the future application of these components in using of individual β-ionone as a promising kairomone lure in tobacco beetle monitoring.

3.4 Conclusions

The effectiveness in use of kairomone lure combining with the pheromone lure for *Lasioderma serricorne* has been different in each country. The development of kairomone lure is necessary. This study aims to find the effective food attractants for using as kairomone lure in monitoring the population of tobacco beetle. Mulberry leaf tea showed the highest attractive activity to *L. serricorne* among 30 host-plant materials. The headspace-SPME-GC-MS analysis of volatile constituents appeared 13 compounds. Phytol, β-ionone and methyl palmitate were the main volatile constituents. β-Ionone exhibited the highest attractive activity to *L. serricorne*, with 60% response index at 0.001 mg dose, while phytol, the major component, showed low attractive activity. However, β-ionone did not show significantly different to commercial kairomone lure. The combination of phytol and β-ionone did not show the synergistic activity. The high efficacy of individual β-ionone might be the promising kairomone lure used for monitoring the population of tobacco beetle in product store that suitable at least in Thailand.

CHAPTER IV

CONCLUSIONS

Thailand is an agricultural country. Nowadays, agriculture in Thailand has tended to be organic farm. Thus, biological strategies have become a significantly promising alternative for Thai farmers *via* IPM. A biological strategy for insect attractants plays an important role in protection of stored products from insect infestation and using for increasing crop production. Attractants, a kind of odorants, may consist of pheromones, natural food lures, and oviposition lures.

Pheromones and plant-derived volatiles are the effective odorants for insect attraction using in both pre- and post-harvest managements. The use of trail pheromone of stingless bee in pre-harvest crop may effective for their pollination in crop field, while plant-derived volatiles may help to attract storage insect pest into trap when combined with their sex pheromones.

This study aims to analyze the main constituents of pheromone from labial gland of a stingless bee, *Tetragonula pagdeni*, a common species of Thailand, for pre-harvest use, and to find plant-derived volatiles from food-host materials that act as kairomone attractant to *Lasioderma serricorne*, an important insect pest, in use for post-harvest products.

The first part of this study revealed that there were 6 hydrocarbons containing in the labial gland extract of *T. pagdeni*, mainly as pentatriacontane, a long straight chain hydrocarbon. Among them, three unidentified compounds appeared in scent-marked filter paper extract on which the stingless bees rub. One of them displayed the trail-following attractant to this stingless bee species. This compound was a main component in both labial gland and scent-marked filter paper extracts. This possible trail scent was the compound of peak no. **2** that might be the short-chain unsaturated hydrocarbon.

The other study, mulberry leaf tea exhibited the highest attractive activity to *L. serricorne* among 30 plants tested. Thirteen volatile compounds containing in this tea were analyzed by headspace-SPME-GC-MS method. Phytol, β-ionone and methyl palmitate were main volatile constituents. β-Ionone exhibited the highest attractive

activity to *L. serricorne*, with 60% response index at 0.001 mg dose, while phytol, the major component, showed low attractive activity. However, β-ionone did not show significantly different to commercial kairomone lure. The combination of phytol and β-ionone did not show the synergistic activity.

This research is the first report in Thailand on the use of volatile odorants from mulberry lea tea to attract *L. serricorne*, and the odorous compound from labial gland as trail pheromone for attracting *T. pagdeni* to food source. These might be the promising alternative tools for pre- and post-harvest managements that suitable at least in Thailand.

4.1 Proposal for Future Work

This research was the pioneer work in studying about trail-following substances of a stingless bee in Thailand. The use of odorants to promote the trail-following activity of stingless bees in crop field pollination should have many supporting studies. General biology, species variation, and their communication may have influence to constituents of labial gland extracts and their behavior. Further study should be conducted in the area without the wind, for example in green house, aims for stable in volatility of substances. The integrated characterization techniques should be used for identify the active substances in small amount of labial gland extracts. And commercial compounds should be confirmed.

For the study of tobacco beetle, the development of potential traps for management this storage insect pest is less in Thailand. Research in attractant trap for use in domestic might be reducing cost. Food preference of this beetle is varied. This study found the new effective food attractants that might be specific to tobacco beetle occurring in Thailand. Further study should involve the test for the effectiveness in combining of this kairomone substance with sex pheromone of tobacco beetle. Other components of trap such as control release materials and the adhesive plate should be study.

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APPENDIX

Figures A1-A3 were mass spectra of the 3 identified constituents of labial gland extract from *Tetragonula pagdeni*.

