การวิเคราะห์หาชนิดและปริมาณสารสำคัญที่มีฤทธิ์ต้านซักในบัวบก

นางสาว พรพักตร์ ศิระธนารัณฑ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชเคมี ภาควิชาเภสัชเคมี
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2550

QUALITATIVE AND QUANTITATIVE DETERMINATIONS OF ANTICONVULSANTS IN *CENTELLA ASIATICA* (L.) URBAN

Miss Pornpak Sirathanarun

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmaceutical Chemistry
Department of Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2007

Thesis Title	QUALITATIVE AND QUANTITATIVE DETERMINATIONS OF ANTICONVULSANTS IN *CENTELLA ASIATICA* (L.) URBAN
By	Miss Pornpak Sirathanarun
Field of Study	Pharmaceutical Chemistry
Thesis Advisor	Assistant Professor Chamnan Patarapanich, Ph.D.
Thesis Co-advisor	Associate Professor Suwanna Laungchonlatan

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pornpen Pramyothin, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Assistant Professor Mitr Pathipvanich , Ph.D.)

.....Thesis Advisor

(Assistant Professor Chamnan Patarapanich, Ph.D.)

.....Thesis Co-advisor

(Associate Professor Suwanna Laungchonlatan)

.....Member

(Associate Professor Mayuree Tantisira, Ph.D.)

.....Member

(Assistant Professor Pornchai Rojsitthisak, Ph.D.)

พรพักตร์ ศิระธนารัณฑ์ : การวิเคราะห์หาชนิดและปริมาณสารสำคัญที่มีฤทธิ์ต้านชักในบัวบก (QUALITATIVE AND QUANTITATIVE DETERMINATIONS OF ANTICONVULSANTS IN *CENTELLA ASIATICA* (L.) URBAN) อ. ที่ปรึกษา : ผศ. ดร. ชำนาญ ภัตรพานิช, อ. ที่ปรึกษาร่วม : รศ. สุวรรณา เหลืองชลธาร, 129 หน้า.

การศึกษาชนิดของสารที่มีฤทธิ์ต้านชักในบัวบกในการสกัดด้วยเอทิลอะซิเทต โดยการแยกสารสำคัญด้วยวิธีคอลัมน์โครมาโทกราฟี สารที่สกัดได้จะถูกนำไปทดสอบฤทธิ์การต้านชักทางเภสัชวิทยาด้วยการกระตุ้นการชักจากสารเพนทิลีนเตตตระโซล สารสำคัญที่มีฤทธิ์ต้านชักถูกนำไปพิสูจน์เอกลักษณ์ด้วยวิธีสเปกโทรสโกปีข้อมูลจากไออาร์และเอ็นเอ็มอาร์ยืนยันว่าสารสำคัญที่มีฤทธิ์ต้านชักที่สกัดได้จากบัวบก คือ เวรีลองเซนแฟตตีแอซิด และด้วยเทคนิกการวิเคราะห์ด้วยจีซีเอ็มเอสพบว่าสารสำคัญประกอบด้วยสายโซ่ซึ่งมีคาร์บอนจำนวน 12-28 อะตอม ซึ่งมีองค์ประกอบหลักที่มีสายโซ่ซึ่งมีคาร์บอนจำนวน 16 และ 28 อะตอม

ได้พัฒนาวิธีทินแลโครมาโทกราฟี เพื่อวิเคราะห์เวรีลองเซนแฟตตีแอซิดในบัวบก โดยใช้แผ่นซิลิกาเจลเป็นวัฏภาคคงที่ และ เฮกเซนต่อเอทิลอะซิเทตต่อเกลเชียลอะซิติกแอซิด (14:6:1) เป็นวัฏภาคเคลื่อนที่ โดยมีสารโอลิเอโนลิกแอซิด เป็นสารมาตรฐานอินเทอร์นอล การตรวจวัดจุดของสารโดยฉีดพ่นด้วยสารละลาย 3 เปอร์เซ็นต์ฟอสโฟโมลิบดิกแอซิดในเมทานอล และสแกนด้วยเดนซิโทมิเตอร์ ที่ความยาวคลื่น 550 นาโนเมตร วิธีวิเคราะห์ดังกล่าวได้ผ่านการตรวจสอบความใช้ได้ตามข้อกำหนดของไอซีเอชซึ่งพบว่าปริมาณร้อยละของการกลับคืนอยู่ในช่วง 98.68-102.99 และ ร้อยละของความเบี่ยงเบนมาตรฐานสัมพัทธ์ไม่เกิน 2

วิธีที่ได้พัฒนาขึ้นถูกนำมาใช้ในการวิเคราะห์ตัวอย่างพืชบัวบกที่เก็บจาก 12 แหล่งในประเทศไทยได้แก่ จังหวัดปราจีนบุรี, จังหวัดนครศรีธรรมราช (พันธุ์บ่อล้อพื้นเมือง และ พันธุ์บ่อล้อการค้า), จังหวัดอุบลราชธานี, จังหวัดตราด, จังหวัดระยอง, จังหวัดชลบุรี, จังหวัดนครราชสีมา, จังหวัดสุโขทัย, จังหวัดเชียงใหม่, จังหวัดเชียงใหม่ (ก้านเขียว) และ จังหวัดพิษณุโลก พบปริมาณเวรีลองเซนแฟตตีแอซิดสูงสุด 0.27 เปอร์เซ็นต์ (จังหวัดระยอง), 0.24 เปอร์เซ็นต์ (จังหวัดสุโขทัย และจังหวัดนครราชสีมา) นอกจากนี้ ได้ศึกษาหาปริมาณสารสำคัญในรอบปีจากบัวบกที่เก็บได้จาก จังหวัดนครปฐม จังหวัดอุบลราชธานี และ จังหวัดนครศรีธรรมราช จากการศึกษาพบว่าบัวบกจากจังหวัดนครปฐมที่เก็บในช่วงเดือนพฤศจิกายนมีปริมาณเวรีลองเซนแฟตตีแอซิดสูงสุด บัวบกจากจังหวัดอุบลราชธานีมีปริมาณสารสำคัญสูงสุดในช่วงเดือนกันยายน และ บัวบกจากจังหวัดนครศรีธรรมราชมีปริมาณสารสำคัญสูงสุดในช่วงเดือนพฤษภาคม จากข้อมูลที่ได้ในการศึกษาครั้งนี้สามารถนำไปใช้ในการคัดเลือกสายพันธุ์บัวบก และวางแผนการเพาะปลูกเพื่อให้ได้ซึ่งปริมาณสารสำคัญเวรีลองเซนแฟตตีแอซิดสูงสุด

ภาควิชา...เภสัชเคมี..... ลายมือชื่อนิสิต.....พรพักตร์ ศิระธนารัณฑ์.....

สาขาวิชา...เภสัชเคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา...2550..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

477 65845 33 : MAJOR PHARMACEUTICAL CHEMISTRY
KEY WORD : CENTELLA ASIATICA/ ISOLATION/ VERY LONG-CHAIN FATTY ACID/ VLFAs/ TLC-DENSITROMETRIC METHOD

PORNPAK SIRATHANARUN : QUALITATIVE AND QUANTITATIVE DETERMINATIONS OF ANTICONVULSANTS IN *CENTELLA ASIATICA* (L.) URBAN. THESIS ADVISOR : ASST. PROF. CHAMNAN PATARAPANICH, Ph.D., THESIS COADVISOR : ASSOC. PROF. SUWANNA LAUNGCHONLATAN, 129 pp.

Ethyl acetate extract of the aerial part of *Centella asiatica* (L.) Urban (CA) was found to possess the anticonvulsant activity in the PTZ test model. Using the fractionation of the extract by conventional chromatographic technique together with the standard PTZ antiepileptic screening model, it was found that saturated very long-chain fatty acids (VLFAs) were the active principle in CA. Spectroscopic techniques were used to elucidate the chemical structure, IR and NMR data confirmed that active principle are VLFAs with the GC-MS technique to analyze the composition of the VLFAs, revealed that the sample contain $C_{12} - C_{28}$ chain length acids, which contain C_{16} and C_{28} chain length acids as main component.

A thin-layer chromatographic (TLC) method was developed to determine VLFAs by using silica gel plate GF_{254} as stationary phase and hexane: ethyl acetate: glacial acetic acid (14:6:1) as developing solvent, oleanolic acid as internal standard. The detection of the TLC spot was developed by spraying with 3% phosphomolybdic acid in methanol and scanned with densitometer at wavelength 550 nm. This method was validated according to ICH guideline. The percentage recovery was in the range of 98.68-102.99 and percentage of RSD was not more than 2.

The developed method was applied to analyze the CA that collected from 12 locations of Thailand, such as Prachin Buri, Nakhon Si Thammarat (Ban Bo Lo local growth and Ban Bo Lo trade), Ubon Ratchathani, Trat, Rayong, Chon Buri, Nakhon Ratchasima, Sukhothai, Chiang Mai (green petioles), Chiang Mai and Phitsanulok. The maximum content of VLFAs was founded 0.27% w/w (Rayong), 0.24% w/w (Sukhothai and Nakhon Ratchasima). Furthermore, the content of those compounds in CA that collected from the commercial crops of Nakhon Pathom, Ubon Ratchathani and Nakhon Si Thammarat provinces. The maximum VLFAs of CA in sample collected from Nakhon Pathom province was observed also annually determine in November, from Ubon Ratchathani in September and from Nakhon Si Thammarat province was observed in May. Information obtained may be utilized as criteria for plant varieties selection and breeding programs for commercial cultivars of optimal VLFAs content for further isolation of pure VLFAs.

Department: Pharmaceutical chemistry Student's signature: Pornpak Sirathanarun
Field of study: Pharmaceutical chemistry Advisor's signature:
Academic year: 2007..... Co-advisor's signature: Suwanna Laungchonlatan

Acknowledgements

I wish to express my sincerely indebted to my thesis advisor, Assistant Professor Dr. Chamnan Patarapanich, and my thesis co-advisor, Associate Professor Suwanna Laungchonlatan, for their support, valuable advice, continual guidance, kindness, and understanding throughout my graduate study period.

I am sincerely grateful to Assistant Professor Dr. Mitr Pathipvanich, Head of the Department of Pharmaceutical Chemistry for his valuable advice, kindness during my graduate study and I also would like to thank the staffs of the Department of Pharmaceutical Chemistry for their facilities and helpfulness.

I also would like to thank Assistant Professor Dr. Pornchai Rojsitthisak, Associate Professor Dr. Mayuree Tantisira and Associate Professor Dr. Phensri Thongnopnua for their kind suggestion and discussion to complete this thesis.

I also would like to thank Associate Professor Dr. Mayuree Tantisira and Miss Aree Wanasuntronwong for their kind anticonvulsants test in Department of Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

My great appreciation is extended to Dr. Piya Chalermglin, Miss Jirapan Sritongkul from Thailand Institute of Scientific and Technological Research and Mr. Niyom Chaeukerd for their kindness cooperation and supplying *Centella asiatica* (L.) Urban samples throughout my study.

I also thank to all of my friends for their kind assistance, helpfulness and friendship throughout this study.

Finally, I would like to express my appreciation to my dear parent for their love and understanding.

CONTENTS

Page

LIST OF TABLES

Table **Page**

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF SCHEMES

Scheme **Page**

LIST OF ABBREVIATIONS

AA	Asiatic acid
AR	Analytical reagent
^{o}C	Degree Celsius
CA	*Centella asiatica* (L.) Urban.
cm	Centrimeter
GC	Gas Chromatography
g	Gram
IR	Infrared
l	Liter
MA	Madecassic acid
mg	Milligram
ml	Milliliter
mm	Millimeter
min	Minute
MS	Mass Spectroscopy
MW	Molecular weight
NMR	Nuclear Magnetic Resonance
nm	Nanometer
No.	Number
PTZ	Pentylenetetrazole
Std	Standard

SD	Standard deviation
sec	second
%R	Percent recovery
rpm	sound per minute
TLC	Thin Layer Chromatography
UAE	Ultrasonic-assisted extraction
UV	Ultraviolet
VLFA	Very long-chain fatty acid
WS	Working standard
µg	Microgram
µl	Microliter

CHAPTER I

INTRODUCTION

Epilepsy is a common neurological disorder associated with the alteration in psychological, emotional and educational parameter. Treatment with conventional antiepileptic drugs (AEDs), such as phenobabital, gabapentin, phenyltoin, valproate and zonisamide, were associated with side-effects, dose-related and chronic toxicity. Therefore, phytomedicines can potentially play as important role in the development of new antiepileptic drugs.

Natural products from plants remedies have contributed significantly in discovery of ADEs with novel structures and better safety and efficacy profiles. Now, various phytochemical and pharmacological studies have been carried out on these anticonvulsant plants **(1)**. More-over, the number of patients and medical practitioners in the industrialized world which use herbal medicines as a supplement to as substitute for prescription drugs are increased. Herbal medicines are often considered to be a gentle and safe alternative to synthetic drugs.

Centella asiatica (L.) Urban. (CA) or Gotu kola is a plant in family Apiaceae (Umbeliferae) and widely distributed in Asia such as China, India, Indonesia and Thailand. This plant continues to be used as crude medicinal herb within the framework of folk medicine as an effective remedy. It has been used for treatment of skin disease **(2)**, inflammation **(2)**, mental illness **(3)**. The substances found in CA were triterpenoid glycosides (such as asiaticoside, madecassoside, brahmoside,

brahminoside) **(4)**, triterpenes (such as asiatic acid, madecassic acid, madasiatic acid) **(4)**, flavonoids (such as rutin, kaempferol, quercetin, myricetin and apigenin) **(5)** and polyacetylenes (such as 2,9-pentadecadiene-4,6-diyn-1-ol acetate, pentadeca-1,9-diene-4,6-diyne-3,8-diol,8-monoacetate,pentadeca-1,9-diene-4,6-diyne-3,8-diol ,diacetate, pentadeca-1,8-diene-4,6-diyne-3,10-diol, 10-monoacetate and pentadeca (1,8)-diene-4,5-diyne-3,10-diol) **(6)**. The active substances of CA were proposed to be the triterpenoids the constituents of which includes : asiaticoside (AS), madecassoside (MS), asiatic acid (AA), madecassic acid (MA) **(7-9)**.

In 2005, Anusara Vattanajun, *et al* reported the anticonvulsant activity of CA's ethyl acetate extract (10). This extract is matrix of natural compounds including chlorophyll, AA, MA and some other compounds. However, identification, isolation and quantification of the anticonvulsant active principle has never been reported.

The purpose of this research works is to develop the method of extraction, identification and analysis method of the anticonvulsant active principle in CA. To isolate and identify the target compounds, conventional column chromatography were used to fractionate the matrix base on the polarity basis, each fractions collected were tested for anticonvulsants activity by using pentylenetetrazole (PTZ) model. The active fraction was then subjected to further purification to obtain pure substance which was then confirmed to test for anticonvulsants activities.

The extraction techniques have been widely investigated. The traditional extraction methods, such as reflux and soxhlet, have been used in many studies. This method is time-consuming and required relatively large quantities of solvents. The

other method, such as ultrasonic-assisted extraction (UAE), was a fast and efficient method for extracting chemical constituents from plant matrices **(11)**.

To quatify the anticonvulsant active principle, an appropriate analytical method which can simultaneously, detect those analyte must be developed. The chromatographic method is usually applied to quantitation of the analyte in the complex matrix of natural extract. Thin layer chromatographic (TLC) method is also popular due to the simplest and rapid analysis. The distribution and variation of the anticonvulsant active principle in many CA accessions, collected from various locations will be monitored and compared. The amount of the active principle in the CA extract will also be monitored for the purpose of setting the specification of plant and its extract.

For this TLC analysis, due to the lack of chromophoric functionality of the bioactive analyte structure, a phosphomolybdic acid spray reagent was then used to develop for colorimetric analysis by the densitometry. The TLC-densitometry was selected, developed and validated according to ICH guidelines (2005). Finally the developed method will be applied to analyze the interesting compounds in plant samples.

CHAPTER II

LITERATURE REVIEW

Centella asiatica (L.) Urban., the synonyms *Hydrocotyle asiatica* (L.) is known as Bua-bok, Gotu Kola, Asiatic Pennywort, Indian Pennywort, Indian Water Navelwort, Mandukparni, *etc*. It is a cultivated plant in the family Apiaceae or Umbelliferae that found in subtropical and topical climates of Africa, North and South America and Asia such as Thailand, Indonesia, Sri Lanka, India, and China **(12-14)**.

Figure 2.1 Picture of *Centella asiatica* (L.) Urban.

2.1. Botanical description

A slender trailing herb, stems long, prostrate, emerging from the leaf-axils of a vertical rootstock, filiform, often reddish, with long internodes and rooting at the

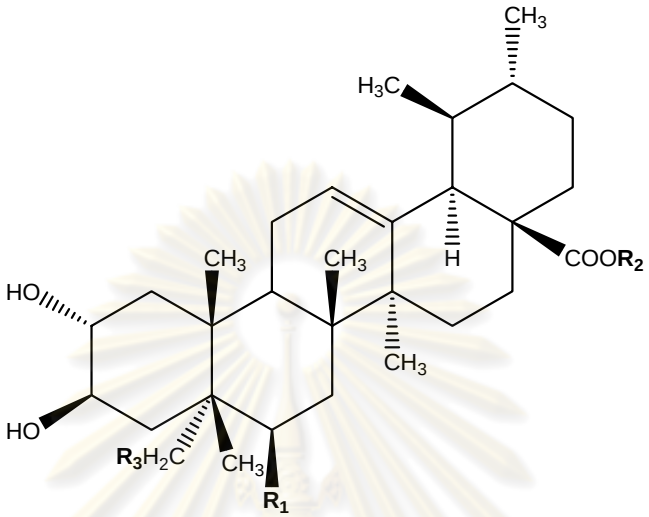
nodes; leaves thin, long-petioled, several from the rootstock and 1-3 from each node of the stems, 1.3-6.3 cm diameter, orbicular reniform, more or less cupped, entire, crenate or lobulate, glabrous; petioles very variable in length, 7.5-15 cm long or more, channelled; stipules short, adnate to the petioles forming a sheathing base **(7-9)**.

2.2. Chemical constituents

2.2.1 Triterpenes

The major principles in CA are the triterpenes (agylcones) and triterpene ester glycosides (glycosides). Most common triterpenes aglycone are asiatic acid, madecassic acid, and madasiatic acid and common triterpene ester glycosides are asiaticoside, asiaticoside A, asiaticoside B, madecassoside, brahmoside and brahminoside. **(4,9,15-18)**. The chemical structure of this triterpene are shown in Table 2.1.

Table 2.1 Chemical structure of major triterpenes from *Centella asiatica*



Compounds	R_1	R_2	R_3	Formular	Molecular Weigh
Asiatic acid	H	H	OH	$C_{30}H_{48}O_5$	488
Madecassic acid	OH	H	OH	$C_{30}H_{48}O_6$	504
Madasiatic acid	OH	H	H	$C_{30}H_{48}O_5$	488
Asiaticoside	H	glu-glu-rham	OH	$C_{48}H_{78}O_{19}$	958
Madecassoside	OH	glu-glu-rham	OH	$C_{48}H_{78}O_{20}$	974
Brahmoside	OH	rham-glu-arab	OH	$C_{47}H_{78}O_{19}$	946
Brahminoside	OH	2glu-rham-arab	OH	$C_{58}H_{88}O_{24}$	1108

2.2.2 Essential oil

The aerial parts of CA contain 0.1% of essential oil which composes of 80% sesquiterpenoids such as β-caryophyllene, α-humulene and germacrene-D, elemene and bicycloelemene, trans-farnesene **(19)**.

2.2.3 Flavone derivatives

Kaempferol-3-glucoside and quercentin-3-glucoside have been found **(20)**.

2.2.4 Phytosterols

Stigmasterol, sitosterol have been found **(21)**.

2.2.5 Amino acids

Free amino acids found in the leaf and stem are glutamate, serine and alanine. Amino acids mostly found in the root are aspartate, glutamate, serine, threonine, alanine, lysine, histidine and amino butylate **(7, 20)**.

2.3 Medicinal and pharmacological activities

2.3.1 Central Nervous system

The ethyl acetate extract of CA (EACA) was given orally to mice one hour prior to the injection of pentylenetetrazole (PTZ). The isobolographic analysis, the principle method applicable for understanding the real nature of drug interactions was used to analyze the interaction between EACA and conventional antiepileptic drugs (phenyltoin, valproate and gabapentin) in the PTZ test in mice.

In results, when the antiepileptic drug was given alone, the medium effective dose of phenyltoin, valproate and gabapentin were found to be 13, 104 and 310 mg/kg body weigh, respectively, where as the corresponding values in the presence of EACA were 5, 29 and 79 mg/kg body weight. By the isobolographic analysis, the results obtained indicated an additive effect among all combinations test. In relation to neurotoxicity, combination of gabapentin and EACA demonstrated a broader margin between the effective dose and the neurotoxic dose while the other two combinations did not. Only EACA, given orally, were able to demonstrated anticonvulsant activity or protect the mice against PTZ-induced convulsion in a dose dependent manner exhibiting the ED_{50} of EACA 673 mg/kg body weight at the pretreated time of 1 hour. The TD_{50} of EACA was found to be 415 mg/kg body weight **(10)**.

The alcoholic extract and alkali extract of CA, were compared to the extract of the other plants, with the test in leptazole-induced seizure (LIS) model and strychnine-induced seizures (SIS) model respectively. The outcome showed inactive

for antiepilepsy but was sedative effect in alcoholic extraction **(1)**. CNS depressant activity of CA has been reported and was attributed to the glucosidal saponin; brahmosides **(24)**.

A water extract of CA (300 mg/kg body weight orally in rat) was used in the PTZ-kindled seizures model and results showed improvement in the learning deficit induced by PTZ kinding as evidenced by decreased seizure score and increased latencies in passive avoidance behavior. However, low dose of CA (100 mg/kg body weight) showed improvement only in the learning deficit due to the kindling and improving the seizure score **(22)**.

The water extract of CA was evaluated for the psychotic effect. The mices were feed orally with CA aqueous extract (200, 500, 700 and 1000 mg/kg body weight) for 15 days to select an effective dose for psychotic on intellecual performance leaning and memory. Animals were tested in radial arm maze to assess the learning and memory performance. Mices were treated orally with 200 mg/kg body weight of CA extract for 15 days from day 15 to day 30 post partum and psychotic effect was evaluated on the 31st day and 6 months postpartum. The behavioral (open field, dark/bright arena, hole board and radial arm maze test), biochemical (acetylcholinesterase activity) and histological studies (dendritic aborization) were carried out. Performance of juvenile and young adult mice was significantly improved in radial arm maze and hole board test, but locomotors activity did not show any change compared to control. Treatment resulted in increased acetylcholinesterase activity in the hippocampus. Dendritic arbonization of hippocampal CA3 neurons was also increased in terms of 6 months. Results of this

investigated how that CA extract can influence the neuronal morphology and promote the higher brain function of mice **(3)**.

The aqueous, methanolic and chloroform extract of CA were investigated for their effect on cognitive functions in rats. Animals were used to study the effect on learning and memory by using shuttle box, step through, step down and elevated plus maze paradigms. Only the aqueous extract of whole plant (200 mg/kg body weight for 14 days) showed an improvement in learning and memory in both shuttle box and step through paradigms of learning and memory. Therefore, the experiment was conducted with aqueous extract using 100, 200 and 300 mg/kg body weight doses in different paradigms of learning and memory. All doses of aqueous extract increased the number of avoidances in shuttle box and prolonged the step through latency in step through apparatus in a dose dependent manner, while only two dose 200 and 300 mg/kg body weight of extract showed significant increase in the step down latency in step down apparatus and transfer latency in elevated plus maze. Among doses of aqueous extract tested on oxidative stress parameters, only 200 and 300 mg/kg body weight showed a significant decrease in the brain level of malondialdehyde with simultaneously significant increase the level of glutathione. There was a significant increase in the level of catalase at the 300 mg/kg body weight but no significant change in superoxide dismutase level was observed. The present findings indicated that the aqueous extract of CA has cognitive enhancing effect and on antioxidant mechanism is involved **(23)**.

A standardized extract of CA, was test to 28 healthy elderly volunteers in randomized, placebo-controlled and double-blind study, investigated the effect on

cognitive function. The volunteers received the plant extract at a various dose ranging 250, 500 and 750 mg once daily for 2 months. Cognitive performance was assessed using the computerized test battery and event-related potential whereas mood was assessed using Bond-Lader visual analogue scales prior to the trial and after single, 1 and 2 months after treatment. The results showed that the high dose of the plant extract enhanced working memory and increased component amplitude of event-related potential. Improvements of self-rated mood were also found following the CA treatment **(25)**.

2.3.2 Wound-healing activity

A wound is a disruption of tissue integrity that is typically associated with a loss of substance. The wound healing process is generally independent of the form of injury. It is convenient to divide the overall process into three overlapping phases (inflammatory phase, proliferative phase, maturational phase).

The titrated extract of CA (TECA) contains asiatic acid, madecassic acid, asiaticoside and madecasoside **(9)**. The *In vitro* study, each of these compounds stimulated the production of human collagen I, a protein involved in wound healing. Stimulation of collagen synthesis in foreskin fibroblast monolayer cultures by an extract from CA has also been reported. Asiaticosides accelerated the healing epidermis of superficial postourgical wounds and ulcers by accelerating cicatrical action. Asiaticoside stimulates the epidermis by activating the cells of the Malpighian layer in porcine skin and by keratinization *in vitro*. Topical applications of

asiaticoside promoted wound healing in rats and significantly increase the tensile strength of newly formed skin. Extracts of CA, and in particular its major triterpene ester glycoside, asiaticoside, are valuable in the treatment of hypertrophic scars and keloids. Asiaticoside has been reported to decrease fibrosis in wounds, thus preventing new scar formation. The mechanism of action appears to be two fold; by increasing the synthesis of collagen and acidic mucopolysaccharides and by inhibiting the inflammation phase of hypertrophic scar and keloids. It has further been proposed that asiaticoside interferes with scar formation by increasing the activity of myofribroblasts and immature collagen **(26)**.

In clinical trials study, an extract of CA in a 1% or 2% powder accelerated healing of wounds. A formulation containing asiaticoside as the main ingredient healed 64% of soiled wounds and chronic or recurrent along that was resistant to usual treatment. In an open clinical study, treatment of 20 patients with soiled wounds and chronic or recurrent atony with a galenical formulation containing 89.5% CA healed 64% and produced improvement in another 16% of the lesions studied. Local application of an extract of the drug to second- and third-degree burns expedited healing, prevented the shrink and swelling caused by infection **(9)**.

Twenty-two patients with chronic infected skin ulcers were treated with a cream containing 19% extract of CA. After 3 weeks of treatment, 17 of the patients were completely healed and the ulcer size in the remaining 5 patients was decreased. Another trial using the same cream preparation demonstrated similar results. A standardized extract of CA was reported to treat ulcer cruris (indolent leg ulcer)

effectively in clinical trials. In double-blind study, no significant effect on healing was observed in patients with ulcer cruris after oral treatment with asiaticoside **(9)**.

2.3.3 Antitumor activity

Asiatic acid (AA) form CA extracts decreased viability and induced apoptosis in human melanoma SK-MEL-2 cells in a time- and dose- dependent manner. AA also markedly increased intracellular reactive oxygen species level and enhanced the expression of Bax but not BCl-2 protein in the cells. In addition, AA-induced activation of caspase-3 activity in a dose-dependent manner. Pretreatment with Trolox, an antioxidant, significantly blocked the induction of Bax and activation of caspase-3 in AA-treated cells. Furthermore, Ac-DEVD-CHO, a specific caspase-3 inhibitor, and Trolex prevented the AA-induced apoptosis **(27)**.

Effects of the water extract of CA on formation of azoxymethane (AOM)-induced aberrant crypt foci (ACF) and intestinal tumor genesis in rat were investigated. Significantly decreased the number of larger ACF in the large intestine in the early stage, while the number of methylated DNA adducts was not decreased compared with that in the AOM-treated group. The extract at a dose of 100 mg/kg body weigh significantly reduced the multiplicity of neoplasm in the small intestine (p<0.05) **(28)**.

2.3.4 Anti-gastric ulcers activity

The anti-gastric ulcers activity of water extract of CA, containing asiaticoside (AS) as a major component (about 4% w/w assay by HPLC) was studied. The healing effect on acetic acid induced gastric ulcers was compared with asiaticoside in rats. Different concentrations of CA and AS were orally administered to rat with ulcers. The size of the ulcers was found to be reduced at day 3 and 7 in a dose-dependent, with a concomitant attenuation of myeloperoxidase activity at the ulcer tissues. The expression of basic fibroblast growth factor, an important angiogenic factor, was also unregulated in the ulcer tissues in rats treated with CA or AS **(29)**.

For ethanol induced gastric lesions, water extract of CA was used to test effect to prevent this symptom in rats. Oral administration of CA (0.05 g/kg, 0.25 g/kg and 0.5 g/kg body weight) before ethanol administration significantly inhibited gastric lesions formation (58% to 82% reduction) and increased mucosal mycloperoxidase (MPO) activity in a dose dependent manner. CA prevented ethanol induced gastric mucosal lesions by strengthening the mucosal barrier and reducing the damaging effects of free radical **(30)**.

2.3.5 Antioxidative activity

Extract of CA in various solvents were used to test the antioxidative effect in many experiment. Lipid peroxidation was monitored by measuring the malondildehyde (MDA) level in blood. Activities of free radical-scavenging enzymes

were determined using H_2O_2 decomposition and nitrobluetetrazolium reduction, respectively. Results shown that CA extract, powder, and α-tocopherol lowered MDA level than did the other rats **(31)**.

Crude methanol extract of CA oral treatment for 14 days significantly increased the antioxidant enzymes, like superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx) **(32)**.

The phenolic compounds in root, leaf and petiole of CA extracts were evaluated, compared with natural antioxidant (α-tocopherol) and synthetic antioxidant (butylated hydroxytoluene, BHT). The results showed that both leaf and root of CA had high antioxidative activity, which was as good as that of α-tocopherol. The total phenolic content varied from 3.23 to 11.7 g/100 g dry sample, and showed strong association (r^2=0.90) with antioxidative activity **(33)**.

The various extracts of different parts of CA (leaves, petioles and roots) using three type of solvents (ethanol, water and light petroleum), were evaluated for antioxidative activity using a linoleic acid model system and the thiobarbituric acid test. The ethanol extract of all part of CA exhibited significantly higher antioxidative activity than water extract, while the light petroleum ether showed negative activity. Both the ethanol and water extract increased the activity, when increased the concentration of the extract. When concentration of ethanol extract was more than 3000 ppm, the antioxidative activity was not significantly different (p<0.05) from that of α-tocopherol. Roots extract showed highest activity of all parts tested. The antioxidative activities of the ethanol extracts were found to be stable up to 50^0C and exhibited optimum activity at neutral pH **(34)**.

2.3.6 Immunological activity

The pectin extract from CA and its degradation product showed immunostimulating activity to different extent *in vitro*. The results indicated that the carboxyl and acetyl groups were important roles in the expression of immunological activity **(35)**. The other pectin, isolated from CA, was S3A. S3A had no immunological activity, but its derivatives had immunostimulating activities **(36)**. *In vivo* tests, a water-soluble acidic arabinogalactan (HBN) from CA, had remarkable immunoenhancing activities in T- and B-lymphocytes. It increased spleen index and inhibited the level of IgG. HBN's derivatives by $NaIO_4$ oxidation. Smith degradation and enzyme-treatment possessed immunological activities **(37)**.

The methanol extract of CA (containing 0.18% of AS) in five difference concentration levels (dose-response relationship) ranging from 100 to 500 mg/kg body weight were used to test for immunomodulatory activity using carbon clearance, antibody titer and cyclophosphamide immunosuppressant parameters. Significant increase in the phagocytic index and total white blood cell counts were observed and the F ratio of the phagocytic index was also significant **(38)**.

2.4 Applications

2.4.1 Parts used

Aerial parts of CA are used as folk medicine that is available as teas, dried herb, capsules, tablets, gel and ointments **(7, 8)**.

2.4.2 Traditional and modern use

The leaves and stems of the CA are widely used to treat a variety of illness, particularly in traditional eastern medicine. Historically, CA has been used to treat syphilis, hepatitis, stomach ulcers, mental fatigue, epilepsy, diarrhea, fever and asthma. Today, American and European herbalists use CA for disorders that cause connective tissue swelling, such as scleroderma, psoriatic arthritis(arthritis occurring in conjunction with psoriasis), and rheumatoid arthritis **(39)**. Recent studies confirm some of the traditional uses and also suggest possible new application for CA, such as lowering high blood pressure, treating venous insufficiency (pooling of blood in the veins, usually in the legs, boosting memory and intelligence), easing anxiety and speeding wound healing **(12,14)**.

2.5 Overview of the techniques of extraction of analytes from plant material.

Extraction and product recovery are the most imperative steps in the evaluation of target molecules from various plant parts. In the last decade there has been an increasing demand for new extraction techniques, amenable to automation, with shortening extraction times and reducing of organic solvent consuming-preventing pollution in analytical laboratories and reduction of sample preparation costs.

Conventional method, such as marcelation and soxhlet extraction, which have been used for many decades, are very time-consuming and require relatively large quantities of solvents. Soxhlet is a standard technique and the main reference for evaluating the performance of other solid-liquid extraction methods. This extraction method is a general and well-established technique, which surpasses in performance other conventional extraction techniques except for, in limit field of applications, the extraction of thermolabile compounds **(40)**.

Advanced techniques in sample preparation such as ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE) and supercritical fluid extraction (SFE) have been developed to apply in plant extraction. The similarity between these techniques is the possibility of working at elevated temperatures and pressures, which drastically improves the speed of the extraction process **(41)**.

Recently, there have been several reports on the application of ultrasonic methods in the extraction of various phytochemicals. This method required shorter

extraction time, less solvents, higher extraction rate and better products with lower costs than other conventional extraction methods.

2.5.1 Ultrasonic-assisted extraction (UAE)

2.5.1.1 Basic principle of UAE

Ultrasounds are waves with frequencies ranging from 16 kHz to 1 GHz, inaudible to humans. Ultrasonic vibrations are the source of energy facilitating the release of some analytes from the sample matrix. The improvement in extraction efficiency due to ultrasound appears at certain values of so-called acoustic pressure. Among the most important phenomena taking place in the acoustic field is cavitations (generation and collapse of mostly empty cavities), friction at the boundary and interfacial surfaces, and increase in the diffusion rate of analytes **(40, 41)**.

As ultrasound passes through a liquid, the expansion cycles exert negative pressure on the liquid pulling the molecules among from one another. If the ultrasound intensity is sufficient enough for the expansion cycle, then create cavities or micro-bubble in the liquid. This occurs when the negative pressure exceeds the local tensile strength of the liquid, which varies with the type and purity of the liquid. Once formed, these bubbles will absorb the energy from the sound waves and grow during the expansion cycles and recompress during the compression cycles. The increase in pressure and

temperature caused by the compression leads to the collapse of the bubbles, which causes shock wave that passes through the solvent, enhancing the mass transfer within the system **(40-42)**.

The average time of ultrasonic extraction typically ranges from a few to 30 min, although it can be as long as 70 min. The recoveries obtained during this time are comparable to those obtained after a dozen or so hours of soxhlet extraction, carried out at the same temperature. The extraction conditions can be optimized with respect to time, polarity and amount of solvent, and the mass and kind of sample. The advantage of this technique is the possibility of extraction of many samples at once in an ultrasonic bath. The extraction is carried out at room temperature, which makes it suitable for the extraction of thermally labile analytes. The need for separation of the extract from the sample following the extraction is a disadvantage of this technique.

2.5.1.2 Applications of UAE

A number of articles have been published dealing with the ultrasonically assisted extraction of different vegetal materials. One of the first citations concerning ultrasonic extraction (1952) was related to hop extraction in an aqueous medium and showed that ultrasonic extraction was comparable with the boiling extraction process. Several references concerning ultrasonically assisted extraction are summarized in Table 2.2 as followed:

Table 2.2 Isolation of substances using ultrasonic-assisted extraction

Analyte	Sample matrix	Time (min)	% Recovery	References
Antraquinones	*Morinda citrifolia*'s roots	60	95.72±0.53	**42**
Rutin	*Euonymus alatus* *Sophora japonica*'s flower bud	30 x 3 60	99.5	**43** **44**
Qurcetin	*Euonymus alatus*	30 x 3	100.3	**43**
Saikosaponin	*Radix Bupleuri*	30		**45**
Oil	*Nicotiana tabacum* *Isatis tinetoria*'s seed	20 60	33.8	**46** **47**
Antioxidant	*Rosmarinus officinakis*	15		**48**
Isoflavones	Soybeans	20	80-90	**49**
Ginsenosides	Ginseng roots	120		**50**

Examples of substances isolated by UAE along with extraction time and recoveries are summarized as above; it should be proven to be a powerful tool for the extraction of phytopharmaceutical industry. Although ultrasonic can be enhanced the extraction, it is worth noting that when high frequency ultrasound is employed, the extraction yield did not increase significantly.

It is obvious that reducing the size of vegetal material particles will increase the number of cells directly exposed to extraction by solvent and thus exposed to ultrasonically induced cavitations. This effect can be utilized by

milling the material before extraction. It should be borne in mind however that powerful sonication cab itself serve to mill the vegetal material.

In laboratory scale, ultrasonic-assisted extraction can be easily performed by using the simple ultrasonic cleaning bath as shown in Figure 2.1

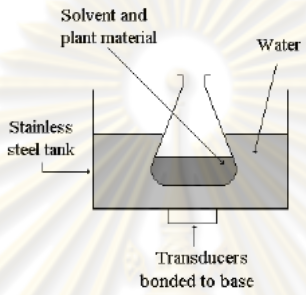


Figure 2.2 Experiment setup for UAE using an ultrasonic bath **(51)**

Pathom somwong **(52)** used the UAE to extract AS, MA, AA and MA from CA sample and determined the content of analytes by high-performance liquid chromatography (HPLC). The UAE method, as compared with soxhlet extraction method, showed increasing the amount of active principle and decreasing the extraction time.

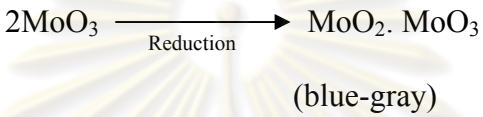
2.6 Overview of the analytical method for determination of active compounds in CA

There are several methods for qualitative and quantitative determination of active constituents of CA. Titrimetric method was conventionally used to determine the triterpene acids (AA, MA) and glycosides (AS, MS). Thin-layer chromatographic (TLC) method and HPLC method were developed to determined AA, MA, AS and MS. Bungon Kongthong **(53)** developed and validated a suitable analytical method for determined AA, MA, AS and MS in the extract of CA by TLC-densitometer and HPLC. However, the analytical method for determine anticonvulsant principle in CA has never been reported.

TLC is a simple and fast analytical method. It was used qualitatively and quantitatively in combination with densitometric scanner for analyte ingredient in foods, medicines and active compounds from plant **(54-57)**.

Spraying reagent can be used to spray some compound which do not have chromophore, phosphomolybdic acid, was used for reducing substances, steroids, bile acid, bile acid conjugates, lipids, phospholipids, fatty acids, fatty acid methyl ester, triglycerides, phenols, indole derivatives, prostaglandins and components of essential oils. The reagent solution was prepared by dissolved 2-20 g of phosphomolybdic acid in 100 ml of ethanol. The solution is stable for only 10 days even in dark. The TLC plate, when spray with the reagent solution, was heated to 120^0C for 20 min in the oven **(58)**. The color

reaction of phosphomolybdic acid and the organic substances is shown in chemical reaction belowed. A large number of organic substance can be oxidized with phosphomolybdic acid, whereby a portion of the Mo(VI) is reduced to Mo(IV), which forms blue-gray mixed oxides with the remaining Mo(VI) **(58)**.



Presently, TLC-densitometric method using with phosphomolybdic acid as spray reagent become a method of choice for analysis of active principle in plants. Breda Simonovsky and Irena Vork **(59)** determined glycoalkaloids from potato (α-solanine and α-chaconine) using chloroform-methanol-2% aqueous NH_4OH (70:30:5) as a developing phase. Six different detection reagents ; Dragendorff's reagent, phosphomolybdic acid reagent, paraformaldehyde-phosphoric acid, Ce (IV) sulfate-sulfuric acid and sulfuric acid-ethanol reagents were applied for quantification by densitometry and compared the results form each spraying reagents. In this study modified Dagendorff's reagent was the most sensitivity for analytical method by fluorescence densitometer.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals and reagents

1.1 Acetonitrile, AR grade (Fishers)

1.2 Dichloromethane (Lab scan)

1.3 Ethyl acetate (Lab scan)

1.4 Filter paper, Number1 (Whatman)

1.5 Glacial acetic acid (Scharlau)

1.6 Hexane (E. Merck)

1.7 Isopropyl alcohol (Lab scan)

1.8 Methanol, AR grade (Lab scan)

1.9 Oleanolic acid (98%, Chengdu Hawk Bioengineering)

1.10 Phosphomolybdic acid, Hydrate (Fluka)

1.11 Sodium Hydroxide (E. Merck)

1.12 Sulfuric acid concentrate (Lab scan)

1.13 TLC plate silica gel 60 F254 (E. Merck)

2. Instruments

2.1 Hot air oven (OMRON)

2.2 Electric mill (Retsch Muhle)

2.3 Rotary evaporator (BUCHI)

2.4 pH meter (Metrohm 744)

2.5 Ultrasonic bath (BANDELIN: Sonorex digital 10P)

2.6 Centrifuge (HETTICH Zentrifugen ; EBA 20)

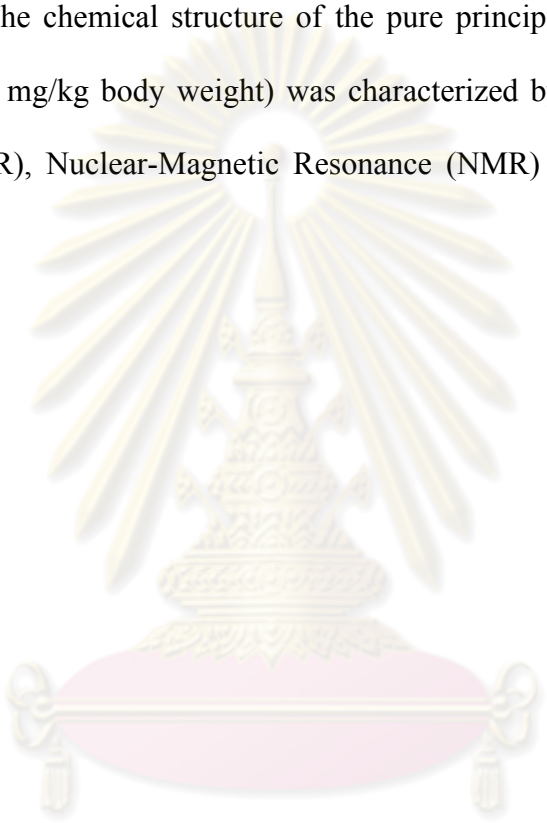
2.7 Densitometer (Shimadzu: CS9301PC)

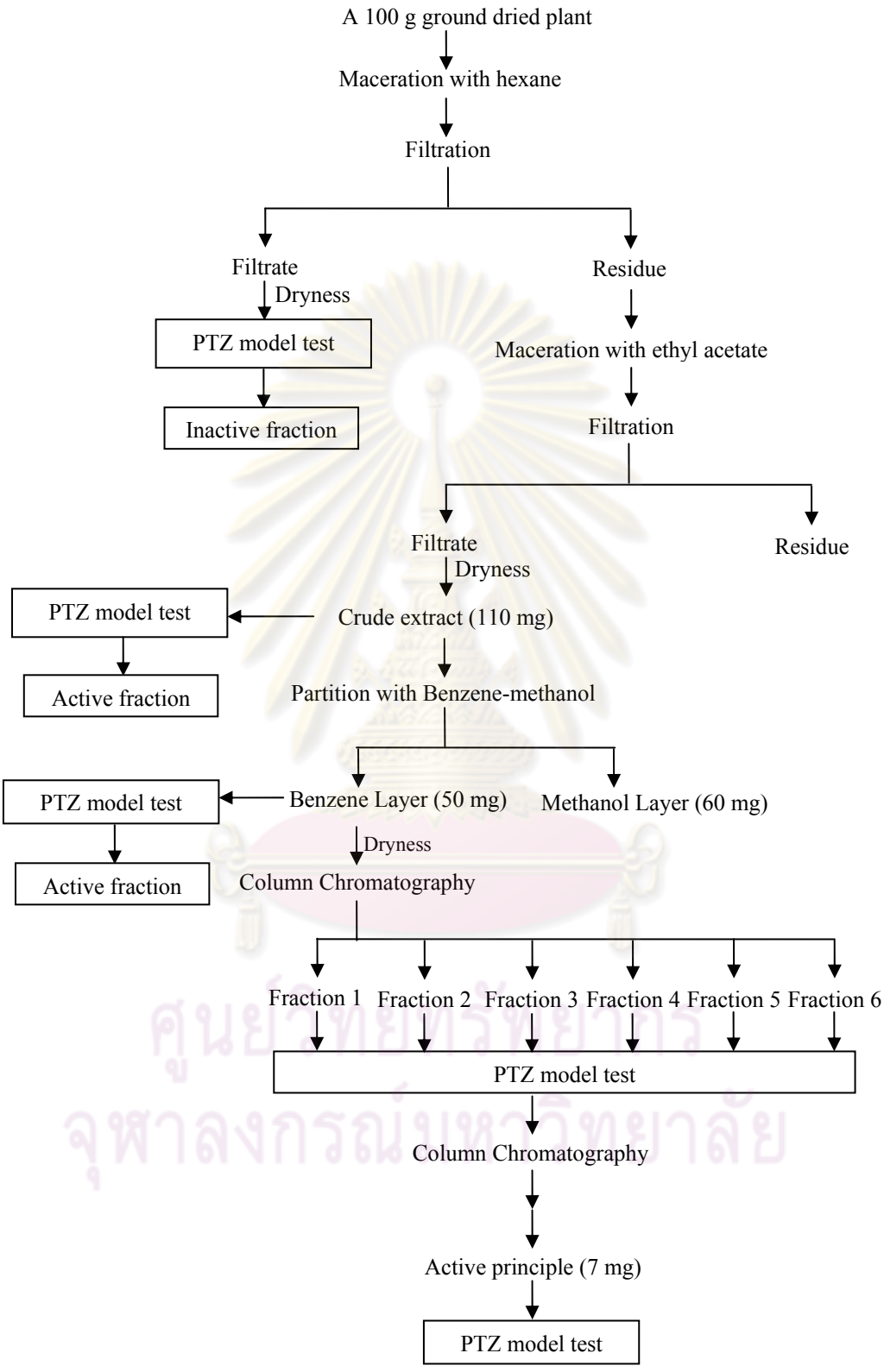
3. Extraction, isolation and determination of the anticonvulsant principle from CA

3.1 Extraction of anticonvulsant principle from CA

Diagram of extraction and isolation of anticonvulsant agent is shown in Scheme 3.1. The whole dried ground plant of *Centella asiatica* (L.) Urban (CA) (100 g) was macerated twice with hexane ten liters for three days and then filtered. The residue of CA was macerated again with ethyl acetate and then filtered. The filtrate of ethyl acetate fraction was concentrated to dryness under reduced pressure. This fraction was test for the anticonvulsant activity by using PTZ model (673 mg/kg body weight). The active fraction was partition with benzene and methanol. The benzene fraction had anticonvulsant activity in the same model test (30 mg/kg body weight).