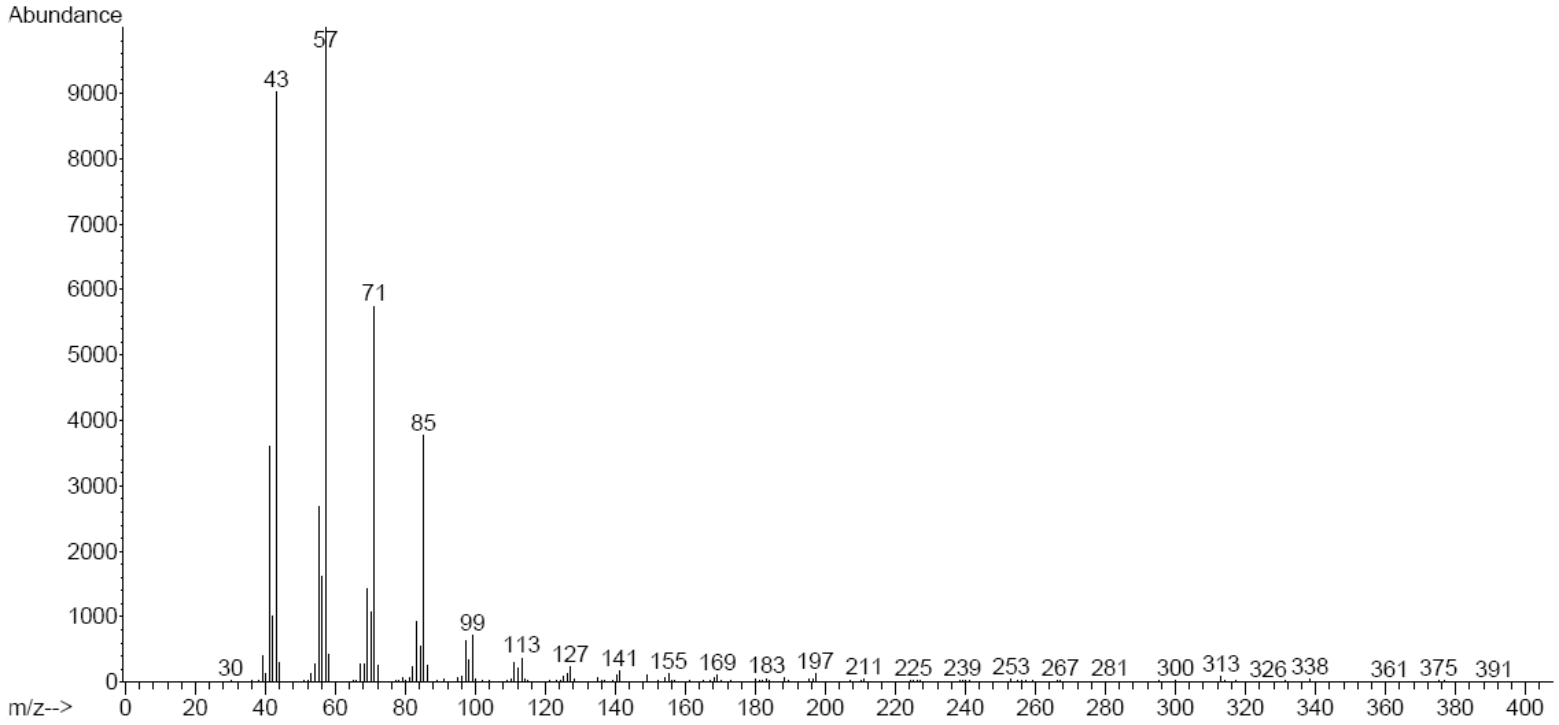

Figure A1 Mass spectrum of tetracosane composing in labial gland extract of *T. pagdeni*.

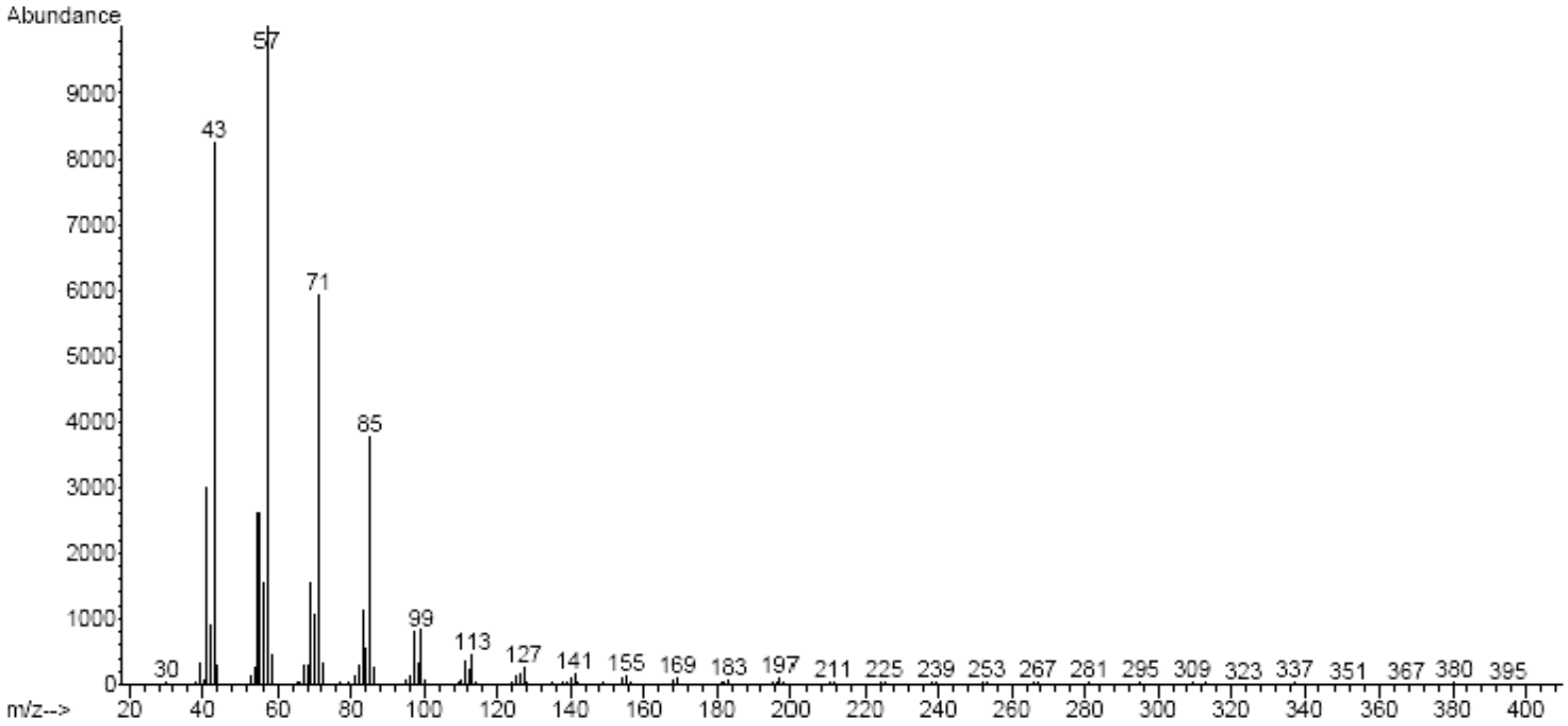

Figure A2 Mass spectrum of pentatriacontane composing in labial gland extract of *T. pagdeni*.