A 50 mg of dried benzene fraction was chromatographed over silica gel column and eluted with mixture of hexane-ethyl acetate with increasing polarity to separate pure compound. To identify and isolate the target compound was test for anticonvulsant activity using PTZ model in each fraction. The chemical structure of the pure principle having anticonvulsant activity (1 mg/kg body weight) was characterized by Infrared Spectroscopic method (IR), Nuclear-Magnetic Resonance (NMR) and Mass Spectroscopy (MS).



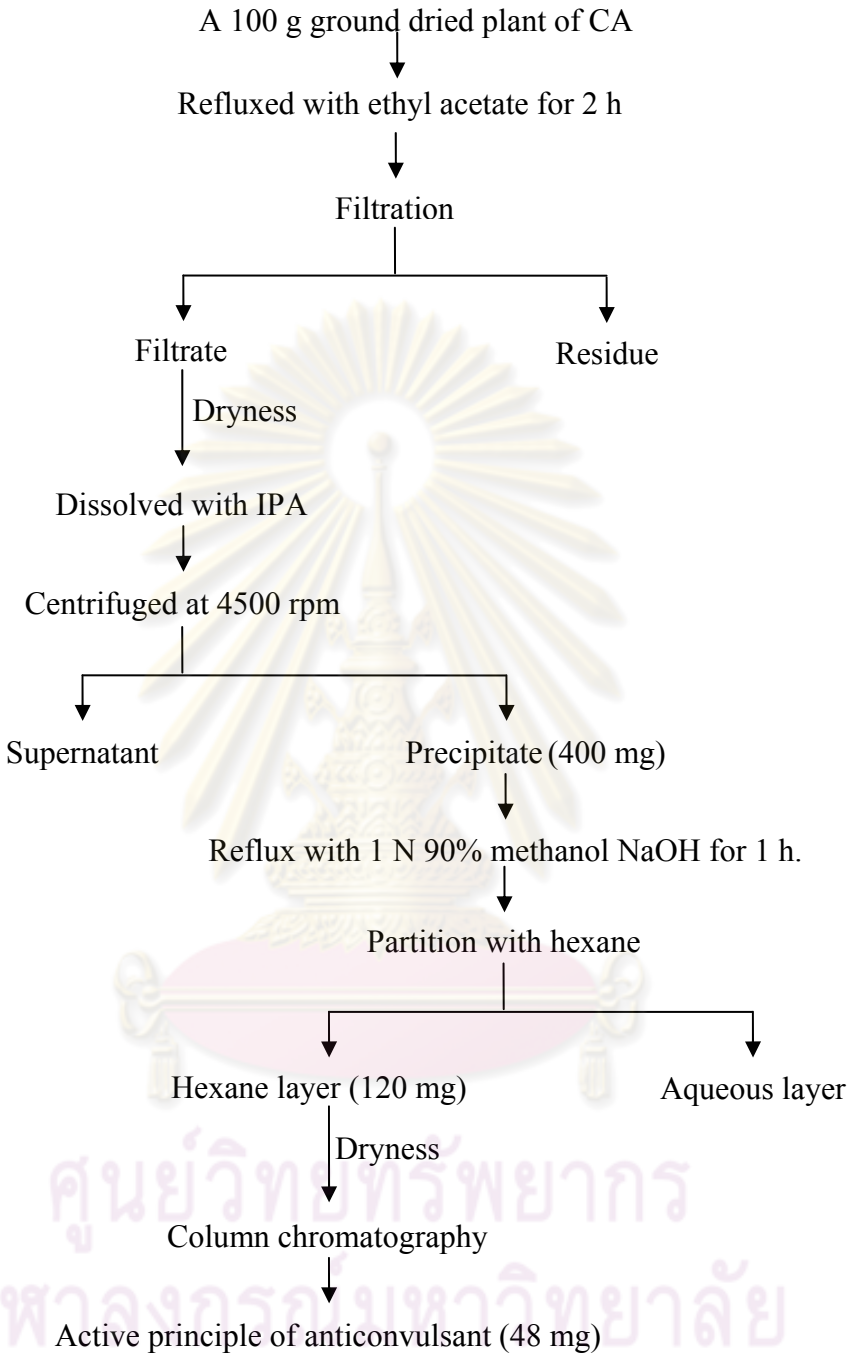


Scheme 3.1 Extraction and isolation scheme for anticonvulsant principle from CA

Scheme 3.2 is the diagram of modified method of extraction and isolation of active principle of anticonvulsant. The whole dried ground plant of CA (100 g) was refluxed with ten liters of hexane-ethyl acetate (8:2) for two hours and then filtered. The filtrate was concentrated to dryness under reduced pressure and then dissolved with isopropyl alcohol. The isopropyl alcohol fraction was centrifuged at 4500 rpm for 10 min to separate solution from precipitate. The precipitate was refluxed with 1 N 90% methanol sodium hydroxide for 60 minutes. The basic solution was partitioned with hexane and the hexane fraction was evaporated to dryness (yields about 120 mg). The active principle of anticonvulsant was obtained in hexane fraction.

A 120 mg of hexane fraction was chromatographed over silica gel column and eluted with the mixture of hexane-ethyl acetate by increasing polarity to separate pure active principle (yields about 48 mg). The target compound was identified by using TLC method compared with active principle of anticonvulsant that isolated from Scheme 3.1.

Repeating the procedure in Scheme 3.2 for 15 times yield the active principle of anticonvulsant about 600 mg, pooled the sample and dissolved in acetonitrile, filtered the insoluble particle and evaporated to dryness.



Scheme 3.2 Modified method of extraction and isolation scheme for anticonvulsant principle from CA

3.2 Identification of anticonvulsant principle

3.2.1 Anticonvulsant activity against PTZ model test

.

The PTZ seizures were elicited by a subcutaneous injection of PTZ 70 mg/kg body weigh to the animals. The end point of this chemoshock test was a generalized clonic seizure with loss of righting reflex within 60 min after injection of PTZ.

The pretreated substance was considered to possess anticonvulsant activity if no generalized clonic seizure with loss of righting reflex occurred within a period of 60 min after injection of PTZ.

Anticonvulsant activity of test substance against PTZ was performed on 2 sets of animals assigned for the oral (given by gavage tube) administration of the test substances. One set of animals was divided into various groups of 6-7 mice each; two groups (normal saline and CMC 0.3 ml/25g body weigh) were used as control groups. The PTZ test was performed after the optimal pretreated time was due. The anticonvulsant test was carried out at Department of Physiology, Faculty of Pharmaceutical sciences, Chulalongkorn University.

3.2.2 Chromatographic method

3.2.2.1 TLC method

Each isolated compounds and ethyl acetate crude extracts was dissolved in ethyl acetate. The solution was applied on a silica gel plate, which was then developed in suitable mobile phase, after that, the R_f value of each compounds were detected with 20% sulfuric acid in methanol.

Spray reagent

A 20 ml of concentrated sulfuric acid was dissolved in methanol and diluted to 100 ml.

TLC condition

Stationary phase : silica gel plate GF_{254} 10 x 10 cm

Developing solvent :

System 1) Ethyl acetate

System 2) Hexane-Ethyl acetate (8:2)

System 3) Hexane-Ethyl acetate-acetic acid (14:6:1)

The developed TLC-plate was sprayed with 10% sulfuric in methanol and heated at 120 ^{o}C for 20 min before detecting the compounds.

3.2.3.2 Gas chromatographic-Mass spectroscopy (GC-MS)

A 2 mg of each isolated active principle was methylation by acid in dry methanol. The fatty acid methyl ester was dissolved in heptane. The solution was injected to GC-MS system and then compared the retention time and mass spectrum to reference standard.

GC-MS system

Column	:	HP-Innowax (60m x 0.32 mm x 0.5µm)
Detector	:	MSD (scan range 50-50 amu)
Injector temp.	:	230 ^{0}C
Mode	:	Split (10:1)
Carrier	:	Helium (1.5 ml/min)
Oven temp. program		
	:	100 ^{0}C (1min) to 180 ^{0}C at rate 20 ^{0}C/min
		Hold 0 min and to 240 ^{0}C at rate 10 ^{0}C/min
		Hold 50 min

3.2.3 Spectroscopic methods

3.2.3.1 Infrared Spectroscopic method (IR)

Approximately 2 or 3 mg of each isolated active principle was mixed and grounded with about 15 mg of previously dried potassium bromide (KBr). The solid mixture was compressed to thin film KBr disc and scanned with IR spectrophotometer.

3.2.3.3 Nuclear-Magnetic Resonance (NMR)

Each isolated active compounds was weighed about 10 mg and dissolved with deuterated chloroform in a NMR tube. The mixture was measured in the NMR spectrospin.

IR spectrum of the active principle indicates the saturated fatty acid nature, which was supported by ^{1}H-NMR and ^{13}C-NMR techniques. For the ^{1}H-NMR the signal of methylene proton α to carboxyl group was found as triplet at 2.35 ppm while those proton β to carboxyl was found as multiplet at 1.65 ppm. The terminal methyl proton was found as triplet at 0.88 ppm. For ^{13}C-NMR spectrum of the isolated VLFAs showed in the upfield region (0-40 ppm) indicated the

aliphartic carbon. The carbonyl carbon appears at chemical ship about 179 ppm, shown in Figure 4.4, 4.5, 4.6, 4.7, 4.8 and 4.9.

The composition of the isolated saturated fatty acids and theirs methyl ester derivative were analyzed by GC-MS technique. The chromatogram indicated the mixture of very long-chain fatty acid (VLFAs) containing C_{12}-C_{28} chain that have octacosanoic acid (C_{28}) and hexadecanoic acid (C_{16}) as the major component, as shown in Figure 4.10, Table 4.1, 4.2 and 4.3.

3.3 Development of Thin-layer chromatographic densitometric analytical method (TLC-densitometric analytical method)

3.3.1 TLC-densitometric system

TLC silica gel plate (20 x 10 cm) and the developing phase of hexane-ethyl acetate-glacial acetic acid (14:6:1) was used in this study. The developed plate was sprayed with 3% phosphomolybdic acid in methanol and 20% sulfuric acid in methanol and heat at 120 ^{0}C for 20 min. The plate was scanned with a densitometer at 550 nm every 5 min interval. Plot the degree of reflection versus time to determine the optimum time to develop stable color.

3.3.2 Internal standard

Several criteria were purposed in finding the suitable internal standard as following; stable compound, no interaction with an analyte, detected by 3% phosphomolybdic acid spray reagent, resolved from the other component in the matrix sample. The following compounds were studied in process of finding internal standard; oleanolic acid, stearyl alcohol, hydroquinone, resorcinol, salicylic acid, 18β-glycyrhetic acid, dipotassium glycyrhezinate, and propyl paraben. Only oleanolic acid gave optimum resolution in the TLC analysis and served as internal standard.

3.3.3 Densitometer parameter

Photo mode	Reflection
Scan mode	Linear
Set zero mode	At start
Beam size	0.4 x 0.5 mm.
Wavelength	550 nm.

The developed plate was sprayed with 3% phosphomolybdic acid in methanol and heated at 120 ^{0}C for 20 min. The plate was kept at room temperature for 10 min before determining the absorbance of VLFAs with densitometer at the wavelength of 550 nm.

3.4 Method validation

3.4.1 Validation of TLC-densitometric analytical method

The developed TLC-densitometric analytical method was validated according to the ICH guideline on following parameters; accuracy, precision, specificity, linearity and range, detection limit, quantitation limit and robustness.

3.4.1.1 Preparation of standard solution

1) VLFAs standard stock solution

Accurately weighed 500 mg of VLFAs and dissolved in ethyl acetate and adjusted the volume to 50.0 ml as a stock standard solution (5.58 mg/ml).

2) Internal standard stock solution (IS)

Accurately weighed 100 mg of oleanolic acid and dissolved in ethyl acetate and adjusted the volume to 100.0 ml. (1.0 mg/ml)

3.4.1.2 Working standard solution

Seven working standard solutions (WS_1, WS_2, WS_3, WS_4, WS_5, WS_6 and WS_7) were prepared according to Table 3.1 and adjusted volume to 10.0 ml with ethyl acetate.

Table 3.1 Preparation of working standard solutions for method validation

Items	Solutions						
	WS_1	WS_2	WS_3	WS_4	WS_5	WS_6	WS_7
Stock standard VLFAs solution (ml)	1.0	2.0	3.0	4.0	5.0	6.0	70
Final volume (ml)	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Concentration of VLFAs working solution (mg/ml)	0.5	1.1	1.6	2.2	2.8	3.3	3.8

3.4.2 Standard calibration curve

Three replications of calibrated standard solutions (CS_1, CS_2, CS_3, CS_4, CS_5, CS_6 and CS_7) were applied to the TLC-densitometric analytical system. The calibrated standard solutions were prepared by pipetting 1.0 ml of each working standard solutions (WS_1, WS_2, WS_3, WS_4, WS_5, WS_6 and WS_7) and added stock internal standard solution 1.0 ml, adjusted volume to 10.0 ml with ethyl acetate, as shown in Table 3.2, and applied to TLC-densitometric analytical system. The standard calibration curve was plotted between peak area ratio (area of VLFAs/area of IS) versus the concentration of standard solution (mg/ml). The equation of the standard calibration curve was determined with the coefficient of determination (r^2), and then $r^2 \geq 0.99$ was accepted.

Table 3.2 Concentration of calibrated standard solutions

Items	Concentration of calibrated standard solutions (mg/ml)						
	CS_1	CS_2	CS_3	CS_4	CS_5	CS_6	CS_7
VLFAs	0.06	0.11	0.16	0.22	0.28	0.33	0.38
IS	0.1	0.1	0.1	0.1	0.1	0.1	0.1

3.4.3 Preparation of sample solutions

3.4.3.1 Stock sample solutions

A 10 g of dried ground plant of CA was refluxed with 90 ml of the mixture of hexane-ethyl acetate (8:2) for 90 minutes. The extract solution was cooled down to room temperature, filtered and adjusted the volume to 100.0 ml with ethyl acetate.

3.4.3.2 Sample solutions

For each five sample solutions, sample solutions (SS_1, SS_2, SS_3, SS_4 and SS_5) were prepared by pipetting 8.0 ml of stock sample solution, then added 1.0 ml of each working standard solutions (WS_1, WS_2, WS_3, WS_4, WS_5) and add stock internal standard solution 1.0 ml, the final volume was 10.0 ml.

Another sample solution (without standard addition, E_0) was prepared by pipetting 8.0 ml of stock sample solution and added 1.0 ml of internal stock standard solution and adjusted volume to 10.0 ml with ethyl acetate.

Table 3.3 Concentration of VLFAs spiked in sample solutions

Items	Concentration of VLFAs spiked in sample solutions (mg/ml)					
	SS_0	SS_1	SS_2	SS_3	SS_4	SS_5
VLFAs	0	0.06	0.11	0.16	0.22	0.28
IS	0.1	0.1	0.1	0.1	0.1	0.1

3.4.4 Accuracy

Six replications of each sample solution (E_0, E_1, E_2, E_3, E_4 and E_5) were applied 2 µl to TLC-densitometric analytical system and calculated the accuracy by using below equation.

The accuracy was determined in term of percent recovery (%R)

$$\%R = (\text{Analytical found}/\text{Actual added}) \times 100$$

3.4.5 Precision

Six replications of each sample solution (SS_0, SS_1, SS_2, SS_3, SS_4 and SS_5) were applied 2 µl to TLC-densitometric analytical system in the same days. The percent recoveries of each of sample solution were calculated as repeatability precision (or intra-day precision) by using the equation below.

Three replications of each sample solution (SS_0, SS_1, SS_2, SS_3, SS_4 and SS_5) were applied 2 µl to TLC-densitometric system for three days. The percent recoveries of each of sample solution in three days were calculated as intermediate precision (or inter-day precision) by using below equation.

The precision was determined in term of percent of coefficient of variation (%CV) or relative standard deviation (%RSD).

$$\%RSD = [SD / \bar{X}] \times 100$$

Where

SD = standard deviation of percent recovery

$\bar{X}$ = mean of percent recovery

3.4.6 Specificity

In this TLC-densitometric analytical system, representative spots should be used to demonstrate specificity. Specificity was demonstrated by the hR_f of the components which elute closest to each other.

The standard and sample solutions were applied 2 µl to TLC-densitometric analytical system. The detecting spot of all analytes in sample solution were compared with developing spot in standard solution.

The specificity was determined by spray reagent (20% sulfuric acid in methanol and 3% phophomolybdic acid in methanol). No other spot from sample solution overlap with the analytical spot in this TLC condition.

3.4.7 Linearity and range

Linearity and range should be evaluated by visual inspection of a plot of signals as a function of analyte concentration. If there should be a linear relationship, test results were evaluated by appropriate statistical methods by calculation of a regression line by the regression analysis. Data from the regression line itself was helpful to provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, slop of the regression line and residual sum of squares were submitted.

Each of sample solution (SS_0, SS_1, SS_2, SS_3, SS_4 and SS_5) was applied to TLC-densitometric analytical system. The graph was plotted between observed concentration and actuated concentration and calculate coefficient of determination (r^2) in term of linearity.

The linearity was determined by a series of three spots of five sample concentrations which were demonstrated as a graph between observed concentrations by analytical found of standard solution and actual concentrations by addition of standard solution.

The range was determined in term of interval concentration which demonstrated as good accuracy (96-104 %R), precision (RSD ≤2%) and linearity ($r^2 \geq 0.99$).

3.4.8 Detection limit (DL)

Detection limit (DL) is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be detected.

The detection limit was expressed as

$$DL = \frac{3.3\ \sigma}{S}$$

Where

σ = the residual standard deviation of a residual sum of squares of calibration curve

S = the slop of the calibration curve

3.4.9 Quantitation limit (QL)

Quantitation limit (QL) is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy (96-104%R) and precision (%RSD$\geq$2).

The quantitation limit was expressed as

$$QL = \frac{10\,\sigma}{S}$$

Where

σ = the residual standard deviation of a residual sum of squares of calibration curve

S = the slop of the calibration curve

3.4.10 Robustness

The evaluation of robustness should be considered during the development phase and depended on the type of procedure under. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

The robustness was susceptible to variation in analytical conditions, these should be suitable controlled. The analytical condition that used to test was variation of developing solution and distance from the origin to the solvent front of the analyte. The variation was twenty percent from the common condition.

3.4.11 Stability of VLFAs solution

A working standard solution of VLFAs, that have concentration about 2.8 mg/ml, was used to prepare the test solution. Pipet the working standard 1.0 ml, add 1.0 ml of IS solution and adjust volume to 10.0 ml with ethyl acetate. The final concentration was 0.28 mg/ml.

The test solution was applied 2 µl to TLC-densitometric system for evaluated the stability of sample solution under the ambient, 25 ^{0}C and 4 ^{0}C during 1, 3, 5 and 7 days.

3.5 Applications

3.5.1 Comparative study of extraction methods

3.5.1.1 Heat reflux

A 10 g of dried ground plant of CA was refluxed with 90 ml of various ratios of the mixture of hexane-ethyl acetate for two hours. The extract was filtered, cooled and finally applied to TLC-densitometry system. The contents of VLFAs were determined.

A 10 g of dried ground plant of CA was refluxed with 90 ml of the mixture of hexane-ethyl acetate (8:2) for 1, 5, 10, 30, 60, 90 120 and 180 min. The extract was filtered, cooled and finally applied to TLC-densitometry system. The contents of VLFAs were determined.

3.5.1.2 Ultrasonic-assisted extraction (UAE)

For the UAE experiments, an ultrasonic bath was used as an ultrasound source. The bath, Sonorex Digital 10P (BANDELIN, GERMANY), was a rectangular container (300 x 240 x 200, mm), which produced HF-frequency at 35 kHz.

The bath power rating was 205 W on the scale 0-100 %. The extraction of CA was performed by adding a 10 g of dried ground CA plant into 90 ml of the mixture of hexane-ethyl acetate (8:2) in the flask. The flask was then partially immersed into the ultrasonic bath, which contained six liters of water. The solvent surface in the flask was kept at the level of the water in the ultrasonic bath and regulated at constant temperature (65°C) to avoid the water temperature rise, caused by ultrasonic exposure. Sampling of extract was carried out at 1, 2, 3, 5, 10, 20, 30 and 60 min and the ultrasonic bath power with the three series of 10%, 50% and 100% of power. The extracts were filtered, cooled and finally applied to TLC-densitometry system. The contents of VLFAs were determined.

The stability of VLFAs during ultrasound exposure has also been investigated used. The solutions were sampling during the extraction time, which carried out for 1, 3, 5, 10, 20, 30 and 60 min with 100% of power. The solutions were cooled, added internal standard, adjusted the volume and then applied to TLC-densitometry system. The contents of VLFAs were calculated.

3.5.2 Collection of plant sample and storage

3.5.2.1 Source of plant sample

CA samples were collected from three locations in Thailand in Ampur Muang of Nakhon Pathom province (source A), Ubon Ratchathani province (source B) and Nakhon Si Thamarat province (source C)

CA samples from Thailand Institute of Scientific and Technological Research that cultivated the plant sample collected from Chaing mai, Chon Buri, Nakhon Si Thamarat, Phisanulok, Nakhon Ratchasima, Prachin Buri, Rayong, Trat and Sukhothai.

3.5.2.2 Sampling times

CA samples collected from Nakhon Pathom province were collected every two month during November, 2005 to September 2006 for annual study.

CA samples collected from Ubon Ratchathani province and Nakhon Si Thammarat province were collected every two month from May, 2006 to March 2007 for annual study.

Each CA samples were refrained from insecticide around 1 week before harvesting and the plants were cuts about 2 inches above the ground. At least 5 kg of fresh plant were collected each collection.

3.5.2.3 Storage of plant sample

Fresh collecting plant was cleaned with clean water to remove soil and other solid particle, and then air dried at room temperature for 3 days and subsequently dried by hot air oven at 50 ^{0}C for 24 hour. The dried plant samples were ground with milling machine. The dried ground plant samples were kept in 3 layers' polyethylene bag and the bag was kept in a plastic box placing in a dry area.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Extraction and isolation of VLFAs

A 100 g of ground dried CA was extracted and isolated of the VLFAs as mentioned in Scheme 3.1 to yield 7.0 mg (0.007% w/w).

A 100 g of ground dried plant was extracted and isolated of the VLFAs working standard as mentioned in Scheme 3.2. VLFAs was finally isolated by column chromatography to yield 44.6 mg (0.045% w/w) of VLFAs as off-white waxy solid.

Finally, 5 kg of collected fresh CA sample, obtained 500 mg of ground dried plant.

4.2 Identification of the isolated VLFAs

4.2.1 Anticonvulsant activity against PTZ model test

The VLFAs that have anticonvulsant activity in PTZ model (1 mg/kg body weight)

4.2.2 Chromatographic method

The isolated VLFAs can be identified by both chromatographic techniques and the spectrophotometric techniques.

Two chromatographic methods (TLC and GC-MS method) were selected by comparing the R_f value, the retention time and mass spectra of the isolated VLFAs to the VLFAs standards.

4.2.2.1 TLC method

TLC method was performed to screen the isolated VLFAs by the system as mentioned in 3.2.1

The R_f values of isolated VLFAs in system 1 (0.55), system 2 (0.23) and system 3 (0.52) was corresponded to the VLFAs standards, as shown in Figure 4.1-4.3.

4.2.2.2 GC-MS method

GC-MS method was performed to confirm the identified of the isolated VLFAs by the system as mentioned in 3.2.3.2.

The retention times and mass spectra of isolated VLFAs were corresponded to the standard fatty acids, as shown in Figure 4.10. The content of fatty acid in VLFAs was shown in Table 4.1, 4.2 and 4.3.

4.2.3 Spectrophotometric methods

Two spectrophotometric methods (infrared spectrometry, and nuclear magnetic resonance spectrometry) were selected for identification of the isolated compound.

4.2.3.1 Infrared spectrometry (IR)

VLFAs showed the characteristic absorption band in the IR spectrum as shown in Figure 4.4; broad band at 3520 cm^{-1} for OH stretching, 2918 cm^{-1} and 2849 cm^{-1} for methylene groups, that was aliphatic and nonstrained cyclic hydrocarbon, 1709 cm^{-1} for carbonyl group, respectively.

4.2.3.2 Nuclear magnetic resonance spectrometry (NMR)

^{1}H-NMR spectrum of VLFAs was shown in Figure 4.5, ^{13}C-NMR spectrum shown in Figure 4.6 and DEPT spectrum shown in Figure 4.7, respectively.

For ^{1}H-NMR spectrum (300 MHz) of the isolated VLFAs, was shown the signal in 0.88 ppm (triplet, terminal methyl), 1.3-1.4 ppm (singlet, large number of methylene protons in long chain), 1.65 ppm (multiplet, methylene that β to carbonyl group) and 2.35 ppm (triplet, methylene that α to carbonyl group)

For ^{13}C-NMR spectrum of the isolated VLFAs was shown in the upfield region (0-40 ppm) indicated the aliphartic carbon. The carbonyl carbon appeared at chemical ship about 179 ppm.

^{13}C-NMR spectrum and DEPT spectrum (500 MHz) of VLFAs that isolated followed Scheme 3.2 were shown in Figure 4.8 and 4.9, respectively.

4.2.4 Determination of percentage purity of VLFAs

Percentage purity of isolated VLFAs was 55.77 when calculated by comparing the peak area of the fatty acid and other compounds from GC spectrum, as shown in Table 4.3.

4.3 Development of analytical methods

4.3.1 Development of Thin-layer chromatographic densitometric analytical method (TLC-densitometric analytical method)

The 3% phosphomolybdic acid in methanol was used as spray reagent. Because of the color intensity of spot chromatogram was stable when kept longer than 30 min at room temperature after heat at 120 ^{o}C for 20 min. While color intensity of spot that developed by 20% sulfuric acid in methanol was unstable. Figure 4.11 shown reflection wavelength of the spot versus peak area of VLFAs after spray with 3% phosphomolybdic acid in methanol. Figure 4.12 showed color faded diagram of color's spot versus time.

TLC combined with densitometer was selected to determine VLFAs in the CA plant sample by using hexane-ethyl acetate-glacial acetic acid (14:6:1) as developing solvent. Figure 4.13 and 4.14 represented TLC spots and TLC densitograms of standard VLFAs, IS and sample of CA extract in the TLC-densitometric analytical method.

Chromatographic condition

TLC plate	:	Silica plate, GF 254, 10 x 20 cm
Developing solvent	:	Hexane-ethyl acetate-glacial acetic acid (14:6:1)
Detection	:	Spray with 3% phosphomolybdic acid in methanol, heat at 120 ^{o}C for 20 min and keep at room temperature for 10 min, Densitometer at wavelength 550 nm.

Densitometer parameter

Photo mode	:	Reflection
Scan mode	:	Linear
Set zero mode	:	At star
Beam size	:	0.4 x 0.5 mm.
Wavelength	:	550 nm

4.3.2 Selection of internal standard

Of all the eight compounds tested for the internal standard, only the oleanolic acid showed a spot appearance in between the spot of VLFAs and the other spot without interference the other compound, as shown in Figure 4.13. Therefore, oleanolic acid was chosen as internal standard.

4.4 Method validation

Accuracy, precision, specificity, linearity and range, detection limit, quantitation limit and robustness were considered in the method validation study.

4.4.1 Standard calibration curve

The standard calibration curve of standard VLFAs were plot between peak area ratio (VLFAs/IS) and concentration of standard VLFAs solutions. The equation coefficient of determination was $y = 5.8542x - 0.4032$. The equation coefficient of determination (r^2) was 0.9959, as shown in Figure 4.15. The concentration of standard calibration curve was in the range 0.11-0.38 mg/ml.

4.4.2 Accuracy

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. The true for accuracy assessment can be obtained in several ways. One alternative is to compare results of the method with results from an established reference method. Secondly, accuracy can be assessed by analyzing a sample with known concentrations, for example, a certified reference material, and comparing the measured value with the true value as supplied with the material. If such

certified reference material is not available, no blank-sample matrix of interest can be spiked with a known concentration by weight or volume, which is selected in this study. After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent.

Accuracy was tested in term of percentage recovery by addition of standard solution in various concentrations. Percent recovery of VLFAs was in the range of 98.64-102.99 and average of percent recovery was 100.27. As presented in Table 4.4. The accuracy test of this method was in the acceptance criteria (96-104 %R). Therefore the method could be used to determine the analytical compounds in CA plant sample.

4.4.3 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision is usually expressed as the variance, relative standard deviation (%RSD) or coefficient of variation of a series of measurements. Precision was considered at two levels: repeatability (intra-day precision) and intermediate precision (inter-day precision).

Intra-day precision

Relative standard deviation was calculated from six replications of sample preparation in one day at various concentrations. It found that %RSD of VLFAs was in the range of 0.46-1.59. Mean %RSD of VLFAs was 0.82. As presented in Table 4.4. These relative standard deviations of intra-day precision were in the range of acceptance criteria (%RSD ≤ 2). Therefore the method could be used to determine the analytical compounds in CA plant sample.

Inter-day precision

Relative standard deviation was calculated from three replications of sample preparation for three days at various concentrations. It found that %RSD of VLFAs was in range of 0.35-1.71. Mean %RSD of VLFAs was 0.67. As presented in Table 4.5. These relative standard deviations of inter-day precision were in the range of acceptance criteria (%RSD ≤ 2). Therefore the method could be used to determine the analytical compounds in CA plant sample.

4.4.4 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

Figure 4.13 and 4.14 represented the TLC spot and TLC densitogram from TLC-densitometer of each compound in standard solution and sample solution. Densitogram of each compound in sample solution was identical to chromatogram in standard solutions.

4.4.5 Linearity and range

Linearity should be evaluated by visual inspection of a plot of signal as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data might be subjected to mathematical transformation prior to the regression analysis.

The linear equations of VLFAs was obtained as following; y = 1.0111x-0.0023. The coefficient of determination (r^2) was 0.9994. These r^2 showed a good relation of standard addition and amount of analytical found.

Ranges of concentration of VLFAs that show good linear correlation between concentration versus response with the acceptable accuracy (96-104 %R) and precision (%RSD $\leq$ 2) was 0.11-0.38 mg/ml, as shown in Table 4.6 and Figure 4.16.

4.4.6 Detection Limit

Detection limit (DL) was determined during the evaluation of the detection of analyte. DL was defined as the lowest concentration that can be detected. The concentration of DL was the same concentration or less than QL.

DL of VLFAs standard solution was resulted 30 µg/ml. This concentration can be detected after spray with 3% phosphomolybdic acid in methanol and heat. The result was calculated by parameter that shown in Table 4.7.

4.4.7 Quantitation Limit

Quantitation limit (QL) was determined during the evaluation of the linear range of calibration curve. QL was defined as the lowest concentration yielding a precision with %RSD less than 2 and accuracy within 4% of the theoretical value (96-104%R).

QL of VLFAs was resulted 0.10 mg/ml. The result was calculated by parameter that shown in Table 4.7. Percent relative standard deviation of six replications was less than 2. Percentage recovery was between 101.39-103.69%. The results were shown in Table 4.8. The calculated QL was shown the linearity in calibration curve, as mentioned in Figure 4.17.

4.4.8 Robustness

The robustness was susceptible to variation in analytical conditions these should be suitable controlled. The analytical condition was varied twenty percent if developing phase and distance from the origin to the solvent front from the common condition.

The results were shown in Table 4.9 and 4.10. The percent recovery of VLFAs in the any condition was in 96-104% and RSD less than 2%. The pattern of spot in the any conditions had the same pattern like the normal condition.

4.4.9 Stability of VLFAs standard solution

VLFAs and IS test solution were prepared. These test solution were evaluated for stability program as followed; under the ambient, 25 ^{0}C and 4°C.

From the data obtained as shown in Table 4.11 and Figure 4.18, the concentration of VLFAs and IS in test solution remain unchanged throughout the period of stability program at least 7 day in any condition. The standard solution was kept in 4 ^{0}C and used in 7 days.

4.5 Applications

4.5.1 Comparative study of extraction methods

4.5.1.1 Heat reflux extraction

When refluxing the CA plant sample with 90 ml of the mixture of hexane-ethyl acetate in various ratios for 120 minutes, as shown in Table 4.12 and Figure 4.19. The mixture of 8:2 hexane:ethyl acetate gave the highest amount of VLFAs in the extract. Therefore, the mixture of 8:2 hexane:ethyl acetate was chosen as the extracting solvent.

To determine the optimum refluxing time as shown in Table 4.13 and Figure 4.20, the percentages of VLFAs per weight of dried plant sample was increased with the increment of refluxing time and remain constant after refluxing the mixture more than 90 min.

Conclusive, the optimum sample preparation was followed; ten gram of dried ground sample was refluxed with 90 ml of 8:2 hexane:ethyl acetate for 90 min.

4.5.1.2 Development of ultrasonic-assisted extraction (UAE)

4.5.1.2.1 Optimization of UAE condition

The mixture of 8:2 hexane:ethyl acetate gave the highest amount of VLFAs in the extract. Therefore, the mixture of 8:2 hexane:ethyl acetate was chosen as the extracting solvent in this experiment.

The ultrasonic bath was performed to extract and carried out with the three series of 10%, 50% and 100% of power at 65°C to determine the optimum extraction time versus the extraction power as shown in Table 4.14 and Figure 4.21. The percentage of VLFAs per weight of dried plant sample was increased with the increment of extraction time and remains constant after extracting more than 20 min. The maximum percentage contents of VLFAs were 0.17 %w/w. Although the maximum power (100%) was increased the cavitations effect during the extraction; thus should be obtained the highest extraction yields with shortened times, unfortunately it was obtained the less contents of VLFAs than the 50% of power.

Therefore, the optimum UAE condition was followed; ten gram of dried ground sample was extracted with 90 ml of 8:2 hexane:ethyl acetate for 20 min using the 50% of ultrasonic power at 65°C.

4.5.1.2.2 Effect of ultrasound on the active compounds

When the extraction was performed by using a high frequency of ultrasound, the extraction time was decreased significantly. However, the degradation of the active contents may possible occurred. Therefore the stability of VLFAs during the UAE was studied and the stability was calculated in term of percentage recovery as shown in Table 4.15 and Figure 4.22.

The result showed that the frequency of ultrasound do not induced degradation during the extraction.

4.5.2 Determination of VLFAs contents in various CA samples

4.5.2.1 Collection and storage of CA samples

CA plant samples were collected on July, September and November of the year 2005 from 12 accessions, cultivated by Thailand Institute of Scientific and Technological Research, as shown in Table 3.4.

CA plant samples were collected during the second week of each month of the year 2005-2006 from the commercial crops in Nakhon Pathom province, which labeled as CA13 as shown in Table 3.5.

CA plant samples were collected during the second week of each month of the year 2006-2007 from the commercial crops in Ubon Ratchathani and Nakhon Si Thammarat province, as shown in Table 3.5.

4.5.2.2 Contents of VLFAs in various CA accession

In this study, a 12 accessions of CA sample were collected from various locations of Thailand which then propregated and cultivated by the post harvest research team of Thailand Institute of Scientific and Technological Research, the sample were prepared to determine the contents of their active compounds.

Each CA dried plant sample was quantitative determined for VLFAs by the TLC-densitometric analytical system as in 3.3 and the percentage contents of this compound were shown in Table 4.16 and Figure 4.23.

From the data obtained, VLFAs in each CA dried sample was found in the range of 0.0324 – 0.2683 % with average at 0.1705%. The maximum content was observed in CA6 which collected from Rayong province and the minimum content was observed in CA11 which collected from Phitsanulok province.

4.5.2.3 Contents of VLFAs in annual study

In this study, CA samples that collected annually from the commercial crops of Nakhon Pathom province (CA13), Ubon Ratchathani province (CA3) and Nakhon Si Thammarat province (CA5) were also determined the content profiles of their active compounds.

Each CA 13, CA3 and CA5 that collected annually was quantitative determined for VLFAs by the TLC-densitometric analytical system as in 3.3 and the percentage contents of this compound was shown in Table 4.17, 4.18 and 4.19, and Figure 4.24.

From the data obtained, the contents of active compounds that observed in CA13 were 0.0442-0.1376 % w/w. The maximum content of VLFAs was observed in sample that collected on November (0.1376% w/w) and the minimum content was observed in sample that collected on May (0.0442% w/w) and July (0.0446% w/w). The results were shown in Figure 4.24 (a).

From the data obtained, the contents of active compounds that observed in CA3 were 0.1451-0.2100% w/w. The maximum content of VLFAs was observed in sample that collected on September (0.2100% w/w) and November (0.2086% w/w) and the minimum content was observed in sample that collected on March (0.1451% w/w). The results were shown in Figure 4.24 (b).

From the data obtained, the contents of active compounds that observed in CA5 were 0.1666-0.2188% w/w. The maximum content of VLFAs was observed in sample that collected on May (0.2188% w/w) and the minimum content was observed in sample that collected on September (0.1666% w/w). The results were shown in Figure 4.24 (c).

The content profiles of CA13 and CA3 were also found the maximum contents of VLFAs in sample that collected on November as presented in Figure 4.24. However, the maximum content of VLFAs of CA3 and CA5 that collected from original sources and presented in annually in this study was lower than other CA samples that cultivated by Thailand Institute of Scientific and Technological Research.

CHAPTER V

CONCLUSION

5.1 Extraction, isolation and characterization of the active anticonvulsant principle in *Centella asiatica* (L.) Urban (CA).

The active anticonvulsant principle in CA, that elucidated by IR, NMR and GC-MS, was Very long-chain fatty acids (VLFAs) containing C_{12}-C_{28}, which containing C_{16} and C_{28} chain acids as main compounds.

The off white waxy solid of VLFAs 0.446 g (yield = 0.045% w/w) was extracted from 1 kg if dried ground CA, which was 55.77 % purity of VLFAs.

5.2 Thin-Layer Chromatographic desitometric analytical method (TLC-densitometric analytical method)

TLC method combined with densitometer was used to determinated of VLFAs in *Centella asiatica* by using hexane-ethyl acetate-glacial acetic acid (14:6:1) as developing solvent. The developed TLC plate was sprayed with 3% phosphomolybdic acid in methanol and heated at 120 ^{o}C for 20 min. The plate was kept at room temperature for 10 min and detected with densitometer at wavelength of 550 nm.

Method validation was studied with accuracy, precision, specificity, linearity and range, detection limit, quantitation limit and robustness. The accuracy presented as percent recovery was found in the range of 94.00-106.0%. The intra-plate precisions measured as %RSD was found less than 3. The linearity was determined by spotting the solutions, that amount range of 0.11-0.38 mg/ml of VLFAs. The good linearity was measured as linear regression (r^2) ≥ 0.999 with insignificant intercept from origin. The QL of VLFAs was determined 0.10 mg/ml and DL was 30 µg/ml in this condition. Robustness for these analytical conditions was flexible in 20% of variation in developing phase and TLC plate length. The data of TLC analytical method validation was shown in Table 5.1.

5.3 Development of extraction method

Heat reflux extraction and ultrasonic-assisted extraction (UAE) were used to extract the interested compound from the dried ground of CA. This study revealed that heat reflux extraction was showed the maximum percent contents of VLFAs as 0.1382 %w/w. The maximum percent content of VLFAs of UAE was showed as 0.1696 %w/w.

By comparing various extraction methods for VLFAs, ultrasonic-assisted extraction (UAE) was more efficient by reflux, which has time consuming techniques. UAE was also required 20 minutes to reach the exhausted extraction. However, the heat reflux extraction technique was required more than 90 minutes to reach the exhausted extraction.

These findings agree with previous studies in that the alternative extraction techniques such as UAE is powerful technique; thus can be applied to large scale of samples.

5.4 Determination of VLFAs in various CA samples

The present study has revealed the presence of considerable content variation of active compounds in the 12 CA accessions collected from different parts of Thailand and cultivated at Thailand Institute of Scientific and Technological Research. The percentage content of VLFAs in various CA samples was showed in Table 4.16. The average of percentage contents of active compound in dried ground plant of various CA samples was 0.1705%. The maximum contents VLFAs that obtained from CA samples was 0.2683%w/w (Rayong province), 0.2411%w/w (Sukothai province), 0.2399%w/w (Nakhorn Ratchasima province) and 0.2143%w/w (Ban Bo Lo province), respectively.

Information on chemical constituents of these accessions can now be further used as the criteria for plant variety selection.

5.5 Determination of VLFAs of CA samples in annual study

Table 4.17 showed percentage contents of VLFAs in the dried ground of CA that collected every 2 month from the commercial crop at Nakhon Pathom province. The maximum contents of VLFAs (0.1376%w/w) was obtained from CA, which collected on November.

Table 4.18 showed percentage contents of VLFAs in the dried ground of CA that collected every 2 month from the commercial crop at Ubon Ratchathani province. The maximum content of VLFAs was 0.2100%w/w found in CA, which also collected on September.

Table 4.19 showed percentage contents of VLFAs in the dried ground of CA that collected every 2 month from the commercial crop at Nakhon Si Thammarat province. The maximum content of VLFAs was 0.2188%w/w found in CA, which also collected on May. .

Information on the chemical content profiles in this study may be utilized as criteria for further used in breeding programs for high biomass of VLFAs in commercial cultivars.

REFERENCES

(1) Nsour W.M., *et al.* Review on phytotherapy in epilepsy. Seizure 9 (2000): 96-107.

(2) Shukla A., *et al*. In vitro and in vivo wound healing activity of asiaticoside isolated from *Centella asiatica*. Journal of Ethnopharmacology 65 (1999): 1-11.

(3) Sulochana B., *et al*. *Centella asiatica* treatment during postnatal period enhances learning and memory in mice. Physiology & Behaior 86 (2005): 449-457.

(4) Kwanchanok Pramongkit. Active constituents of *Centella asiatica* (Linn.) Urban in Thailand. Master's Thesis, Faculty of Pharmaceutical Sciences, Mahidol University, 1995.

(5) Suntornsak L. and Anurukvorakun O. Precision improvement for the analysis of flavonoids in selected Thai plants by capillary zone electrophoresis. Electrophoresis 26 (2005): 648-660.

(6) Fact and comparisons. The review of natural products : the most complete source of natural product information., St. Louis, 2001.

(7) British herbal pharmacopoeia. Bournemouth: Megaron Press, 1983.

(8) British pharmacopoeia. Bournemouth: Megaron Press, 2002.

(9) World Health Organization. Herba Centellae. WHO monograph on selected Medicinal Plants 1 (1999): 77-85.

(10) Vattanajun Anusara, *et al*. Isobolographically additive anticonvulsant activity between *Centella asiatica*'s ethyl acetate fraction and some antiepileptic drugs. J.med Assoc Thai. 88 (2005): s131-s140.

(11) Vilkhu Kaniljit, *et al*. Applications and opportunities for ultrasound assisted extraction in food industry – A review. Innovation Food Science and Emerging Technologies. 9 (2008): 161-169.

(12) Brinkhaus, B., *et al.* *Centella asiatica* in traditional and modern phytomedicine A pharmacological and clinical profile - Part I: Botany, chemistry, preparations. Perfusion 11 (1998): 466-74.

(13) Brinkhaus, B., *et al*. *Centella asiatica* in traditional and modern phytomedicine A pharmacological and clinical profile - Part II: Pharmacological and therapeutical profile, conclusions**.** Perfusion 11 (1998): 508-20.

(14) Brinkhaus, B., Lindner, M., Schuppan, D., and Hahn, E.G. Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. Phytomedicine 7 (2000): 427-428.

(15) Collins, D.J., Pilotti, C.A., and Wallis, A.F.A. Triterpene acids from some Papua New Guinea Terminalia species. Phytochemistry 31 (1992): 881-884.

(16) Inamdar, P.K., Yeole, R.D., Ghogare, A.B., and De Souza, N.J. Determination of biologically active constituents in *Centella asiatica*. J. Chromatogr. A 742 (1996): 127-130.

(17) Sahu, N.P., Roy, S.K., and Mahato, S.B. Spectroscopic determination of structures of triterpenoid trisaccharides from *Centella asiatica*. Phytochemistry 28 (1989): 2852-2854.

(18) Sung, T.V., Lavaud, C., Porzel, A., Steglich, W., and Adam, G. Triterpenoids and their glycosides from the bark of *Schefflera octophylla*. Phytochemistry 31 (1992): 227-231.

(19) Wong, K.C., and Tan, G.L. Essential oil of *Centella asiatica* (L.) Urb. Sch. Journal of Essential Oil Research 6 (1994): 307.

(20) Newall, C.A., Anderson, L.A., and Phillipson, J.D. Herbal medicines. London: Pharmaceutical Press, 1996.

(21) Ramaswamy, A.S., Periyasamy, S.M., and Basu, N.K. Pharmacological studies on *Centella asiatica*. Journal of Research and Education in Indian Medicine 4 (1970): 160.

(22) Gupta Y.K., Kumar M.H. Veerandra and Srivastara A.K.. Effect of *Centella asiatica* on pentylenetetrazole-induced kindling cognition and oxidative stress in rats. Pharmacology, Biochemistry and Behavior. 74 (2003): 579-585.

(23) Kumar M.H Veerendra and Gupta Y.K.. Effect of different extracts of *Centella asiatica* on cognition and markers of oxidative stress in rats. Journal of Ethnopharmacology 79 (2002): 253-260.

(24) Sakina MR. and Dandiya PC. A psychopharmacological profile of *Centella asiatica* extract. Fitoterapia 96 (1990): 291-296.

(25) Wattanathorn Jintanaporn, *et al*. Positive modulation of cognition and mood in the healthy elderly volunteer following the administration of *Centella asiatica*. Journal of Ethnopharmacology 116 (2008): 325-332.

(26) Sedlarik K.M. The processes of wound healing. Wound Forum Online.

(27) Byung Chul Park, *et al*. Asiatic acid induces apoptosis in SF-MEL-2 human melanoma cells. Cancer Letters. 218 (2005): 81-90.

(28) Bunpo P., *et al*. Inhibitory effects of *Centella asiatica* on azoxymethane-induced aberrant crypt focus formation and carcinogenesis in the intestines of F344 rats. Food and Chemical Toxicology. 42 (2004): 1987-1997.

(29) Chuen Lung Cheng, *et al*. The healing effects of *Centella* extract and asiaticoside on acetic acid induced gastric ulcers in rats. Life Science. 74 (2004): 2237-2249.