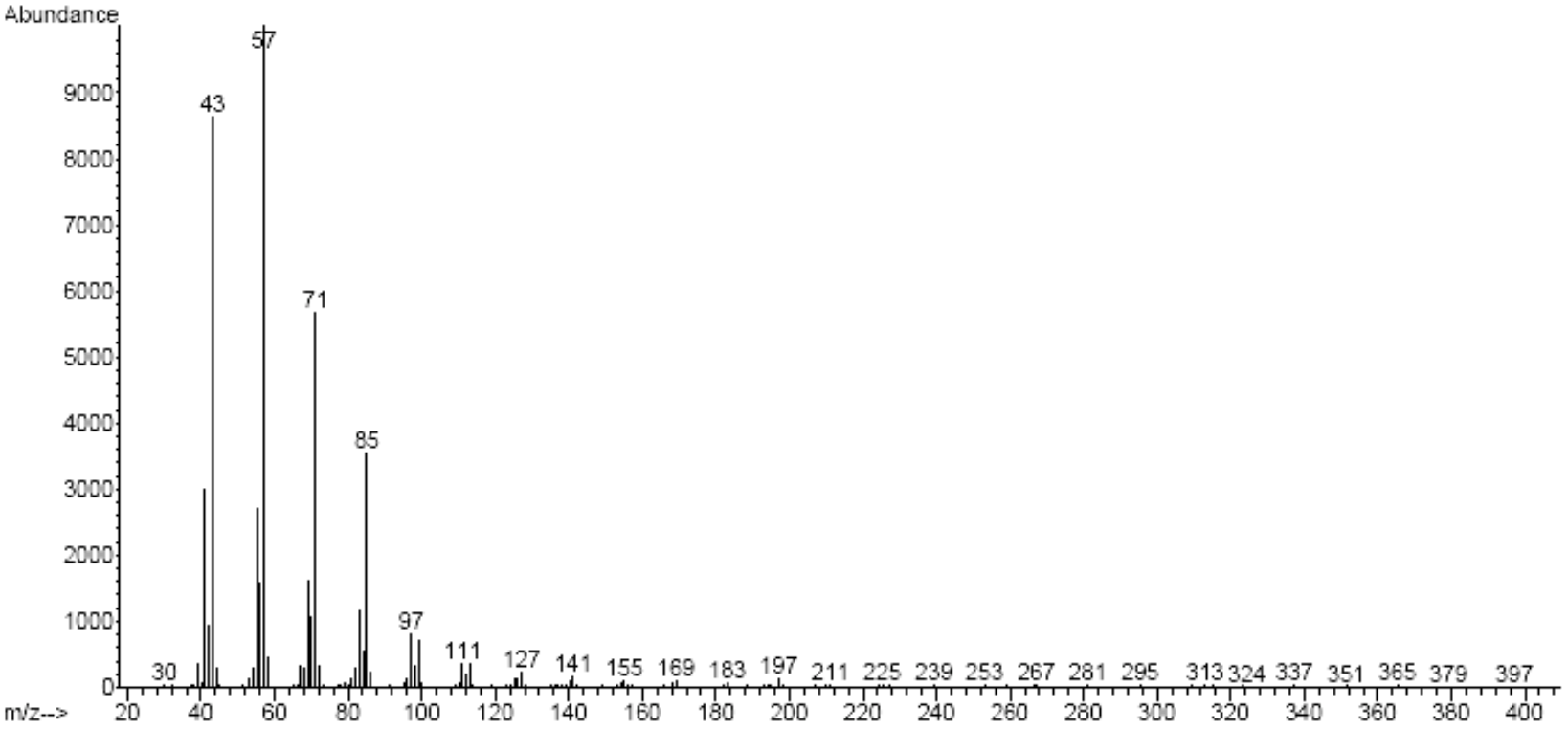

Figure A3 Mass spectrum of hexatriacontane composing in labial gland extract of *T. pagdeni*.

Figures A4-A13 were mass spectrum of the 10 analyzed constituents of mulberry leaf tea by headspace-SPME-GC-MS technique

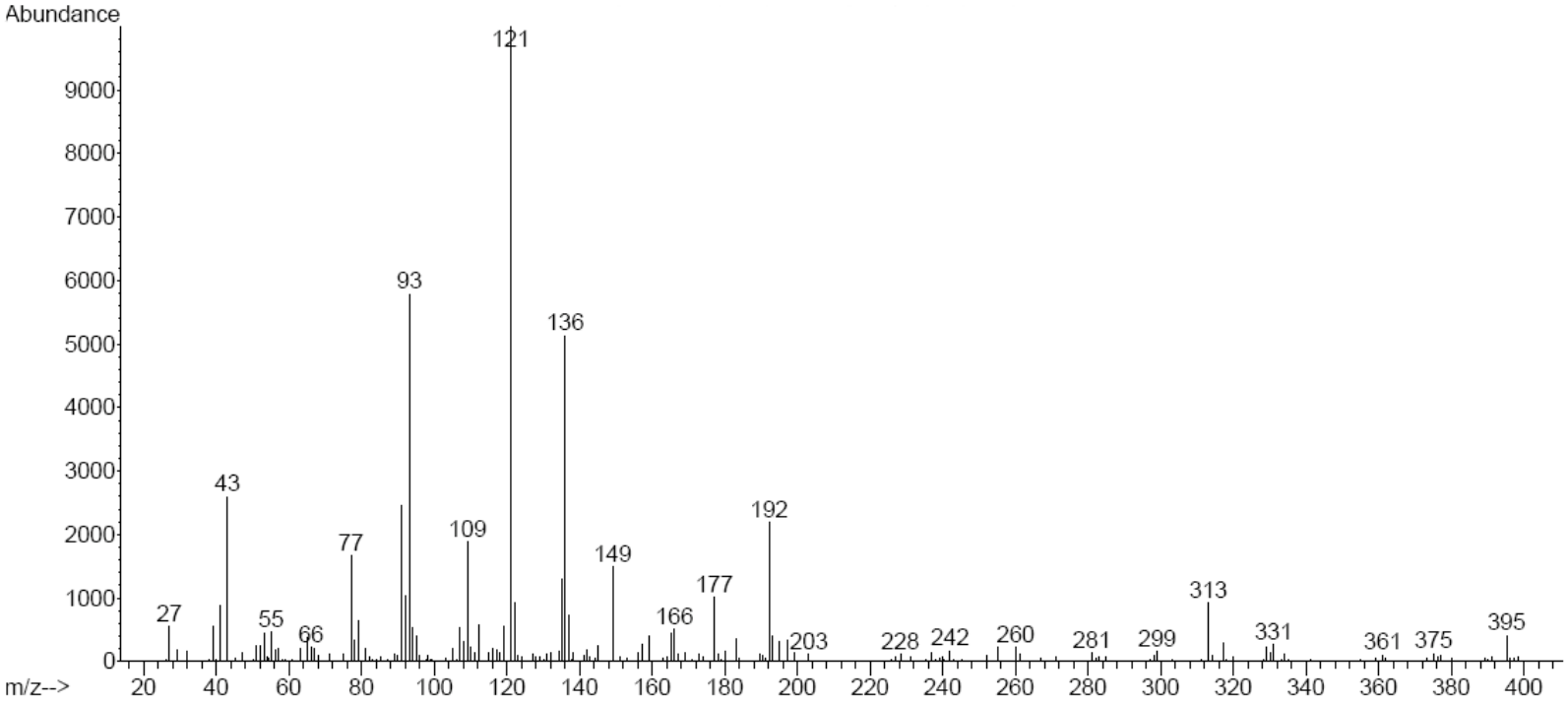

Figure A4 Mass spectrum of α-ionone composing in volatile compound of mulberry leaf tea.

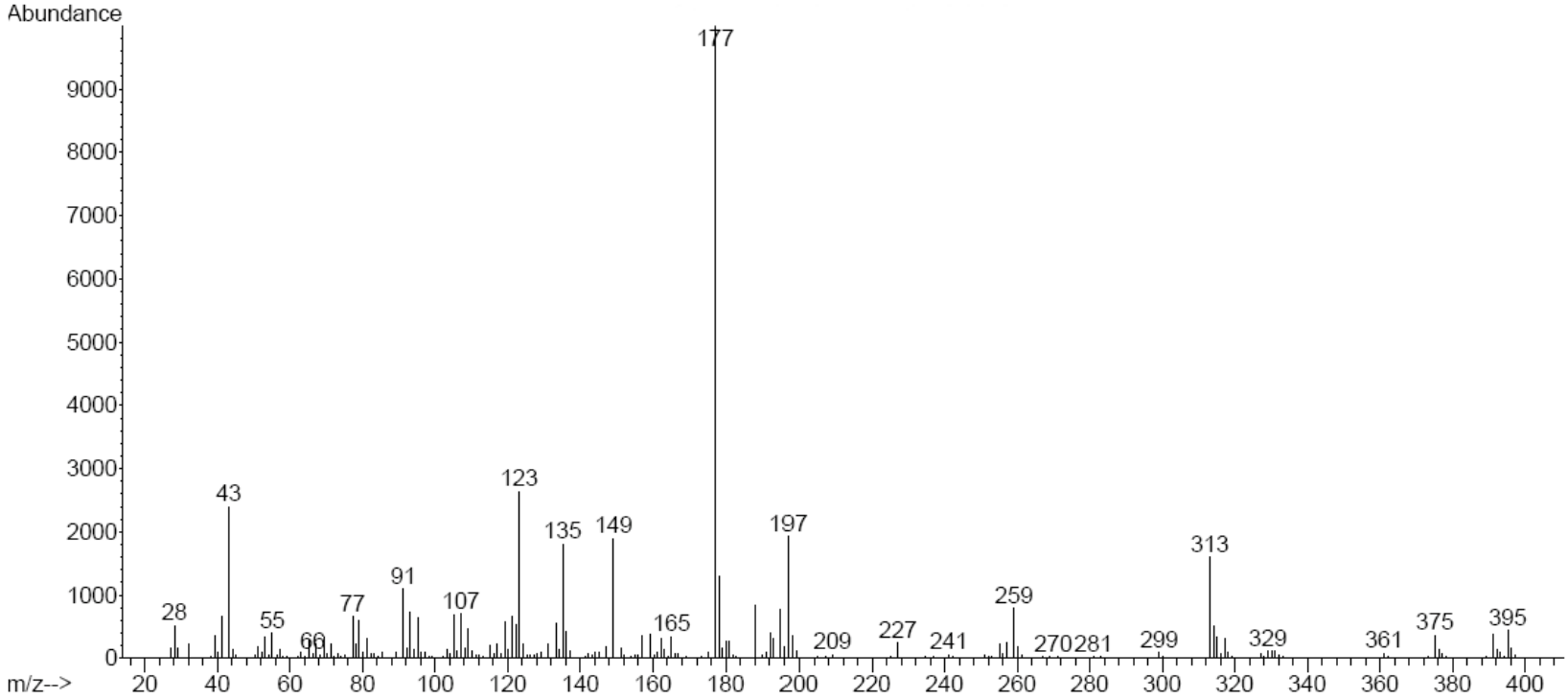

Figure A5 Mass spectrum of β-ionone composing in volatile compound of mulberry leaf tea.

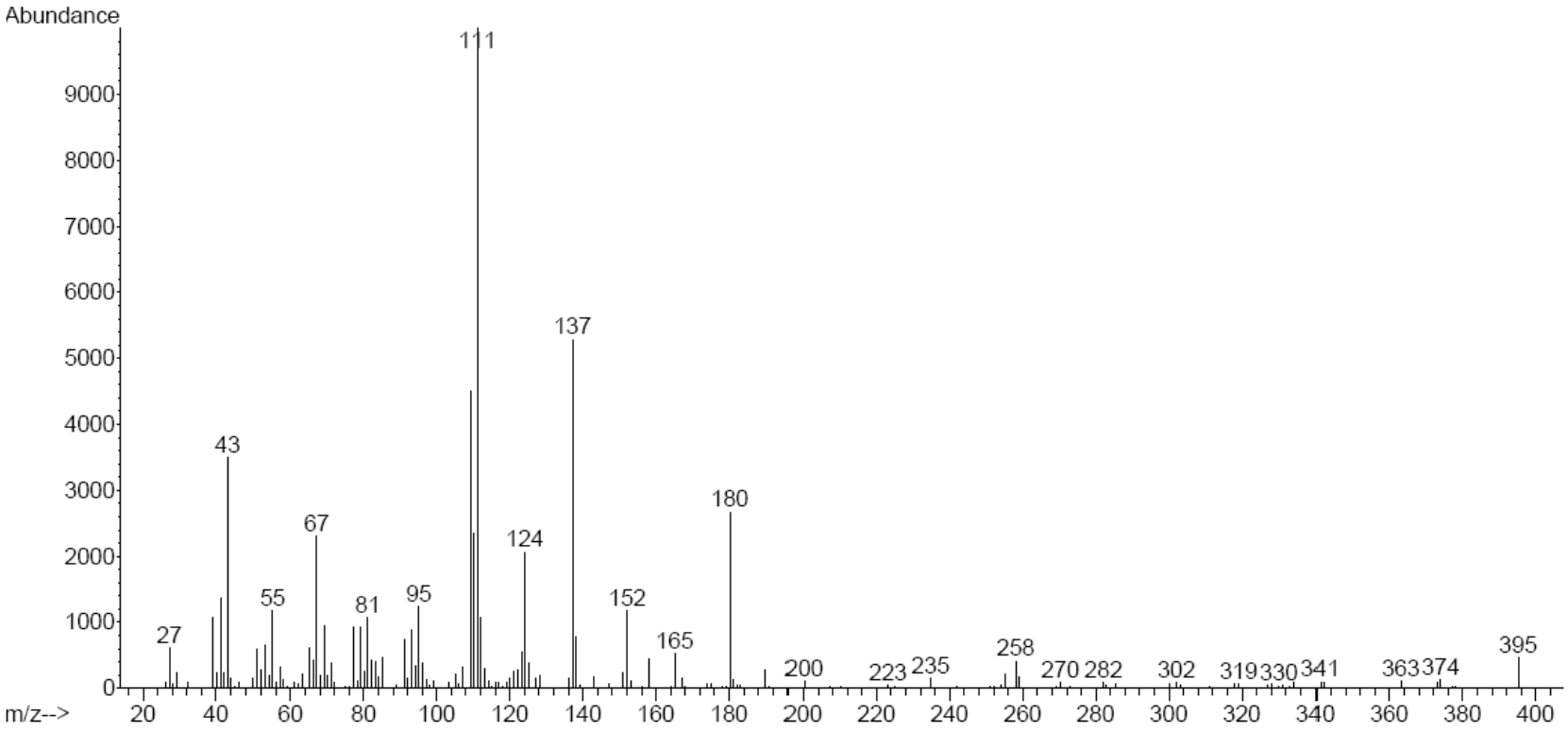

Figure A6 Mass spectrum of dihydroactinidiolide composing in volatile compound of mulberry leaf tea.