(30) Cheng C.L. and Koo M.W.L. Effects of *Centella asiatica* on ethanol induced gastric mucosal lesions in rats. Life Science. 67 (2000): 2647-2653.

(31) Mahanom Hussin, *et al*. Protective effect of *Centella asiatica* extract and powder on oxidative stress in rats. Food Chemistry. 100 (2007): 535-541.

(32) Jayashree G., Muraleedhara G.Krup, Sudarslal S. and Jacob V.B.. Anti-oxidant activity of *Centella asiatica* on lymphoma-bearing mice. Fitoterapia. 74 (2003): 431-434.

(33) Zainol M.K., Abd-Hamid A., Yusof S., and Muse R.. Antioxidative activity and total phenolic compounds of leaf, root and petiole of four accessions of *Centella asiatica* (L) Urban. Food Chemistry. 81 (2003): 575-581.

(34) Hamid A Abdul, Sheh Z. Md., Muse R. and Mohamed S. Characterization of antioxidant activities of various extract of *Centella asiatica* (L) Urban. Food Chemistry. 77 (2002): 465-469.

(35) Xue Song Wang, Liu Liu and Ji Nian Fang. Immunological activities and structure of pectin from *Centella asiatica*. Carbohydrate Polymers. 60 (2005): 95-101.

(36) Xue Song Wang, Qun Dong, Jian-Ping zuo and Ji-Nian Fang. Structure and potential immunological activity of pectin from *Centella asiatica* (L) Urban. Carbohydrate Research. 338 (2003): 2393-2402.

(37) Xue Song Wang, Yun Zheng, Jian Ping zuo and Ji-Nian Fang. Structure factors of an immunoactive acidic arabinogalactem from *Centella asiatica*. Carbohydrate Polymers. 59 (2005): 281-288.

(38) Jayathirtha M.G and Mishra S.H.. Preliminary immunomodulatory activities of methanol extracts of *Eclipta alba* and *Centella asiatica*. Phytomedicine. 11 (2004): 361-365.

(39) Singh Pratibha and Singh J.S.. Recruitment and competitive interaction between ramets and seedings in a perennial medicinal herb, *Centella asiatica*. Basic Appl. Ecol. 3 (2002): 65-76.

(40) Romanik, G., Gilgenast, E., Przyjazny, A. and Kaminski. Techniques of preparing plant material for chromatographic separation and analysis. J.Biochem.Biophys.Methods. 70 (2007): 253-261.

(41) Wang L. and Weller C.L. Recent advances in extraction of nutraceuticals from plants. Trends in Food Science & Technology. 17 (2006): 300-312.

(42) Hemvimol Surasak, Parasant Prasert and Shotipruk Artiwan. Ultrasound-assisted extraction of anthraquinones from roots of *Morinda citrifolia*. Ultrasonics sonochemistry. 13 (2006): 543-548.

(43) Yi Yang and Fan Zhang. Ultrasound-assisted extraction of rutin and quercetin from *Euonymus alatus* (Thunb.) Sieb. Ultrasonics sonochemistry. 15 (2008): 308-313.

(44) Paniwnyk L., Beaufoy E., Lorimer J.P. and Mason T.J.. The extraction of rutin from buds of *Sophora Japonica*. Ultrasonics sonochemistry. 8 (2001): 299-301.

(45) Shuna Zhao, Kin-Chor Kwok and Hanhua Biang. Investigation on ultrasound assisted extraction of saikosaponins form *Radix Bupleuri*. Separation and Purification Technology. 55 (2007): 307-312.

(46) Ivana T. Stanisavljevic, Lazie M.L. and Veljkovic V.B.. Ultrasonic extraction of oil from Tobacco (*Nicotiana tabacum* L.) seed. Ultrasonics sonochemistry. 14 (2007): 646-652.

(47) Romdhane M. and Gourdon C.. Investigation in solid-liquid extraction : Influence of ultrasound. Chemical Engineering Journal. 87 (2002): 11-19.

(48) Alba S., Joyce E., Paniwnyk L., Lorimer J.P. and Moron T.J.. Potential for the use of ultrasound in extraction of antioxidants form *Rosmarinus officinalis* for the food and pharmaceutical industry. Ultrasonics sonochemistry. 11 (2004): 261-265.

(49) Mauricio A. Rostagno, Miguel Palma and Carmelo G. Barroso. Ultrasound-assisted extraction of soy isoflavones. Journal of Chromatography A. 1012 (2003): 119-128.

(50) Jianyoung Wu, Lidong Lin and Foo-tim Chan. Ultrasound-assisted extraction ginseng saponins from ginseng roots and cultured ginseng cells. Ultrasonics sonochemistry. 8 (2001): 347-352.

(51) Vinatiru M., *et al*. The use of ultrasound for the extraction of bioactive principles from plant materials. Ultrasonics sonochemistry. 4 (1997): 135-139.

(52) Somwong Pathom. Isolation, purification and quantitative determination of Asiaticoside, Madecassoside, Asiatic acid and Madecassic acid in varieties of *Centella asiatica* (L.) Urb. Master's Thesis, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, 2006.

(53) Kongthong Bungon. Development of the quantitative determination of Asiaticoside, Madecassoside, Asiatic acid and Madecassic acid in *Centella asiatica* (Linn.) Urban by high-performance liquid chromatography. Master's Thesis, Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, 2004.

(54) Yukari Ikeda, *et al*. Quantitative determination of cardiac glycosides in *Digitalis lanata* leaves by reversed-phase thin-layer chromatography. Journal of Chromatography A. 746 (1996): 255-260.

(55) Shalaby A.R.. Simple, rapid and valid thin-layer chromatographic method for determining biogenic amine in foods. Food Chemistry. 65 (1999): 117-121.

(56) Joerg Stroka and Elke Anklam. Development of a simplified densitometer for the determination of aflatoxins by thin-layer chromatography. Journal of Chromatography A. 904 (2000): 263-268.

(57) Jerome Bouligand, *et al*. Quality control and stability using HPTLC : applications to cyclophosphamide in various pharmaceutical products. Journal of Pharmaceutical and Biochemical Analysis. 38 (2005): 180-185.

(58) Hellmut Jork, Werner Funk, Walter Fisher and Hans Wimmer. Thin-Layer Chromatography : Reagent and Detection Methods. Vol.19 (1990): 376-379.

(59) Breda Simonoska and Irena Vovk. High-performance thin-layer chromatographic determination of potato glycosides. Journal of Chromatography A. 903 (2000): 219-225.

APPENDICES

Table 3.4 Various CA samples cultivated by Thailand Institute of Scientific and Technological Research

Sources of CA	Sample codes	Collecting times
Prachin Buri	CA1	July
Ban Bo Lo (local growth) Nakhon Si Thammarat	CA2	September
Ubon Ratchathani	CA3	July
Trat	CA4	November
Ban Bo Lo (trade) Nakhon Si Thammarat	CA5	July
Rayong	CA6	July
Chon Buri	CA7	July
Pak Chong Nakhon Ratchasima	CA8	September
Sukhothai	CA9	September
Chiang Mai (green petioles)	CA10	November
Phitsanulok	CA11	November
Chiang Mai	CA12	July

Table 3.5 Various CA samples collected from the commercial crops of Nakhon Pathom (CA13), Ubon Ratchathani and Nakhon Si Thammarat province

Sampling times (Month)	Sources of CA and codes		
	Ubon Ratchathani	Nakhon Si Thammarat	Nakhon Pathom
January	CA3-1	CA5-1	CA13-1
March	CA3-3	CA5-3	CA13-3
May	CA3-5	CA5-5	CA13-5
July	CA3-7	CA5-7	CA13-7
September	CA3-9	CA5-9	CA13-9
November	CA3-11	CA5-11	CA13-11

Table 4.1 GC peak area of isolated fatty acids (analyzed by AMARC)

Compounds	Data	
	Retention time (min)	Area
Dodecane	5.241	2640689
Dodecamethyl-pentasiloxane	5.285	3255719
Tetradecane	6.148	2521580
[[4-[1,2-bis[(trimethylsilyl)oxy}ethyl}-1,2-phenylene} bis [trimethyl-silane]	6.362	2138292
2,6,11-Trimethyl-dodecane	6.578	1685094
Octadecamethyl-cyclononasiloxane	7.410	1921615
Hexadecane	7.607	3268757
4-Methyl-hexadecane	8.052	4047872
Heptadecane	8.350	2410841
1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	8.430	1639094
9-Octyl-heptadecane	9.133	3547212
Octadecane	9.183	3910800
Dodecanoic acid, Methyl ester	9.389	7708554
Eicosane	9.590	7342932
4,6-Dimethyl-2-pyrimidinamine	9.980	3495674
1-(4-Fluorophenyl)-2-[(4-hydroxy-6-methylpyrimidin-2-yl) thio] ethan-1-one	10.029	8515208
4-(3-Hydroxy-2,2,,6-trimethyl-7-oxa-bicyclo[4.1.0]hept-1-yl)-but-3-en-2-one	10.078	3276547
2-Methoxy-thiophene	10.141	9062775
2,6-Dimethyl-4-pyrimidinamine	10.329	23078011
4-n-Dodecylresorcinol	10.633	48897804
Divinylbis(cyclopropyl)silane	10.722	20752026
m-Menth-1(7)-ene	11.064	22101527
Methyl tetradecznozte	11.177	35212638
Henecicosane	11.808	6584473
4[-4[-[p-[n-hexyloxyphenyl]butylamino]-1,2-naphthoquinone	11.905	6039245
5,9,13-Trimethyl-tetradecanoic acid, Methyl ester	12.075	872456
Pentadecanoic acid, Methyl ester	12.142	13260574
6,10,14-Trimethyl-2-pentadecanone	12.279	42627074
2,6,10,14-Tetramethyl-hexadecane	12.479	6072068
3,12-Diethyl-2,5,9-tetradecatriene	12.609	5141605
Docosane	12.819	13290215
Hexadecanoic acid, Methyl ester	13.246	191180748
9-Hexadecanoic acid, Methyl ester	13.582	11185979

Table 4.1 GC peak area of isolated fatty acids (analyzed by AMARC) (continue)

Compounds	Data	
	Retention time (min)	Area
Triacontane	13.978	8991170
15-Methyl-hexadecanoic, Methyl ester	14.523	5545428
Phosphorochloridic acid, Methyl ester	15.013	8323029
Tetracosane	15.354	10884313
Octadecanoic acid, Methyl ester	16.064	65085297
2,2'-(1,1,2,2,3,3-hexafluoro-1,3-propanedily)bis-thiophene	16.309	8633477
8-Octadecanoic acid, Methyl ester	16.542	44971124
Pentacosane	17.027	15503096
9,12-Octadecadienoic acid(Z,Z)-, Methyl ester	17.498	23790932
Hexacosane	19.091	13356281
Eicosanoic acid, Methyl ester	20.320	27099617
7,10-Octadecadienoic acid, Methyl ester	21.034	7428829
Heptacosane	21.679	21411719
Heneicosanoic acid, Methyl ester	23.301	9732682
Octacosane	24.944	14020258
Phthalic acid, Hexadecyl methyl ester	25.820	6283767
Docosanoic acid, Methyl ester	27.094	37805842
Nonacosane	29.079	28115658
Tricosanoic acid, Methyl ester	31.936	21644204
Octacosanoic acid, Methyl ester	33.932	273080301
Tetracosanoic acid, Methyl ester	38.135	36126349
Di-n-octyl phthalate	48.408	72604639

Table 4.2 The content of VLFAs calculated base on GC-MS

Compound (methyl ester)	Structure	Molecular weight	% content in fatty acid, methyl ester
Dodecanoic acid	$C_{12}H_{24}O_2$	200	1.07
Tetradecanoic acid	$C_{14}H_{28}O_2$	228	4.90
Pentadecanoic acid	$C_{15}H_{30}O_2$	242	1.85
Hexadecanoic acid	$C_{16}H_{32}O_2$	256	26.63
Octadecanoic acid	$C_{18}H_{36}O_2$	284	9.07
Eicosanoic acid	$C_{20}H_{40}O_2$	312	3.77
Heneicosanoic acid	$C_{21}H_{42}O_2$	326	4.36
Docosanoic acid	$C_{22}H_{44}O_2$	340	5.27
Tricosanoic acid	$C_{23}H_{46}O_2$	354	3.01
Tetracosanoic acid	$C_{24}H_{48}O_2$	368	5.03
Octacosanoic acid	$C_{28}H_{56}O_2$	424	38.04

Table 4.3 Purity of fatty acids in isolated VLFAs base on GC peak area

Compound (methyl ester)	Number of carbon atom	GC peak area	% content in sample, methyl ester
Dodecanoic acid	12	7708554	0.60
Tetradecanoic acid	14	35212638	2.74
Pentadecanoic acid	15	13260574	1.03
Hexadecanoic acid	16	191180748	14.85
Octadecanoic acid	18	65085297	5.06
Eicosanoic acid	20	27099617	2.11
Heneicosanoic acid	21	9732682	0.76
Docosanoic acid	22	37805842	2.94
Tricosanoic acid	23	21644204	1.68
Tetracosanoic acid	24	36126349	2.81
Octacosanoic acid	28	273080301	21.21
Another compound peak area	-	569339094	-
Total	-	1287275900	55.77

Table 4.4 Accuracy test of TLC-densitometric analytical method of VLFAs (intra-day precision)

Added concentration (mg/ml)	Items	Number of sample					
		1	2	3	4	5	6
0.0559	%Recovery	100.15	100.09	99.15	99.32	100.25	99.89
	Average	99.81					
	%RSD	0.46					
0.1118	%Recovery	100.42	99.58	100.70	99.34	99.50	100.10
	Average	99.94					
	%RSD	0.55					
0.1676	%Recovery	100.82	100.27	99.72	101.34	99.62	101.32
	Average	100.52					
	%RSD	0.76					
0.2235	%Recovery	99.12	99.43	102.99	99.02	98.74	100.25
	Average	99.93					
	%RSD	1.59					
0.2794	%Recovery	101.07	101.17	100.80	101.71	100.12	102.21
	Average	101.18					
	%RSD	0.71					
Average of %recovery		100.27					

Table 4.5 Inter-day precision test of TLC-densitometric analytical method of VLFAs

Added concentration (mg/ml)	Items	Day		
		1	2	3
0.0559	%Recovery	99.81	100.80	98.68
	Average	99.76		
	%RSD	0.77		
0.1118	%Recovery	99.94	99.45	100.06
	Average	99.82		
	%RSD	0.32		
0.1676	%Recovery	100.52	99.44	99.92
	Average	99.96		
	%RSD	0.54		
0.2235	%Recovery	99.93	99.75	100.41
	Average	100.03		
	%RSD	0.34		
0.2794	%Recovery	101.18	102.21	102.36
	Average	101.92		
	%RSD	0.63		
Average of %recovery		100.30		
Average of %RSD		0.52		

Table 4.6 Linearity and range test of TLC-densitometric analytical method of VLFAs

Added concentration(mg/ml); x	Mean found concentration(mg/ml); y
0.1175	0.1174
0.1734	0.1733
0.2293	0.2292
0.2851	0.2860
0.3410	0.3409
0.3969	0.4002
$y = 1.0111x-0.0023$, $r^2 = 0.9994$	

Table 4.7 Confirm of linearity standard calibration curve (0.11-0.38 mg/ml)

N	Parameters of calibration curve		
	Slop	Intercept	R^2
1	5.8651	-0.4057	0.9955
2	5.8564	-0.4043	0.9966
3	5.8411	-0.4005	0.9956
Mean	5.8542	-0.4032	0.9959
SD	0.01	0.00	0.00
%RSD	0.21	0.53	0.06

Table 4.8 Quantitation limit of VLFAs by TLC-densitometric analytical method

VLFAs solution (0.11 mg/ml)	sample1	sample2	sample3	sample4	sample5	sample6
Peak area ratio	0.2116	0.2161	0.2155	0.2053	0.2174	0.2582
Peak area ratio average	0.2139					
%Recovery	102.45	103.20	103.69	101.39	103.41	103.41
%Recovery average	102.83					
%RSD	0.77					

Table 4.9 Robustness of TLC-densitometric analytical method; % recovery of VLFAs in varied analytical condition

Developing phase	TLC length (cm)	% Recovery (mean±SD)
Hexane-ethyl acetate-acetic acid (14:6:1)	10.0	100.00 ± 0.00
Hexane-ethyl acetate-acetic acid (14:6:1)	8.0	99.09 ± 0.28
Hexane-ethyl acetate-acetic acid (14:6:1)	8.5	99.31 ± 0.94
Hexane-ethyl acetate-acetic acid (14:6:1)	9.0	99.04 ± 0.53
Hexane-ethyl acetate-acetic acid (14:6:1)	9.5	99.13 ± 0.95
Hexane-ethyl acetate-acetic acid (14:6:1)	10.5	99.29 ± 0.96
Hexane-ethyl acetate-acetic acid (14:6:1)	11.0	99.24 ± 0.54
Hexane-ethyl acetate-acetic acid (14:6:1)	11.5	99.31 ± 1.01
Hexane-ethyl acetate-acetic acid (14:6:1)	12.0	99.26 ± 0.50
Hexane-ethyl acetate-acetic acid (14:6:0.8)	10.0	99.24 ± 0.83
Hexane-ethyl acetate-acetic acid (14:6:0.9)	10.0	99.51 ± 0.96
Hexane-ethyl acetate-acetic acid (14:6:1.1)	10.0	99.38 ± 0.38
Hexane-ethyl acetate-acetic acid (14:6:1.2)	10.0	99.63 ± 0.66
Hexane-ethyl acetate-acetic acid (14:4.8:1)	10.0	101.13 ± 0.93
Hexane-ethyl acetate-acetic acid (14:5.4:1)	10.0	101.59 ± 0.95
Hexane-ethyl acetate-acetic acid (14:6.6:1)	10.0	98.16 ± 0.88
Hexane-ethyl acetate-acetic acid (14:7.2:1)	10.0	98.76 ± 0.90
Hexane-ethyl acetate-acetic acid (11.2:6:1)	10.0	102.39 ± 1.01
Hexane-ethyl acetate-acetic acid (12.6:6:1)	10.0	99.28 ± 0.56
Hexane-ethyl acetate-acetic acid (15.4:6:1)	10.0	99.44 ± 1.00
Hexane-ethyl acetate-acetic acid (16.8:6:1)	10.0	102.12 ± 0.65

Table 4.10 Robustness of TLC-desitometric analytical method; hR_f of TLC spot in varied analytical condition

Table 4.10.1 R_f and hR_f of TLC spot in analytical condition

Spot in TLC	R_f	hR_f
Spot 1	0.39	38.75-40.00
Spot 2 (IS)	0.47	46.25-47.50
Spot 3 (VLFAs)	0.52	51.25-52.50
Spot 4	0.59	58.12-60.62

Table 4.10.2 hR_f of VLFAs and IS TLC spot in varied analytical condition

Developing phase	TLC length (cm)	IS	VLFAs
		hR_f	hR_f
hexane-ethyl acetate-acetic acid (14:6:1)	10.0	46.25-47.50	51.25-52.50
hexane-ethyl acetate-acetic acid (14:6:1)	8.0	43.33-45.00	48.33-50.0
hexane-ethyl acetate-acetic acid (14:6:1)	8.5	44.62-46.15	49.23-50.77
hexane-ethyl acetate-acetic acid (14:6:1)	9.0	47.14-48.57	52.14-53.57
hexane-ethyl acetate-acetic acid (14:6:1)	9.5	46.00-47.33	51.33-52.67
hexane-ethyl acetate-acetic acid (14:6:1)	10.5	46.47-47.65	52.35-53.53
hexane-ethyl acetate-acetic acid (14:6:1)	11.0	47.22-48.33	53.33-54.44
hexane-ethyl acetate-acetic acid (14:6:1)	11.5	48.42-49.47	54.74-55.79
hexane-ethyl acetate-acetic acid (14:6:1)	12.0	46.50-47.50	52.5-53.5
hexane-ethyl acetate-acetic acid (14:6:0.8)	10.0	43.75-45.00	50.00-51.25
hexane-ethyl acetate-acetic acid (14:6:0.9)	10.0	45.00-46.25	50.63-51.87
hexane-ethyl acetate-acetic acid (14:6:1.1)	10.0	50.62-51.87	54.37-55.62
hexane-ethyl acetate-acetic acid (14:6:1.2)	10.0	52.50-53.75	56.25-57.50
hexane-ethyl acetate-acetic acid (14:4.8:1)	10.0	41.25-42.50	45.00-46.25
hexane-ethyl acetate-acetic acid (14:5.4:1)	10.0	42.50-43.75	48.12-49.37
hexane-ethyl acetate-acetic acid (14:6.6:1)	10.0	52.50-53.75	56.25-57.50
hexane-ethyl acetate-acetic acid (14:7.2:1)	10.0	54.37-55.62	58.12-59.37
hexane-ethyl acetate-acetic acid (11.2:6:1)	10.0	48.75-50.00	53.75-55.00
hexane-ethyl acetate-acetic acid (12.6:6:1)	10.0	57.50-48.75	52.50-53.75
hexane-ethyl acetate-acetic acid (15.4:6:1)	10.0	40.62-41.87	45.62-46.87
hexane-ethyl acetate-acetic acid (14:6.8:6:1)	10.0	38.12-39.37	44.37-45.32

Table 4.10.3 hR_f of spot 1 and spot 4 TLC spot in varied analytical condition

Developing phase	TLC length (cm)	Spot 1	Spot 4
		hR_f	hR_f
hexane-ethyl acetate-acetic acid (14:6:1)	10.0	38.75-40.00	58.12-60.62
hexane-ethyl acetate-acetic acid (14:6:1)	8.0	48.33-50.00	60.85-64.17
hexane-ethyl acetate-acetic acid (14:6:1)	8.5	49.23-50.77	60.00-63.08
hexane-ethyl acetate-acetic acid (14:6:1)	9.0	52.14-53.57	59.29-62.14
hexane-ethyl acetate-acetic acid (14:6:1)	9.5	51.33-52.67	58.00-60.67
hexane-ethyl acetate-acetic acid (14:6:1)	10.5	52.35-53.53	59.41-61.76
hexane-ethyl acetate-acetic acid (14:6:1)	11.0	53.33-54.44	60.67-62.78
hexane-ethyl acetate-acetic acid (14:6:1)	11.5	54.74-55.79	62.11-64.21
hexane-ethyl acetate-acetic acid (14:6:1)	12.0	52.5-53.5	59.50-61.50
hexane-ethyl acetate-acetic acid (14:6:0.8)	10.0	39.37-41.87	59.37-61.87
hexane-ethyl acetate-acetic acid (14:6:0.9)	10.0	41.87-43.12	60.62-63.12
hexane-ethyl acetate-acetic acid (14:6:1.1)	10.0	48.12-49.37	64.37-66.87
hexane-ethyl acetate-acetic acid (14:6:1.2)	10.0	46.87-48.12	65.62-68.12
hexane-ethyl acetate-acetic acid (14:4.8:1)	10.0	38.12-39.37	57.50-60.0
hexane-ethyl acetate-acetic acid (14:5.4:1)	10.0	39.37-40.62	61.25-63.75
hexane-ethyl acetate-acetic acid (14:6.6:1)	10.0	48.75-50.00	70.00-72.50
hexane-ethyl acetate-acetic acid (14:7.2:1)	10.0	50.62-51.87	71.87-74.37
hexane-ethyl acetate-acetic acid (11.2:6:1)	10.0	42.50-43.75	63.12-65.62
hexane-ethyl acetate-acetic acid (12.6:6:1)	10.0	41.25-42.50	61.87-64.37
hexane-ethyl acetate-acetic acid (15.4:6:1)	10.0	36.87-38.12	56.25-58.75
hexane-ethyl acetate-acetic acid (14:6.8:6:1)	10.0	34.37-35.62	55.00-57.50

Table 4.11 Stability of VLFAs in solution under 4 ^{0}C **(a)**, 25 ^{0}C **(b)** and ambient condition **(c)**

Sampling time (day)	Concentration of VLFAs (mg/ml) (mean±SD)
0	0.2724 ± 0.01
1	0.2733 ± 0.01
3	0.2717 ± 0.02
5	0.2720 ± 0.00
7	0.2711 ± 0.01

(a)

Sampling time (day)	Concentration of VLFAs (mg/ml) (mean±SD)
0	0.2742 ± 0.02
1	0.2719 ± 0.01
3	0.2712 ± 0.01
5	0.2716 ± 0.02
7	0.2745 ± 0.01

(b)

Sampling time (day)	Concentration of VLFAs (mg/ml) (mean±SD)
0	0.2747 ± 0.02
1	0.2739 ± 0.02
3	0.2771 ± 0.00
5	0.2799 ± 0.00
7	0.2802 ± 0.00

(c)

Tablet 4.12 Efficiency of extracting solvent for extraction of *Centella asiatica*

Extracting solvent	Percentage content of VLFAs (mean ± SD)
100% hexane	1.8760 ± 0.03
90% hexane in ethyl acetate	2.1317 ± 0.02
80% hexane in ethyl acetate	2.2944 ± 0.01
70% hexane in ethyl acetate	2.0307 ± 0.04
60% hexane in ethyl acetate	1.8697 ± 0.01
50% hexane in ethyl acetate	2.0613 ± 0.01
40% hexane in ethyl acetate	2.0839 ± 0.01
30% hexane in ethyl acetate	1.8595 ± 0.02
20% hexane in ethyl acetate	1.9676 ± 0.04
10% hexane in ethyl acetate	1.9075 ± 0.02
100% ethyl acetate	1.8985 ± 0.02

Table 4.13 Percentage content of extraction of *Centella asiatica* with hexane-ethyl acetate (8:2) at various times (refluxing time)

Refluxing time (min)	Percentage content of VLFAs (mean ± SD)
5	0.0579 ± 0.01
10	0.0692 ± 0.01
30	0.0768 ± 0.01
60	0.1141 ± 0.01
90	0.1382 ± 0.01
120	0.1371 ± 0.00
180	0.1371 ± 0.00

Table 4.14 The effect of ultrasonic extraction time and power on percentage contents of VLFAs

Extraction time (min.)	Extraction contents of VLFAs, %w/w		
	10%of power (mean±SD)	50%of power (mean±SD)	100%of power (mean±SD)
0	0.0562 ± 0.00	0.0562 ± 0.00	0.0562 ± 0.00
1	0.0741 ± 0.01	0.0741 ± 0.01	0.0689 ± 0.01
2	0.0823 ± 0.00	0.0823 ± 0.00	0.0828 ± 0.00
3	0.1006 ± 0.01	0.1021 ± 0.00	0.1040 ± 0.01
5	0.1204 ± 0.00	0.1269 ± 0.01	0.1205 ± 0.00
10	0.1381 ± 0.01	0.1422 ± 0.01	0.1344 ± 0.01
20	0.1533 ± 0.00	0.1696 ± 0.04	0.1571 ± 0.01
30	0.1540 ± 0.00	0.1619 ± 0.00	0.1551 ± 0.01
60	0.1550 ± 0.00	0.1653 ± 0.01	0.1550 ± 0.01

Table 4.15 The stability of VLFAs during UAE, values expressed as concentration of VLFAs.