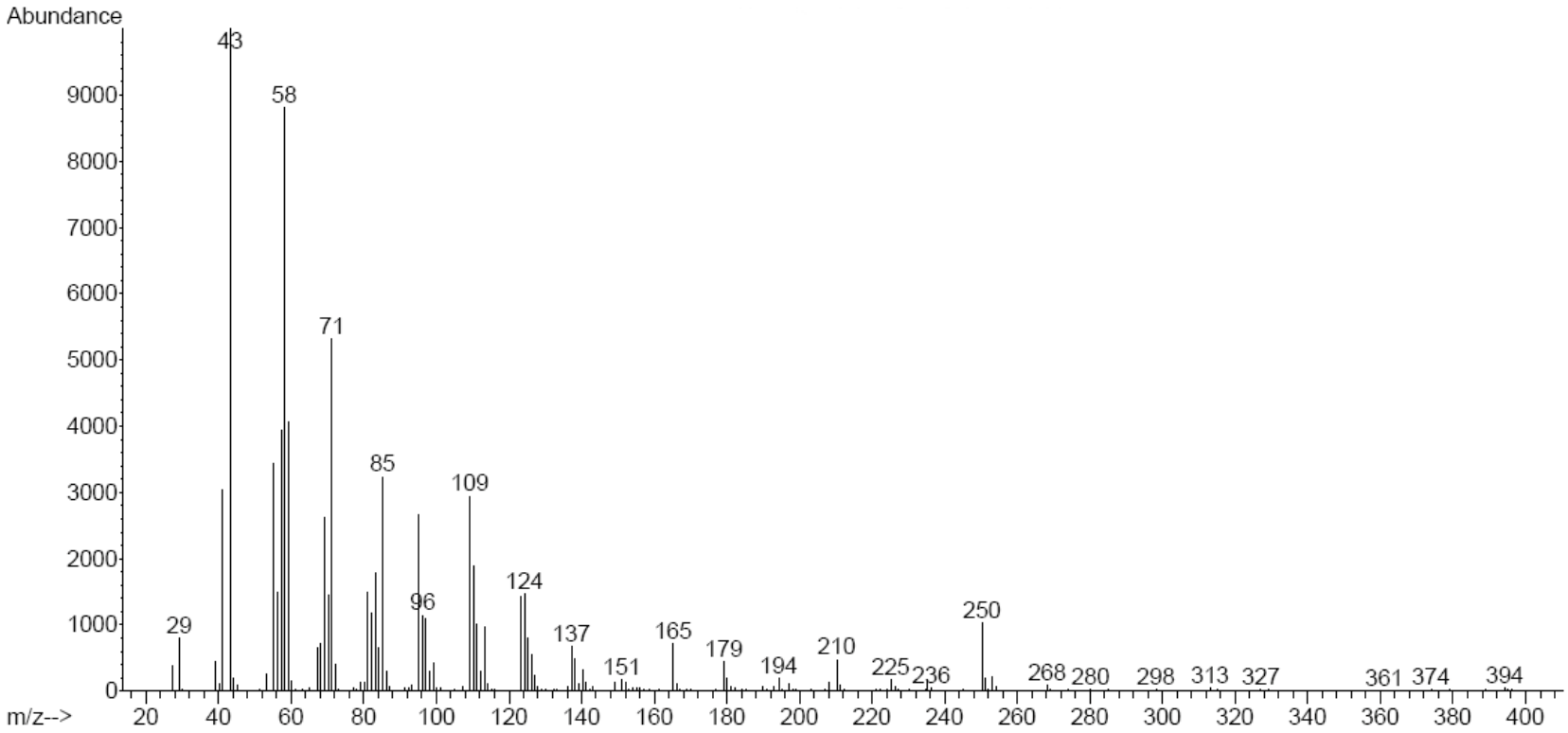

Figure A7 Mass spectrum of 6,10,14-trimethyl-2-Pentadecanone composing in volatile compound of mulberry leaf tea.

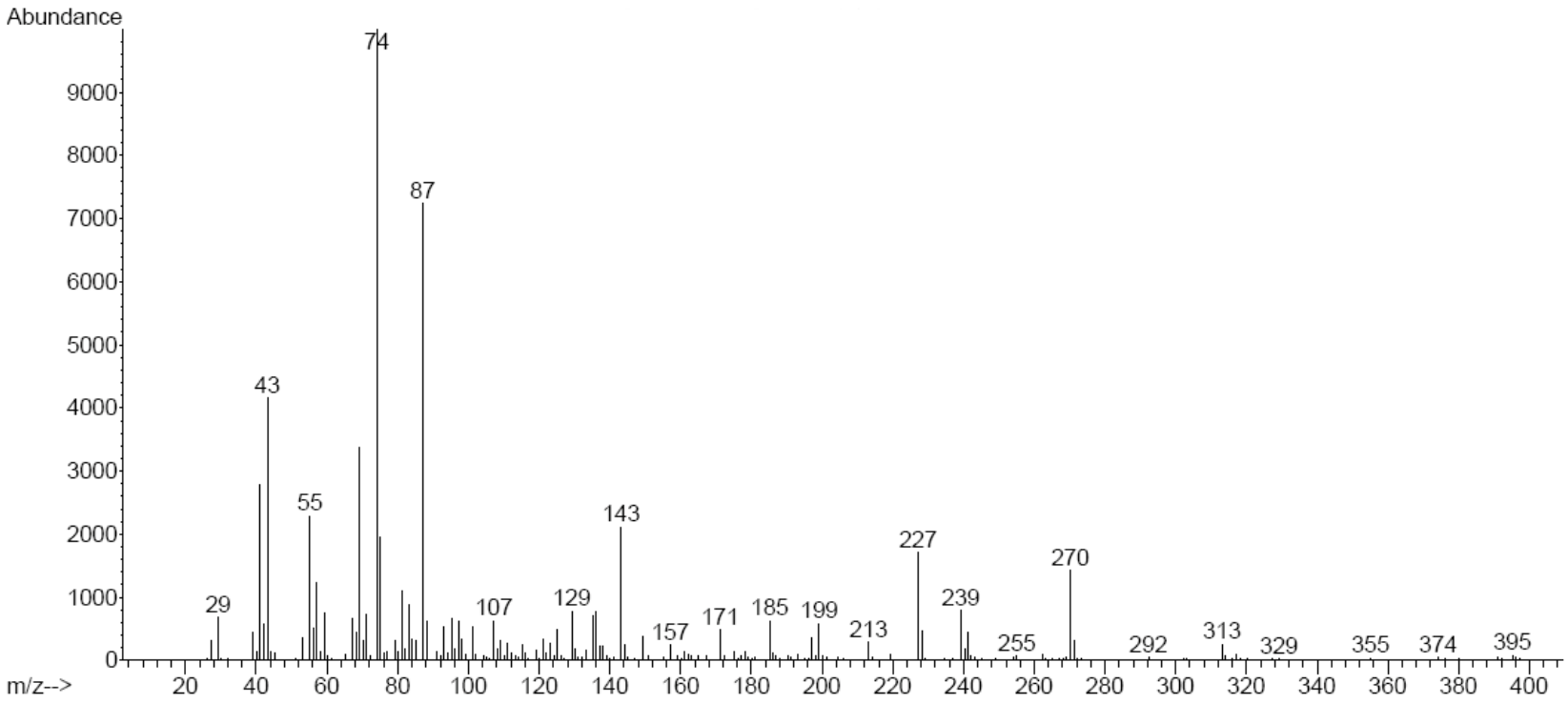

Figure A8 Mass spectrum of methyl palmitate composing in volatile compound of mulberry leaf tea**.**

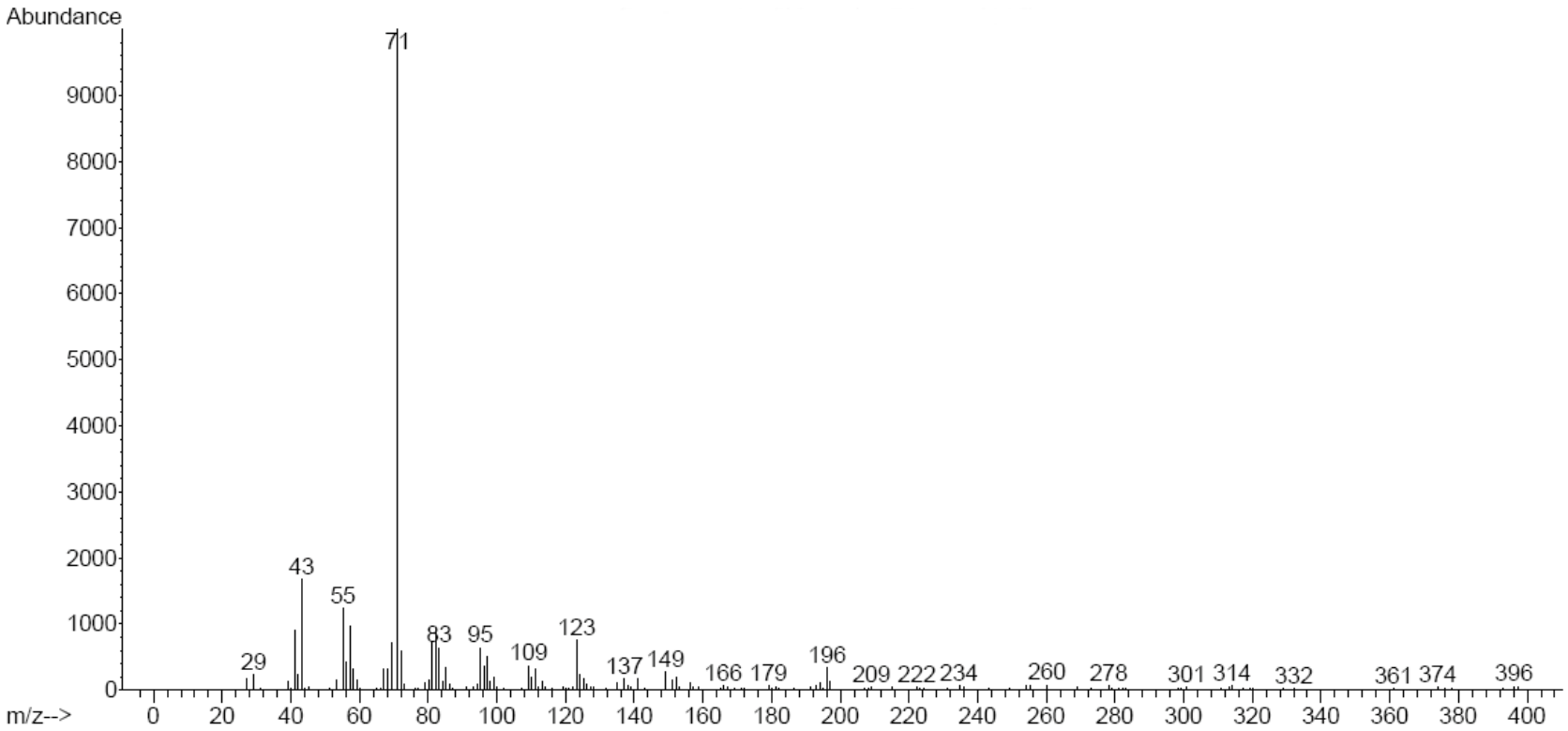

Figure A9 Mass spectrum of isophytol composing in volatile compound of mulberry leaf tea**.**