Extraction time (min.)	Concentration of VLFAs (mg/ml) (mean±SD)
0	0.2992 ± 0.00
1	0.2971 ± 0.00
3	0.3015 ± 0.00
5	0.2993 ± 0.00
10	0.2988 ± 0.00
20	0.3001 ± 0.00
30	0.2982 ± 0.00
60	0.3007 ± 0.00

Table 4.16 Contents of VLFAs in whole plant of various CA from Thailand Institute of Scientific and Technological Research

CA samples	Extraction content, %w/w (mean±SD)
CA1	0.1979 ± 0.00
CA2	0.2143 ± 0.00
CA3	0.2129 ± 0.00
CA4	0.0425 ± 0.00
CA5	0.2006 ± 0.00
CA6	0.2683 ± 0.00
CA7	0.0423 ± 0.00
CA8	0.2399 ± 0.00
CA9	0.2411 ± 0.00
CA10	0.1708 ± 0.00
CA11	0.0324 ± 0.00
CA12	0.1829 ± 0.00

Table 4.17 Contents of VLFAs in whole plant of CA from the commercial crop of Nakhon Pathom province in annual period

Month (sample codes)	Extraction contents, %w/w VLFAs (mean±SD)
January (CA13-1)	0.0673 ± 0.00
March (CA13-3)	0.1136 ± 0.00
May (CA13-5)	0.0442 ± 0.00
July (CA13-7)	0.0446 ± 0.00
September (CA13-9)	0.1012 ± 0.00
November (CA13-11)	0.1376 ± 0.00
Average of percentage content	**0.0974 ± 0.00**

Table 4.18 Contents of VLFAs in whole plant of CA from the commercial crop of Ubon Ratchathani province in annual period

Month (sample codes)	Extraction contents, %w/w VLFAs
January (CA13-1)	0.1673 ± 0.00
March (CA13-3)	0.1451 ± 0.00
May (CA13-5)	0.1863 ± 0.00
July (CA13-7)	0.1691 ± 0.00
September (CA13-9)	0.2100 ± 0.00
November (CA13-11)	0.2086 ± 0.00
Average of percentage content	**0.1811 ± 0.00**

Table 4.19 Contents of VLFAs in whole plant of CA from the commercial crop Nakhon Si Thammarat province in annual period

Month (sample codes)	Extraction contents, %w/w VLFAs
January (CA13-1)	0.1875 ± 0.00
March (CA13-3)	0.1762 ± 0.00
May (CA13-5)	0.2188 ± 0.00
July (CA13-7)	0.1962 ± 0.00
September (CA13-9)	0.1666 ± 0.00
November (CA13-11)	0.1921 ± 0.00
Average of percentage content	**0.1896 ± 0.00**

Table 5.1 Method validation test of TLC-densitometric analytical method

	VLFAs
Accuracy (%R) Mean Range	100.27 98.69-102.99
Precision (%RSD) intra-day inter-day	 0.81 0.52
Range (mg/ml)	0.11-0.38
Linearity R^2 Slope Intercept	 0.9994 1.0111 -0.0023
LOQ (mg/ml)	0.10
LOD (µg/ml)	30

Figure 4.1 Spots on TLC-plate of standard and isolated compounds; system 1

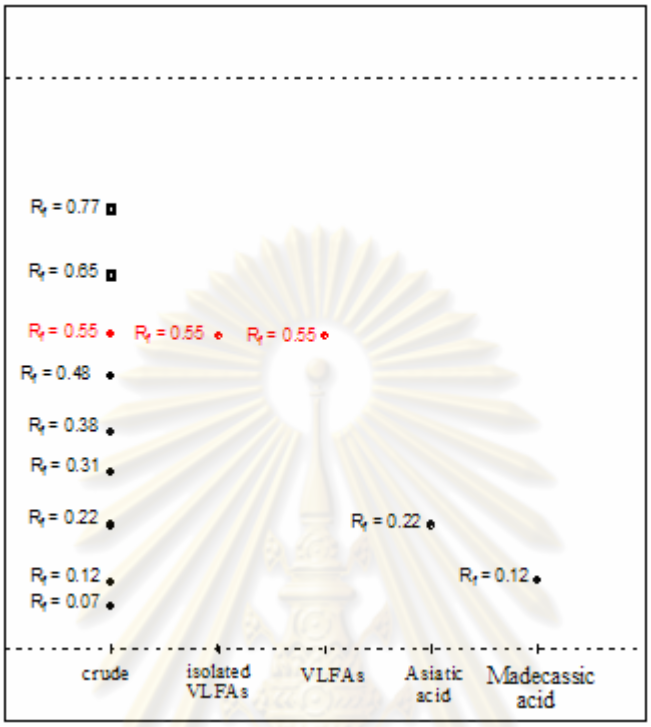


Figure 4.2 Spots on TLC-plate of standard and isolated compounds; system 2

Figure 4.3 Spots on TLC-plate of standard and isolated compounds; system 3

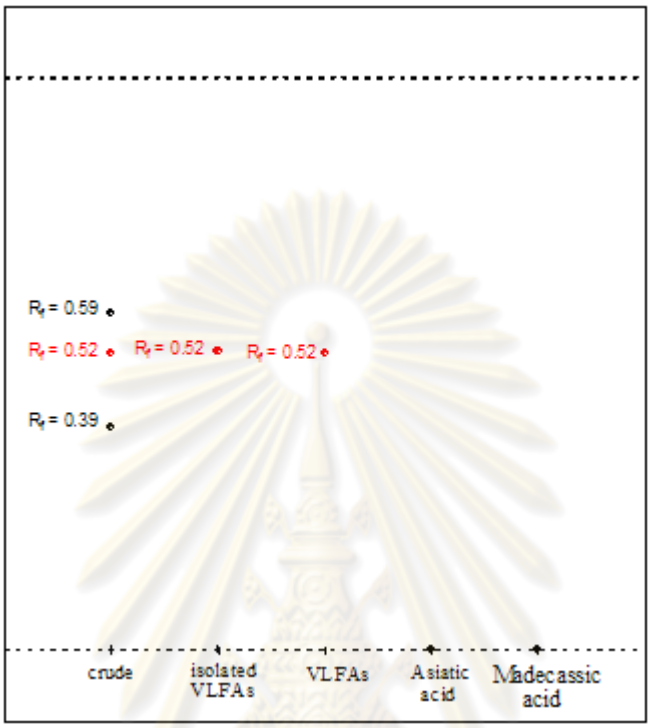
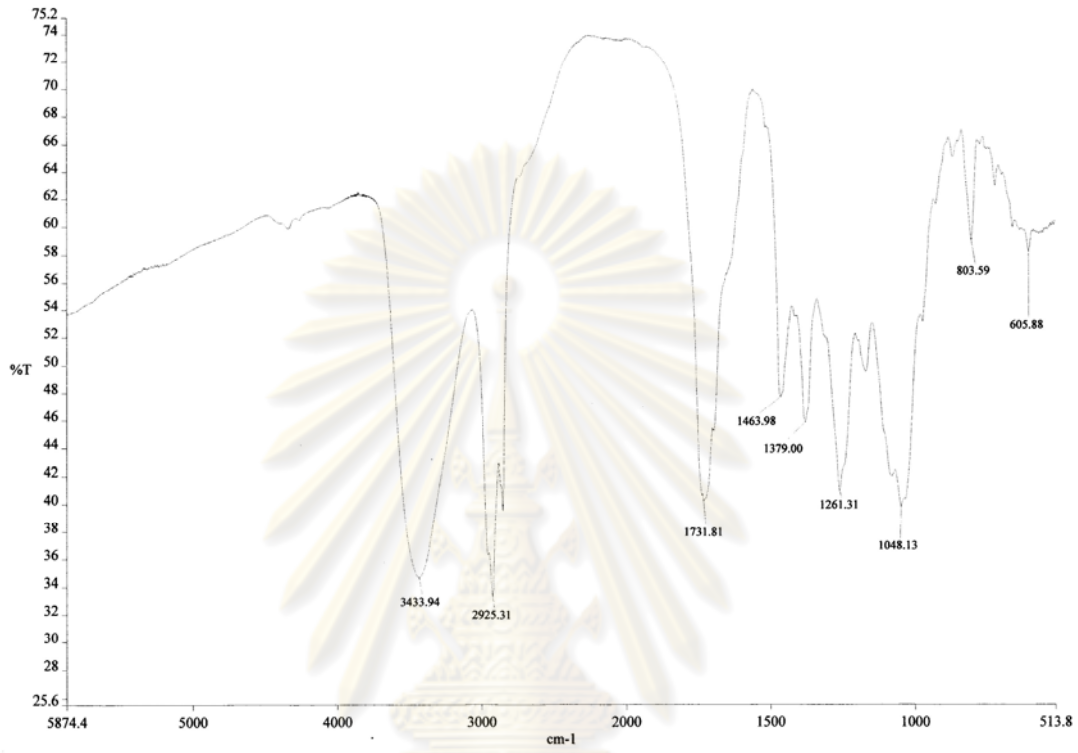
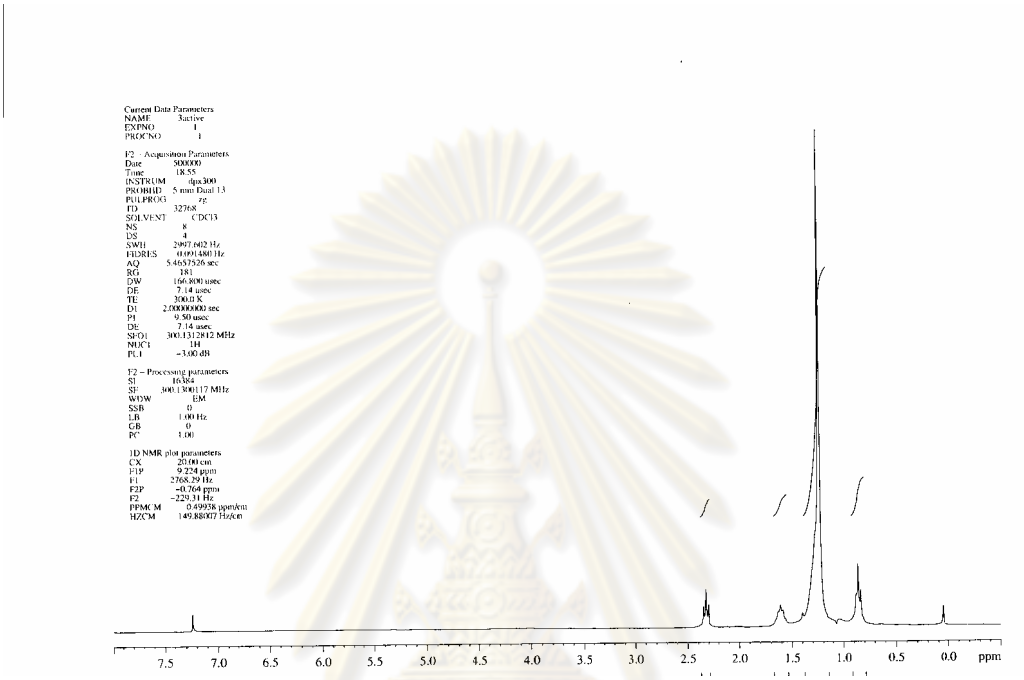


Figure 4.4 IR spectrum of VLFAs



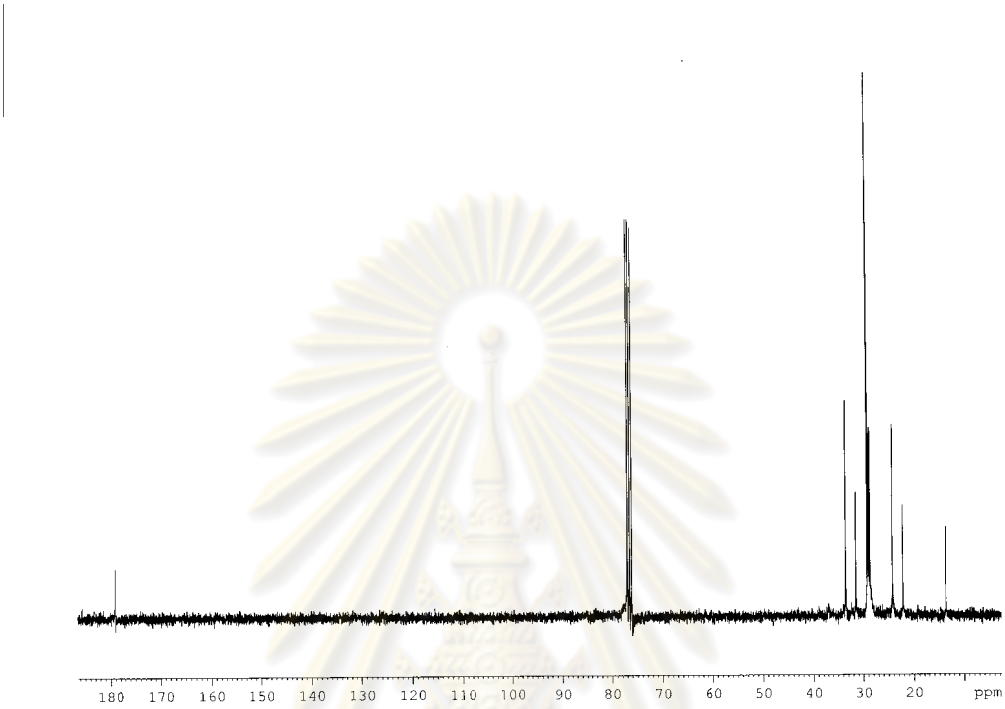
Wave number (cm^{-1})	Functional groups	Vibration
3435	-OH	OH-stretching
2925	$-CH_3$ and $-CH_2-$	CH-stretching
2849	$-CH_3$ and $-CH_2-$	CH-stretching
1731	>C=O	C=O-stretching

Figure 4.5 ^{1}H-NMR of VLFAs



^{1}H chemical shifts δ: ppm	Assigned chemical structure
0.88	Terminal methyl group
1.3-1.4	large number of methylene protons in long chain
1.65	methyl grop of C3
2.35	methylene that α to carbonyl group

Figure 4.6 ^{13}C-NMR of VLFAs



^{13}C chemical shifts δ: ppm	Assigned chemical structure
0-40	Aliphatic carbon
179	The carbonyl carbon