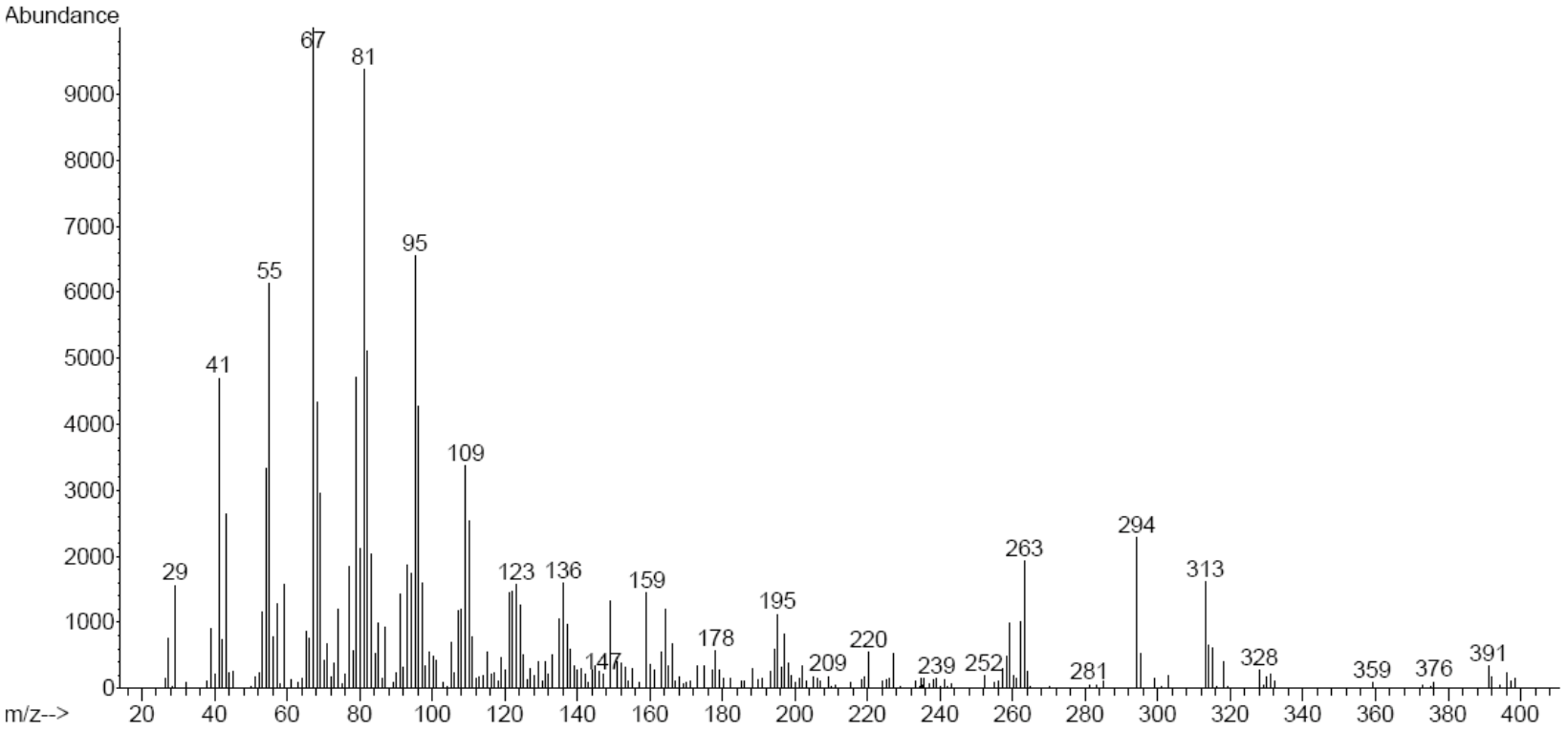

Figure A10 Mass spectrum of methyl linoleate composing in volatile compound of mulberry leaf tea**.**

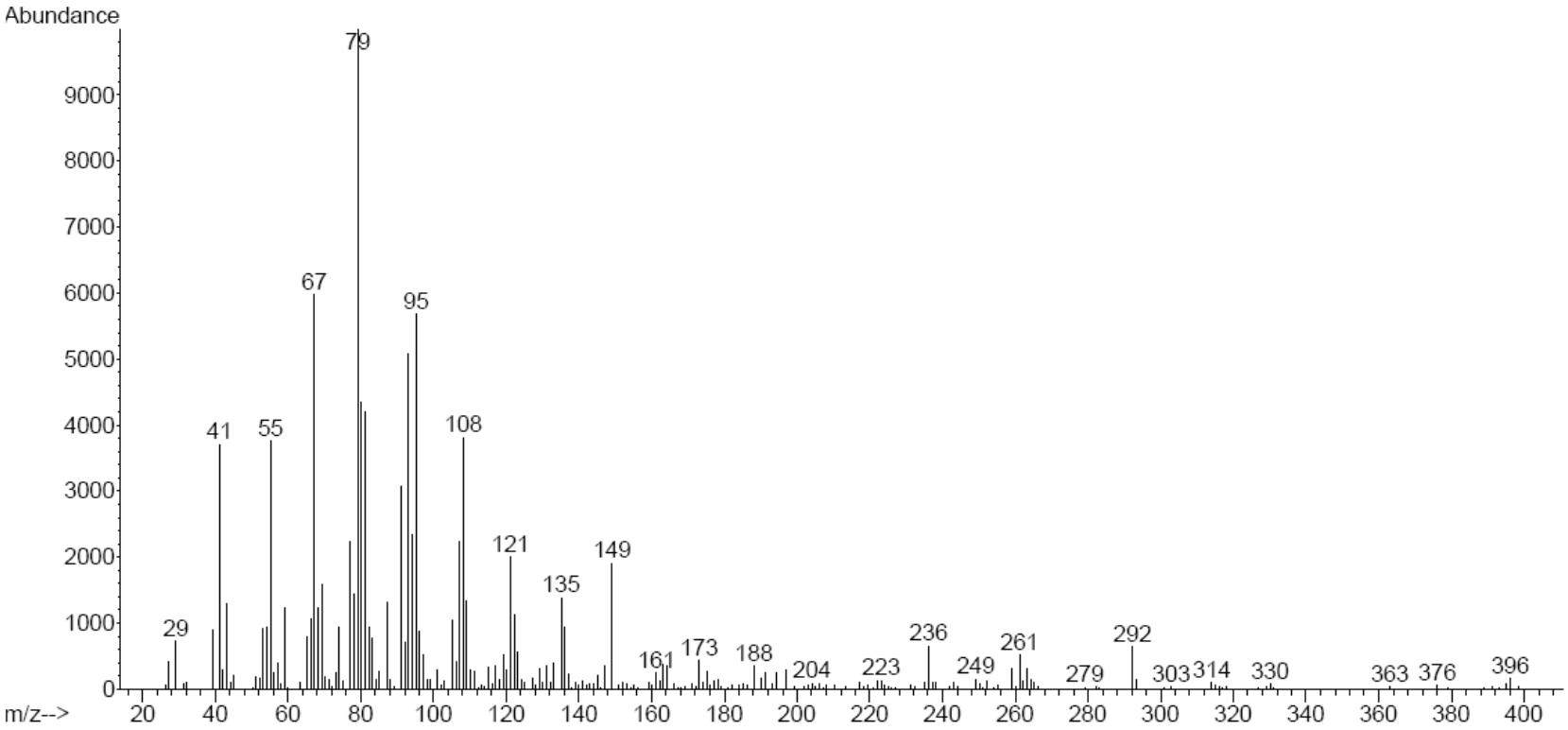

Figure A11 Mass spectrum of methyl linolenate composing in volatile compound of mulberry leaf tea.

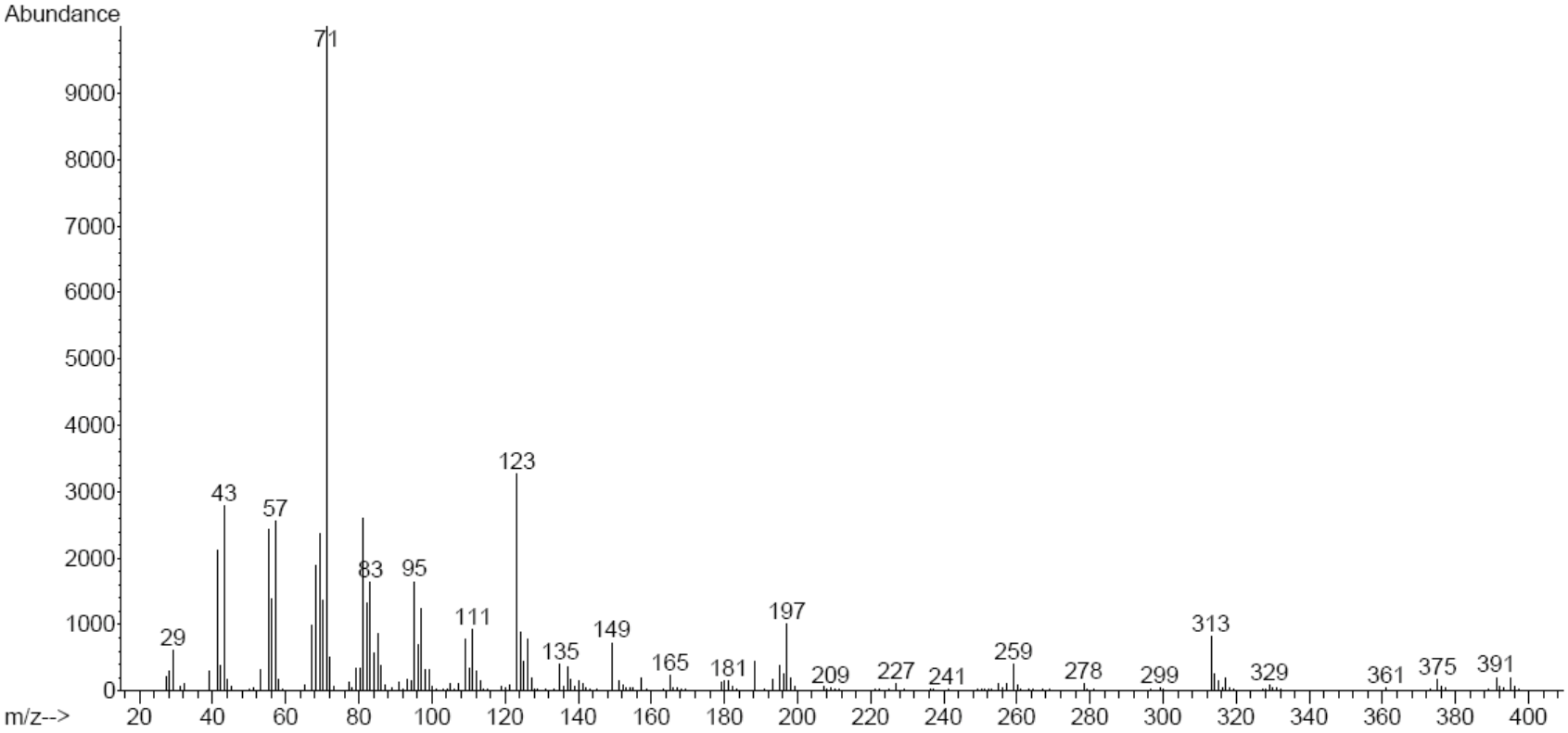

Figure A12 Mass spectrum of phytol composing in volatile compound of mulberry leaf tea**.**

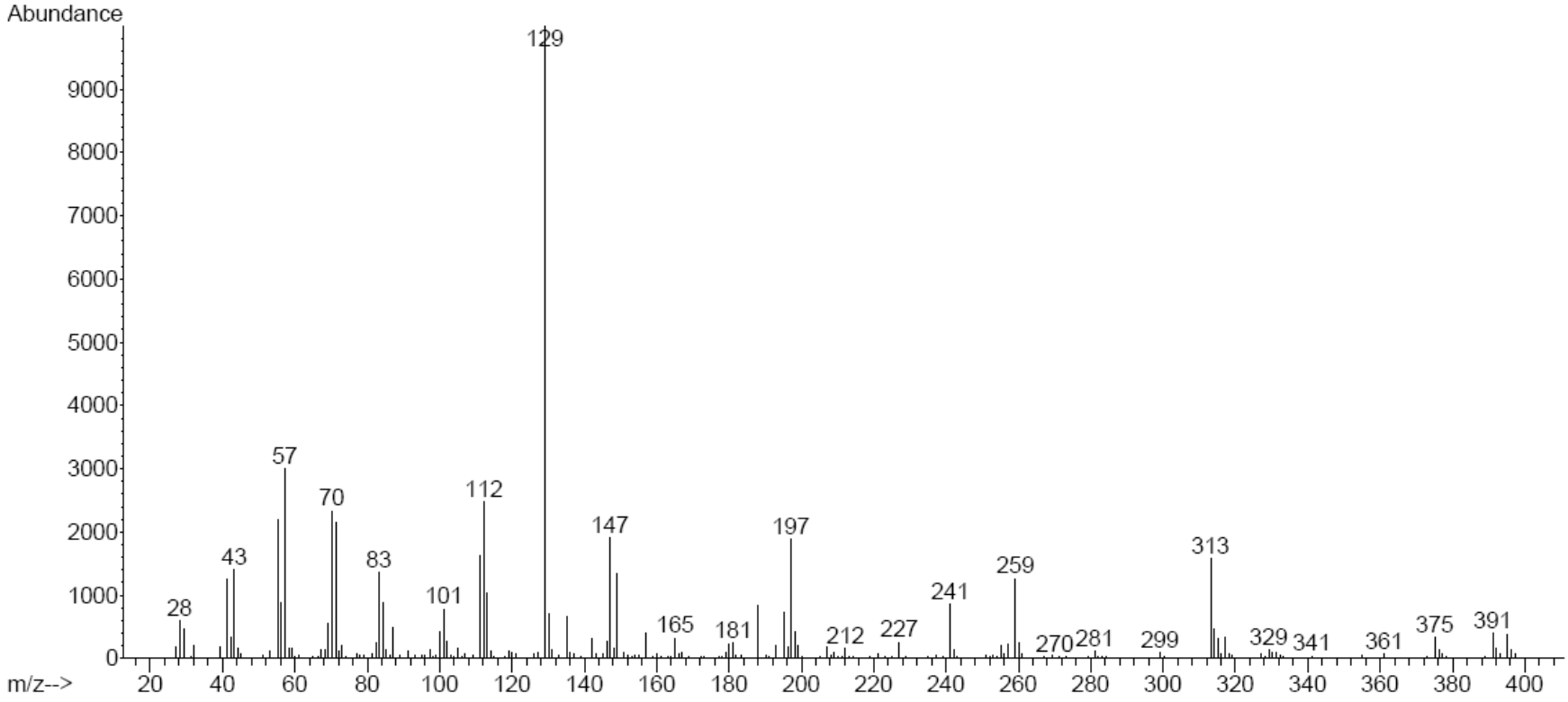

Figure A13 Mass spectrum of *bis*-(2-ethylhexyl) hexanedioate composing in volatile compound of mulberry leaf tea**.**

VITAE

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Academic presentation:

1. Wachiraporn Phoonan, Sureerat Deowanish, and Warinthorn Chavasiri. 2011. Compositions of labial gland extract of Thai stingless bee, *Tetragonula pagdeni* [poster presentation]. *CHE-USDC Congress IV, 14th–16th Sep. 2011*, The Zign Pattaya hotel, Chonburi.

2. Wachiraporn Phoonan. 2011. Compositions of labial gland extract of Thai stingless bees [oral presentation]. *16th Biological science graduate congress, 12th–14th December 2011*, National University of Singapore.