Figure 4.7 DEPT of VLFAs

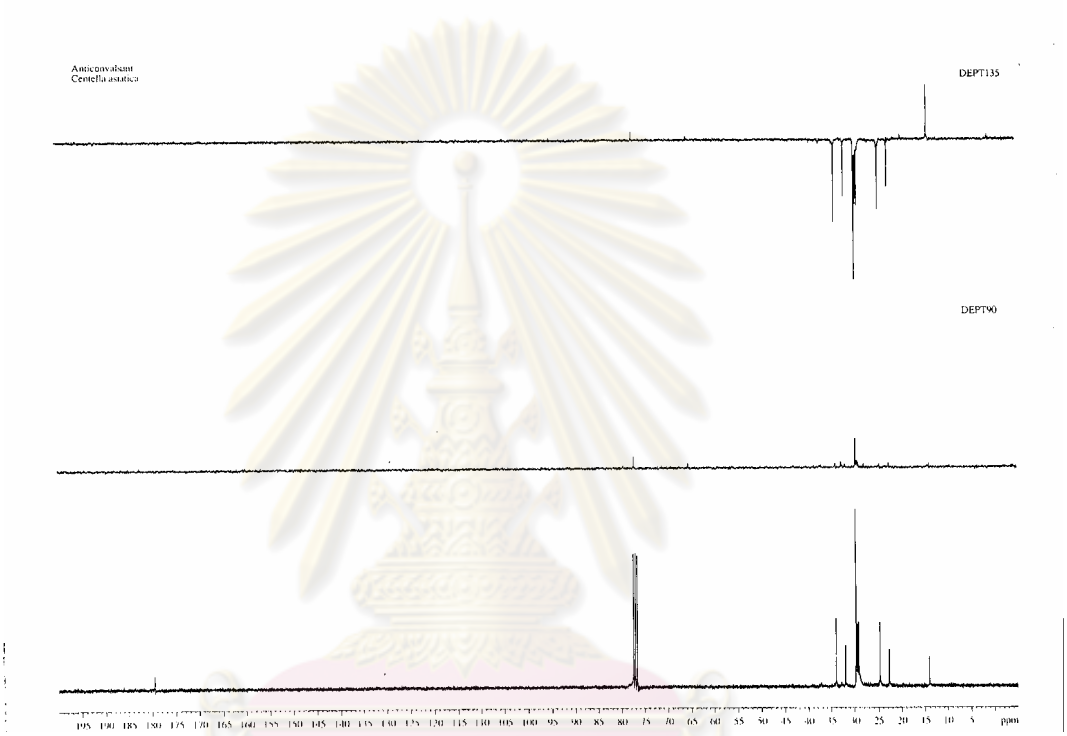


Figure 4.8 ^{13}C-NMR of isolated VLFAs

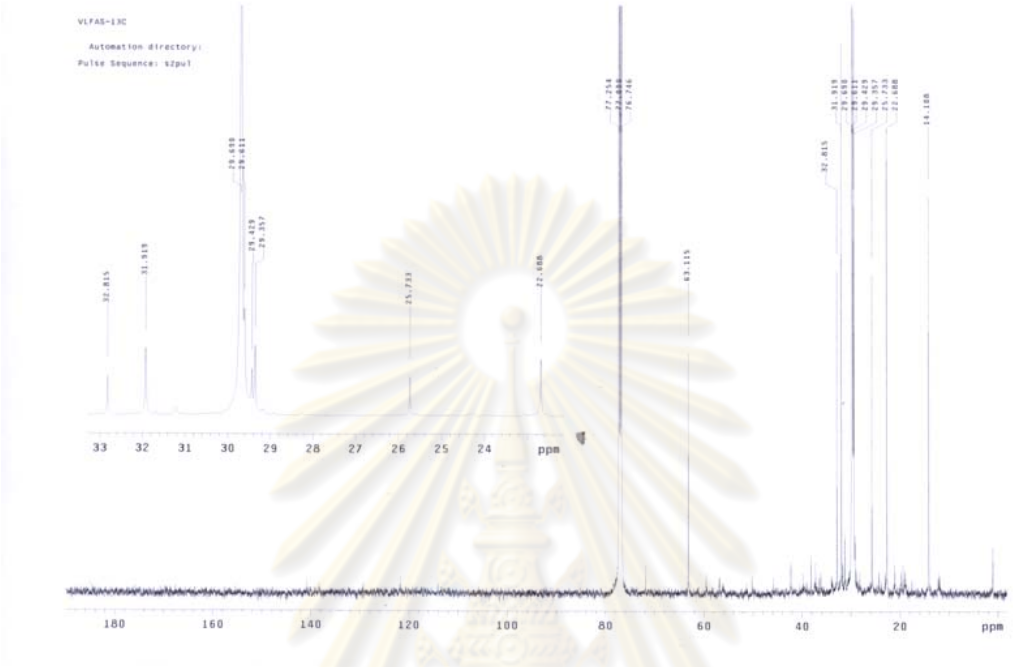


Figure 4.9 DEPT-135 of isolated VLFAs

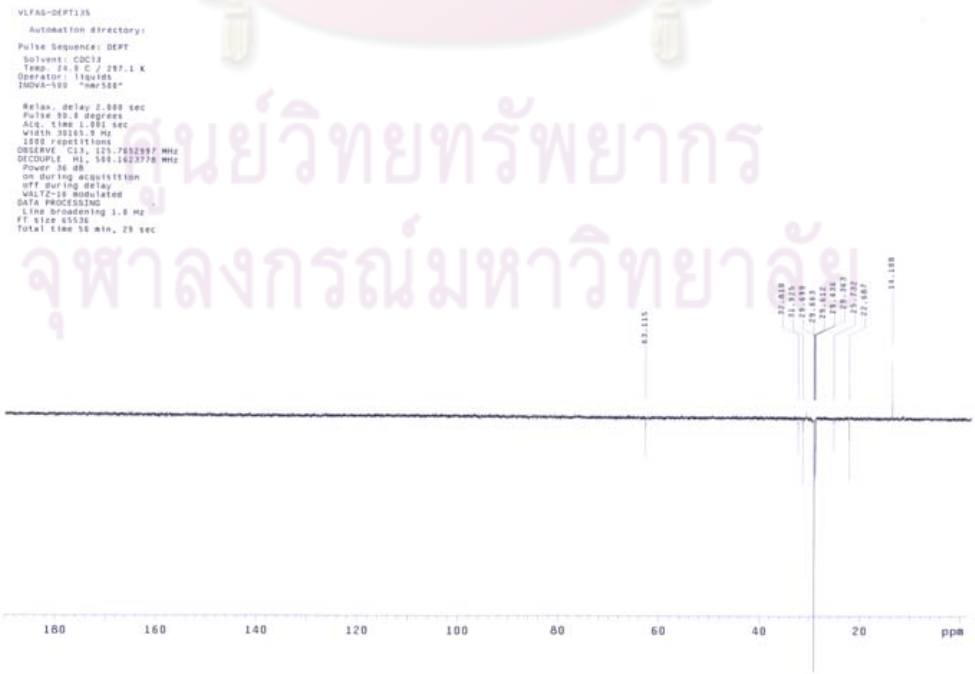


Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acids standard

Figure 4.10.1 GC chromatogram of VLFAs

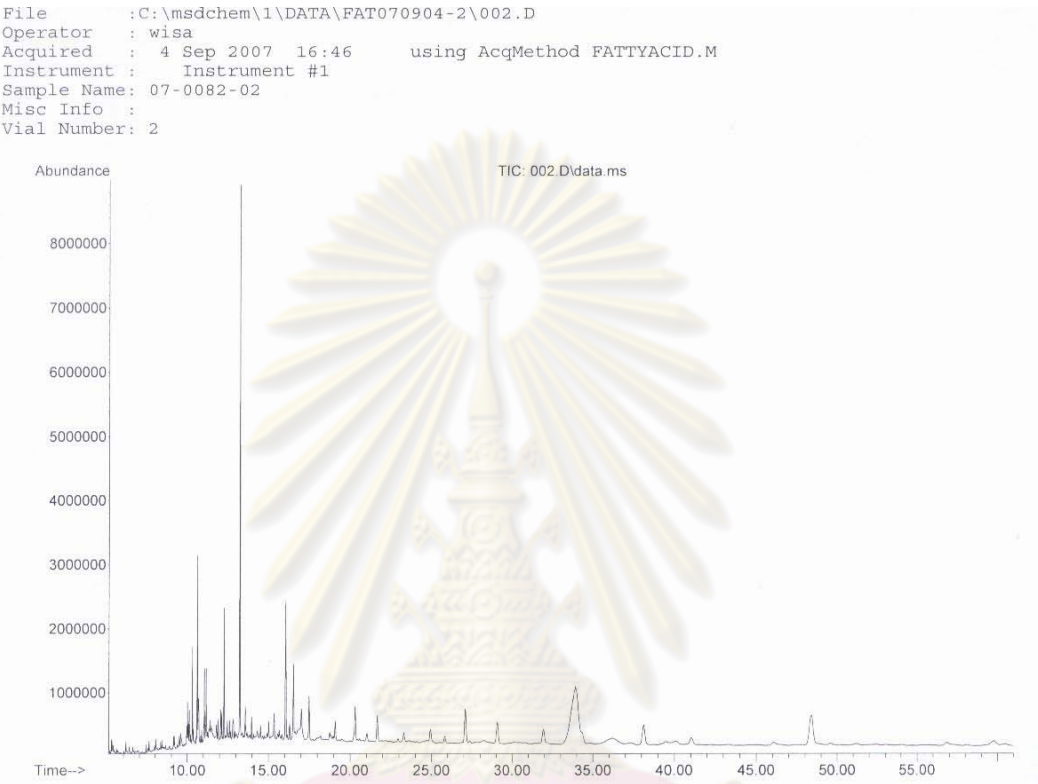


Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acid standard (continue)

Figure 4.10.2 MS pattern of Dodecanoic acid, methyl ester

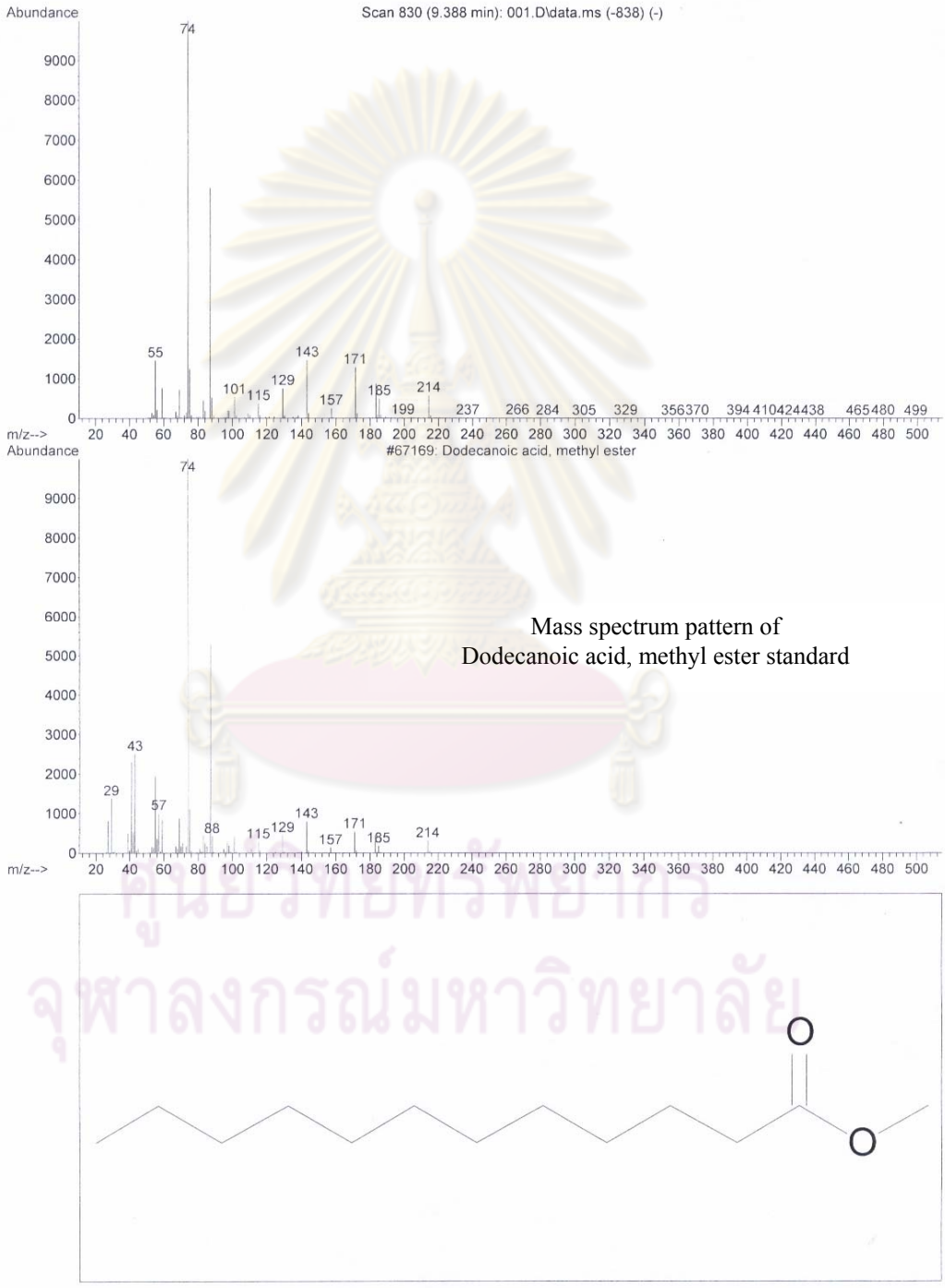
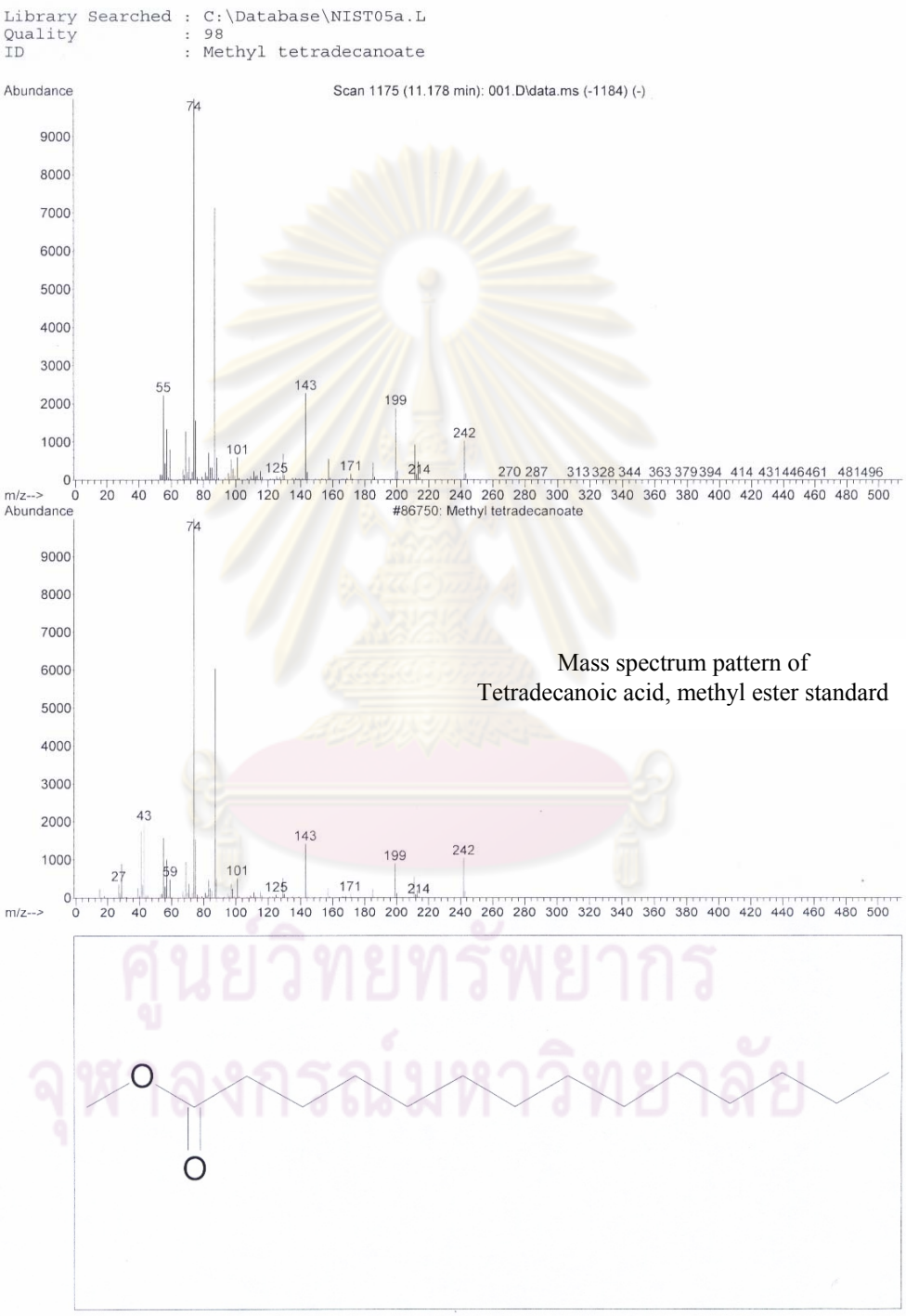


Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acid standard (continue)

Figure 4.10.3 MS pattern of Tetradecanoic acid, methyl ester



Mass spectrum pattern of
Tetradecanoic acid, methyl ester standard

Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acid standard (continue)

Figure 4.10.4 MS pattern of Pentadecanoic acid, methyl ester

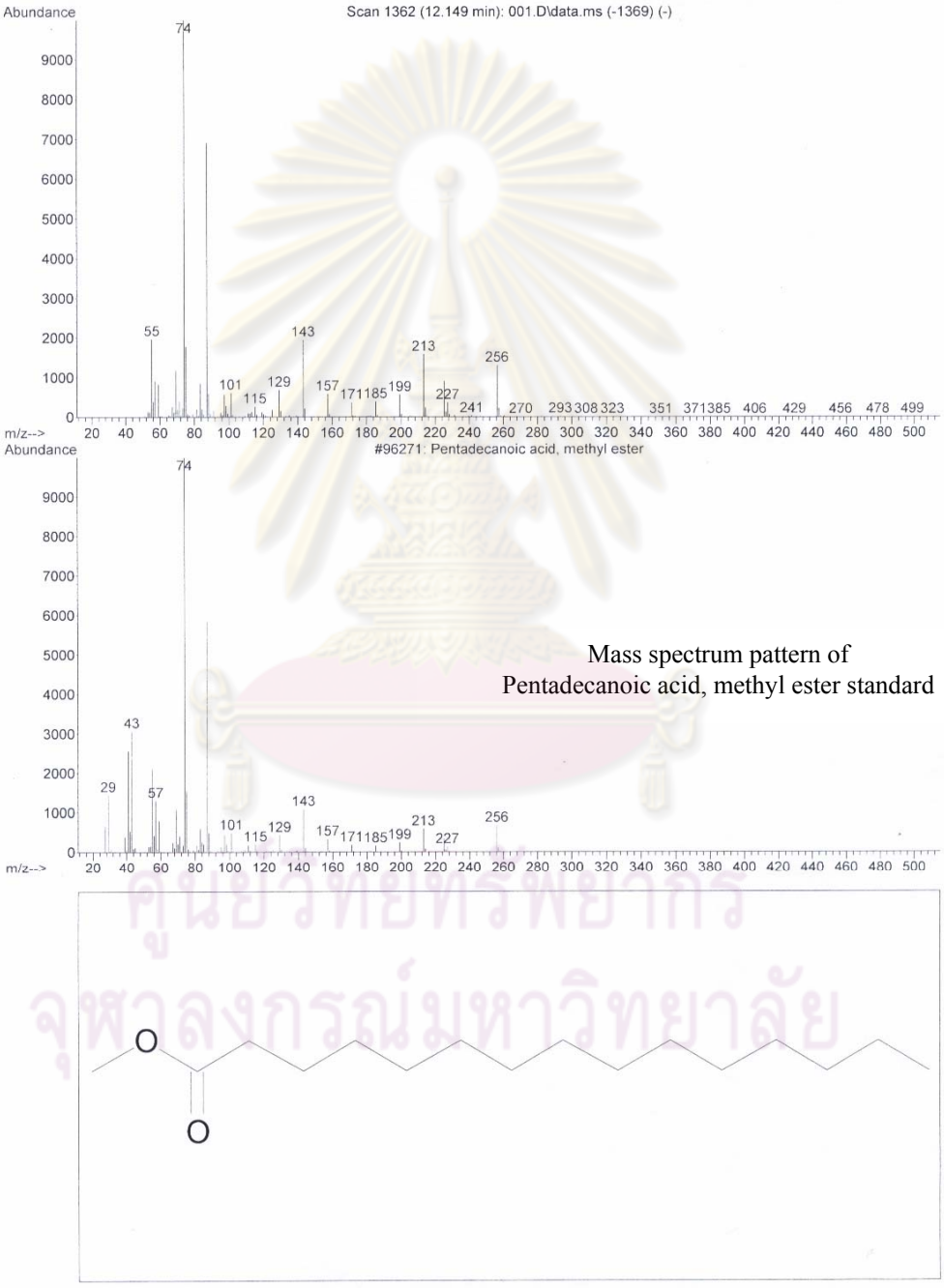
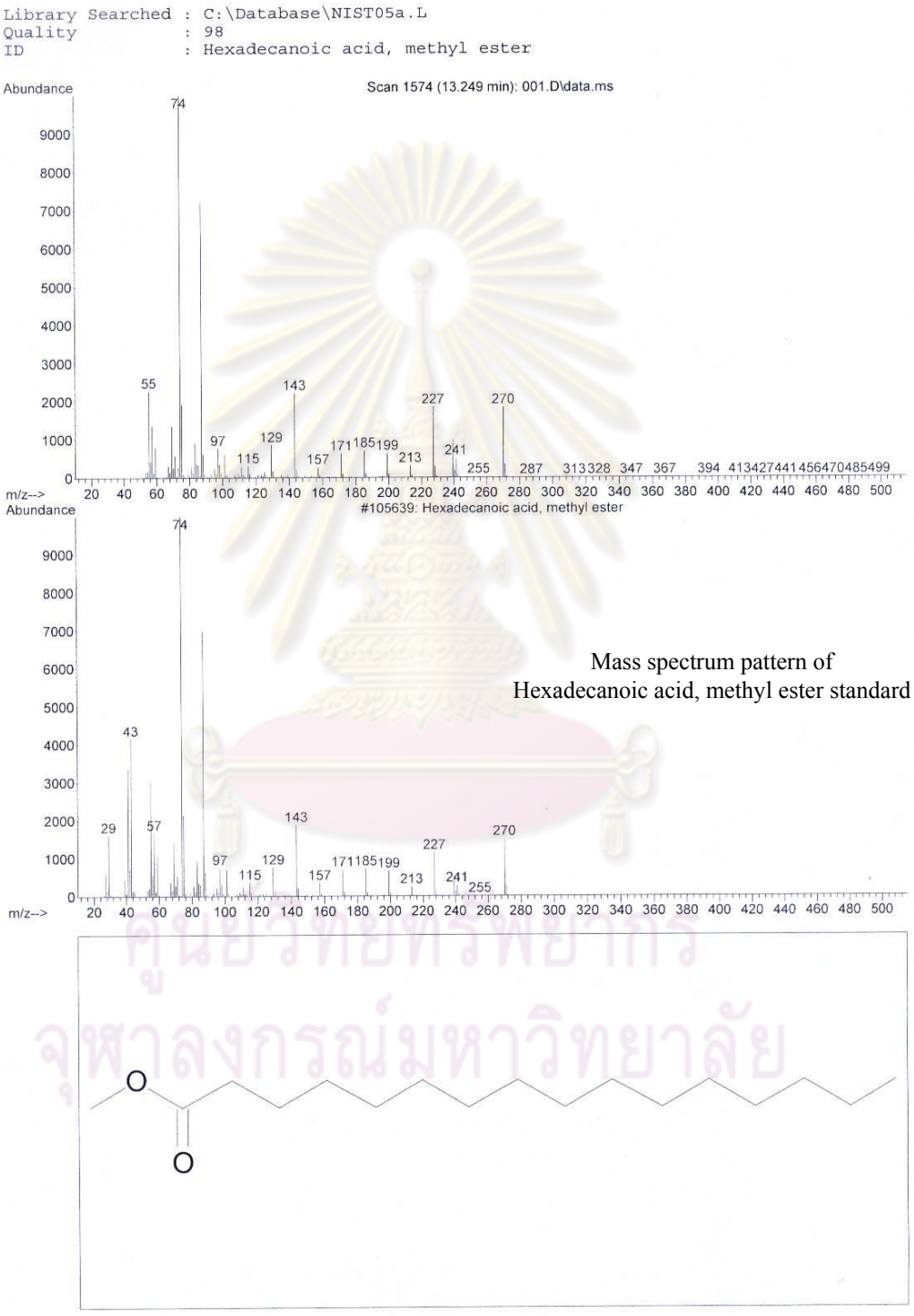


Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acid standard (continue)

Figure 4.10.5 MS pattern of Hexadecanoic acid, methyl ester



Mass spectrum pattern of
Hexadecanoic acid, methyl ester standard

Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acid standard (continue)

Figure 4.10.6 MS pattern of Octadecanoic acid, methyl ester

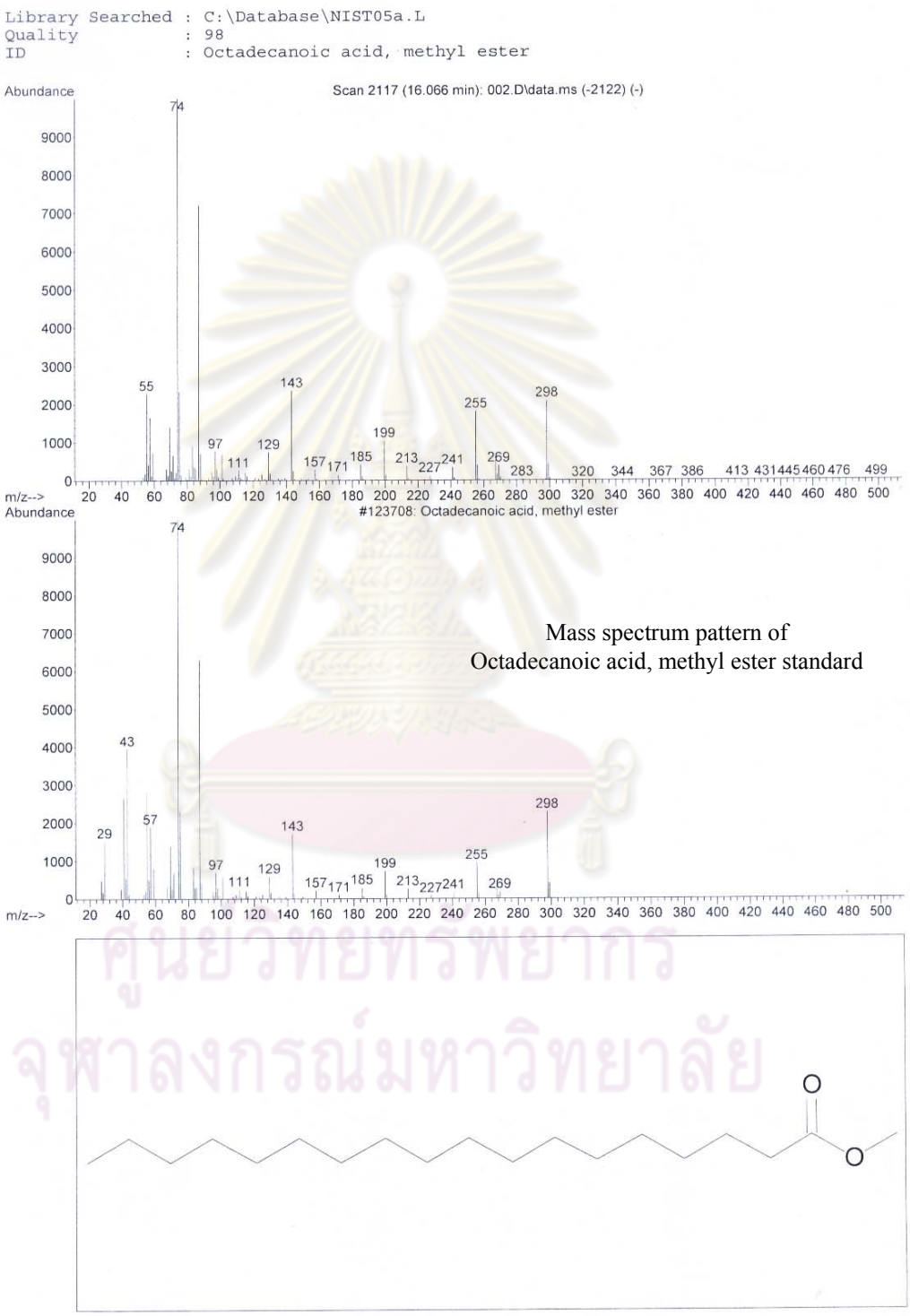


Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acid standard (continue)

Figure 4.10.7 MS pattern of Ecosanoic acid, methyl ester

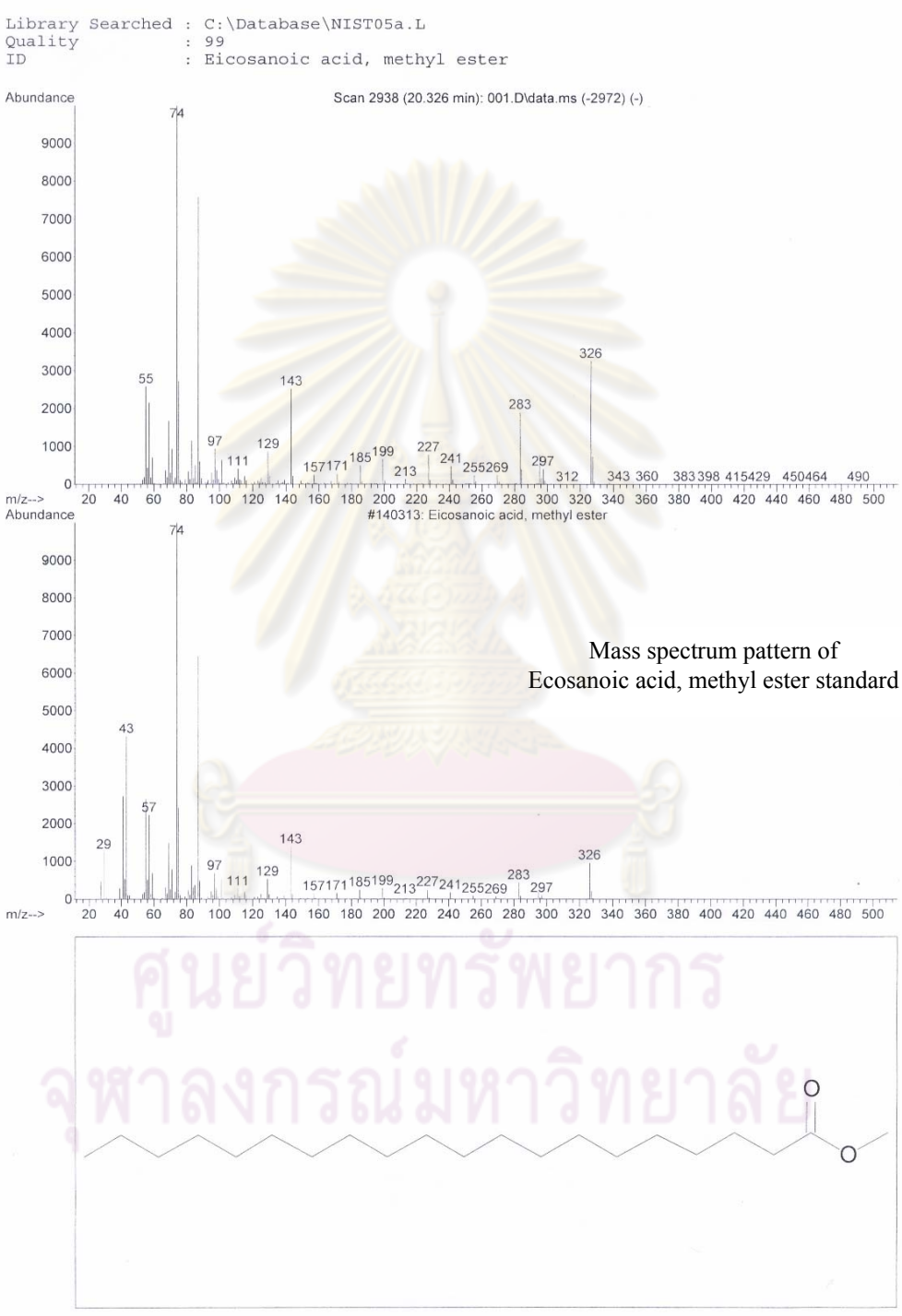
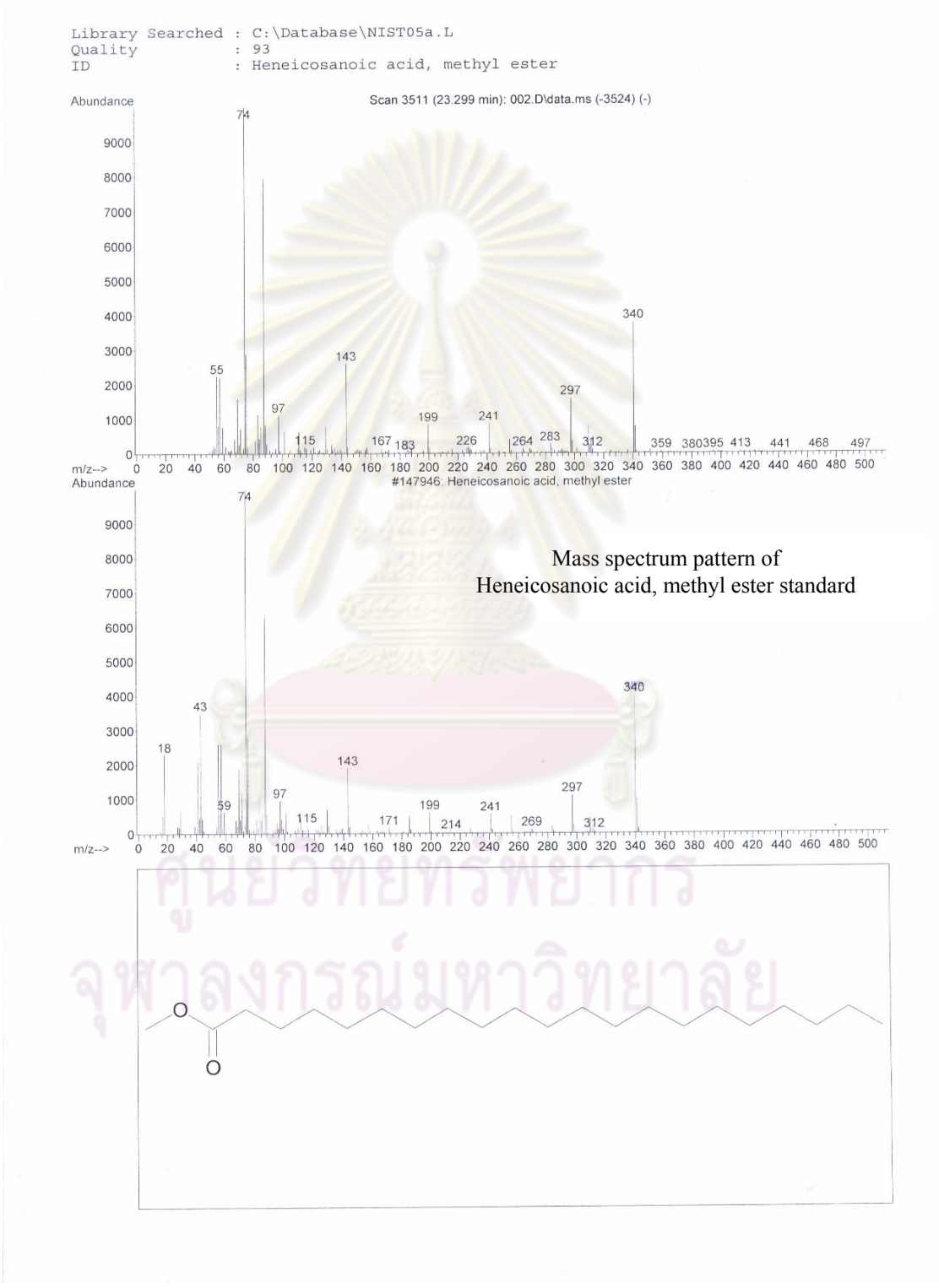


Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acid standard (continue)

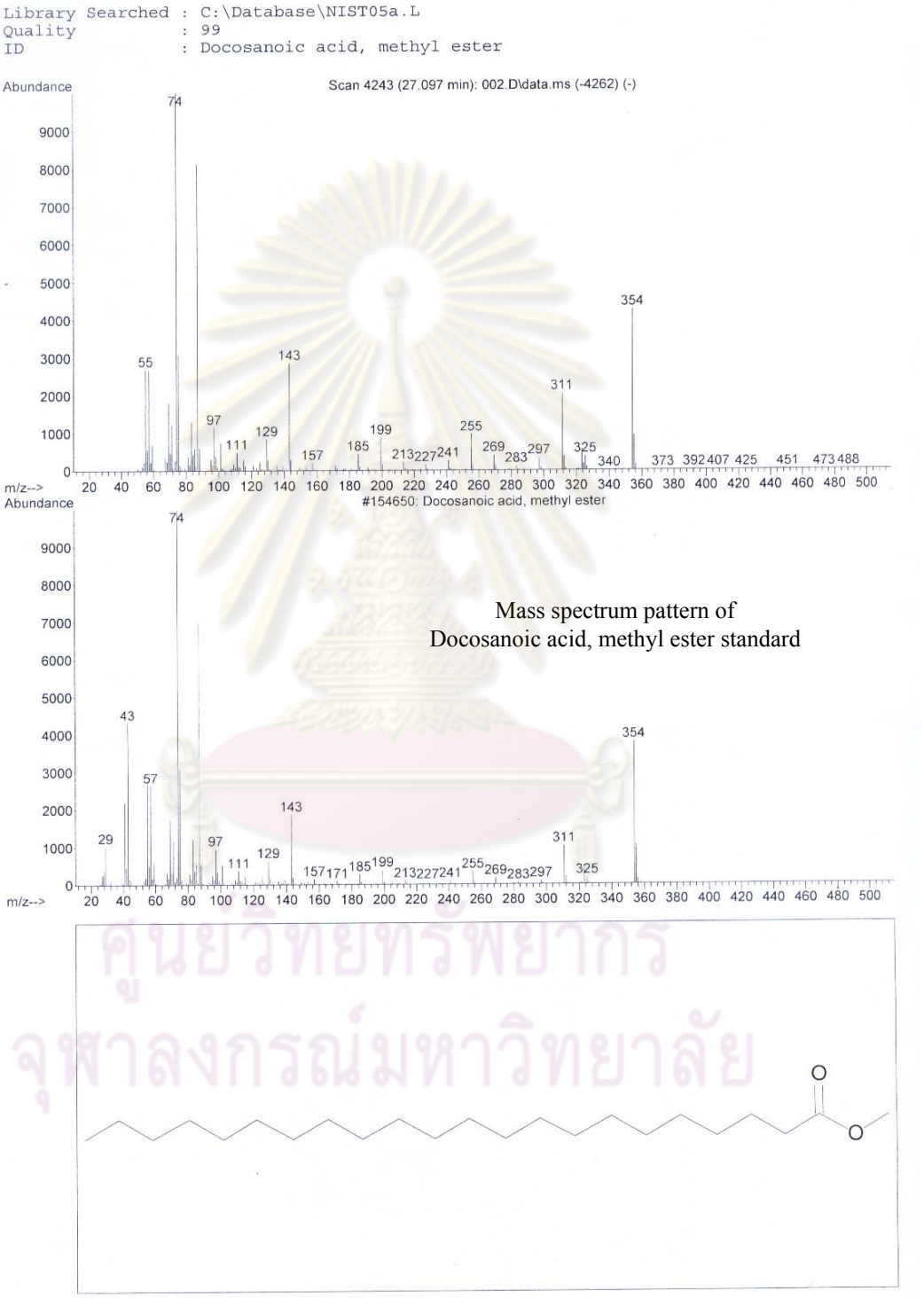
Figure 4.10.8 MS pattern of Heneicosanoic acid, methyl ester



Mass spectrum pattern of
Heneicosanoic acid, methyl ester standard

Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acid standard (continue)

Figure 4.10.9 MS pattern of Docosanoic acid, methyl ester



Mass spectrum pattern of
Docosanoic acid, methyl ester standard

Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acid standard (continue)

Figure 4.10.10 MS pattern of Ttricosanoic acid, methyl ester

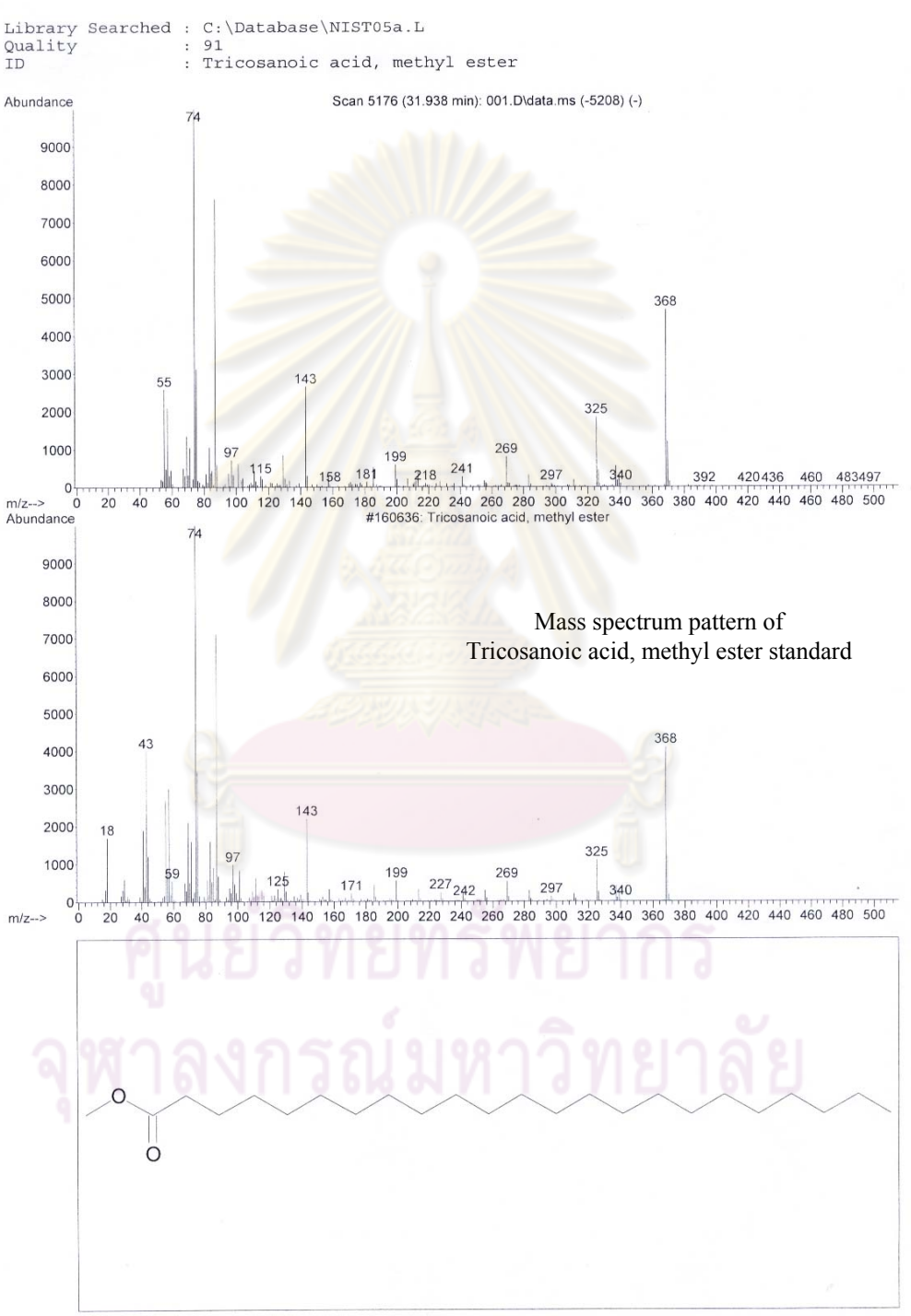
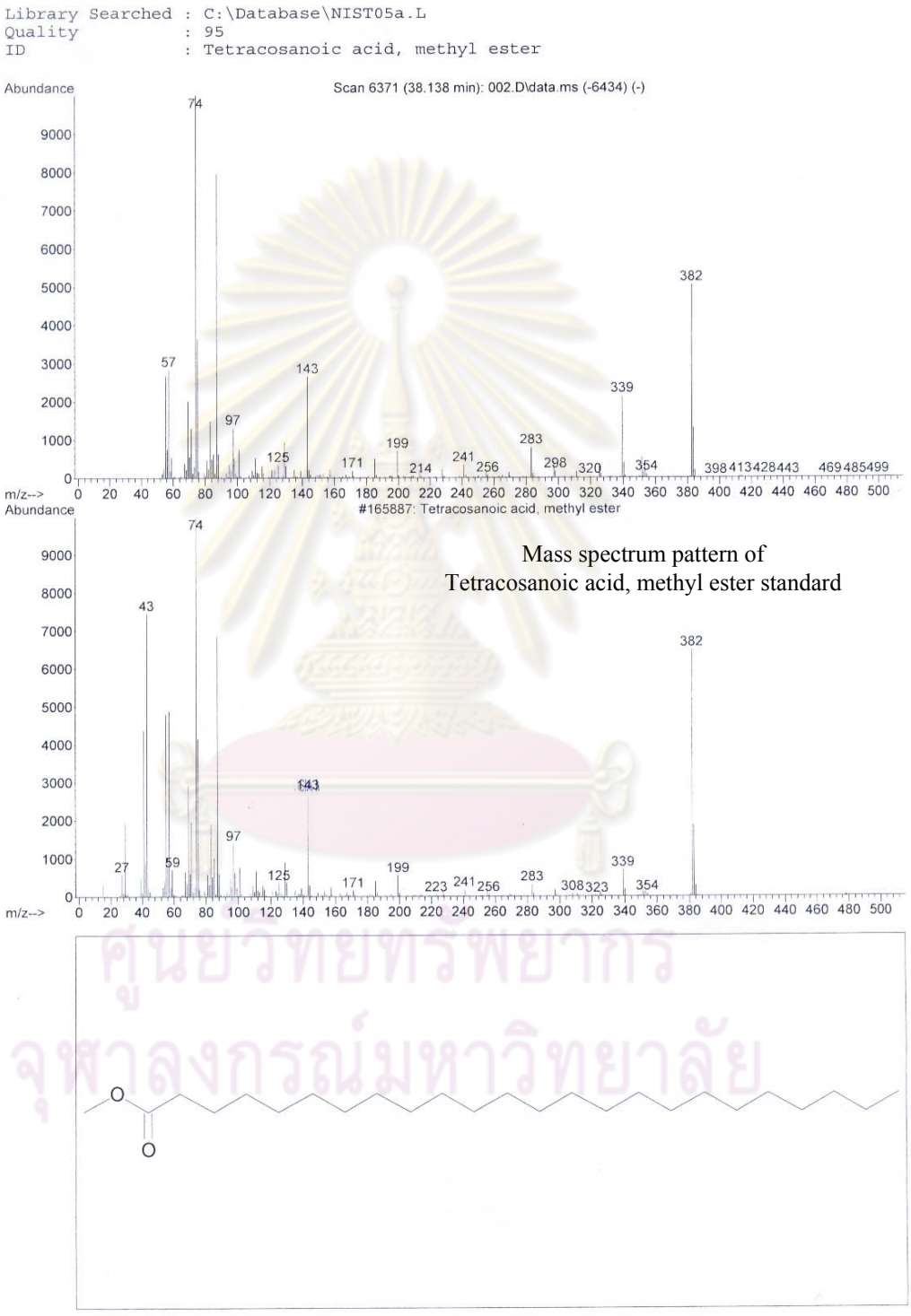


Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acid standard (continue)

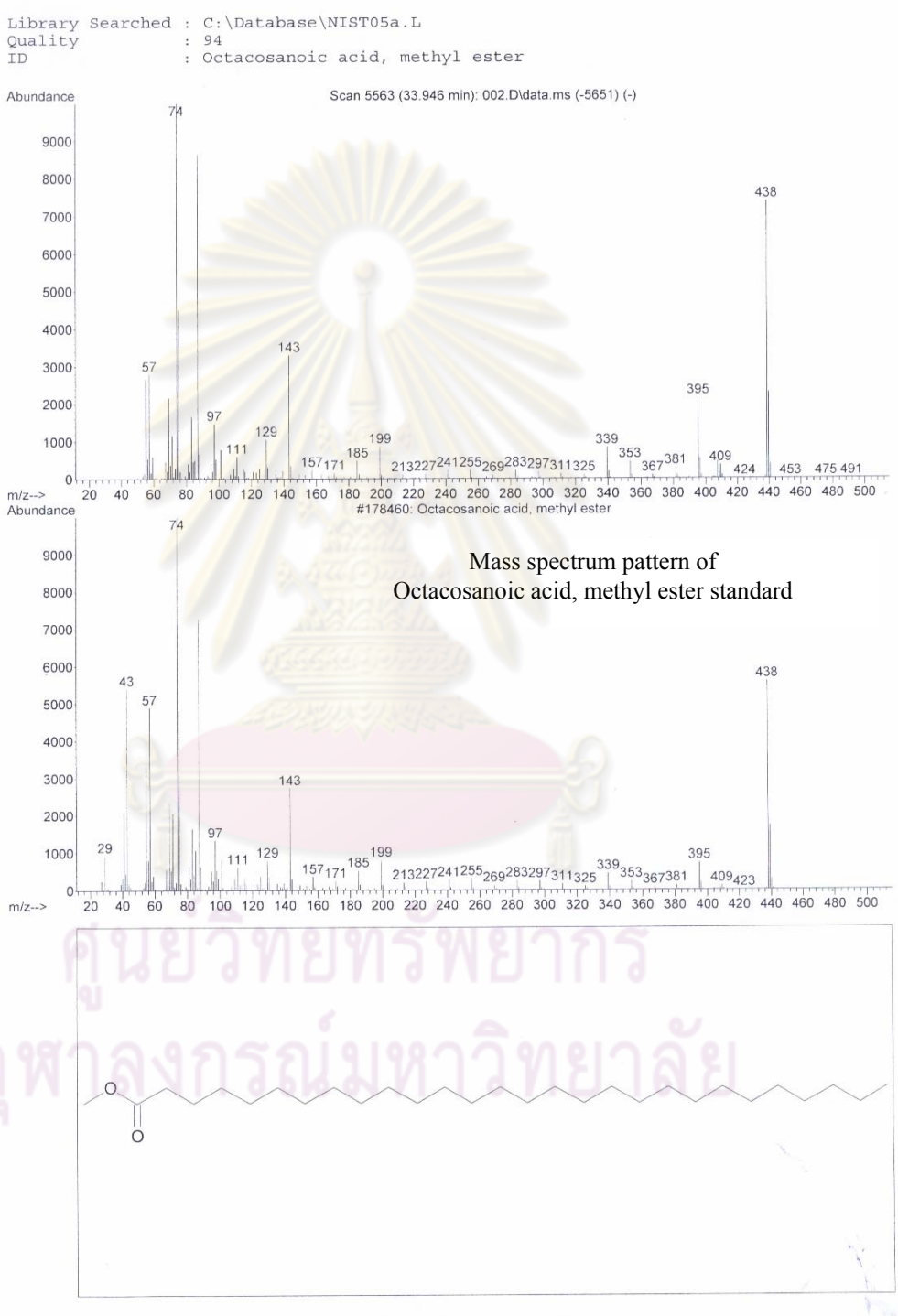
Figure 4.10.11 MS pattern of Tetracosanoic acid, methyl ester



Mass spectrum pattern of
Tetracosanoic acid, methyl ester standard

Figure 4.10 GC-MS chromatogram of VLFAs compared with fatty acid standard (continue)

Figure 4.10.12 MS pattern of Octacosanoic acid, methyl ester



Mass spectrum pattern of
Octacosanoic acid, methyl ester standard

Figure 4.11 Reflection wavelengths versus peak area of VLFAs after spray with 3% phosphomolybdic acid and heat at 120 ^{0}C

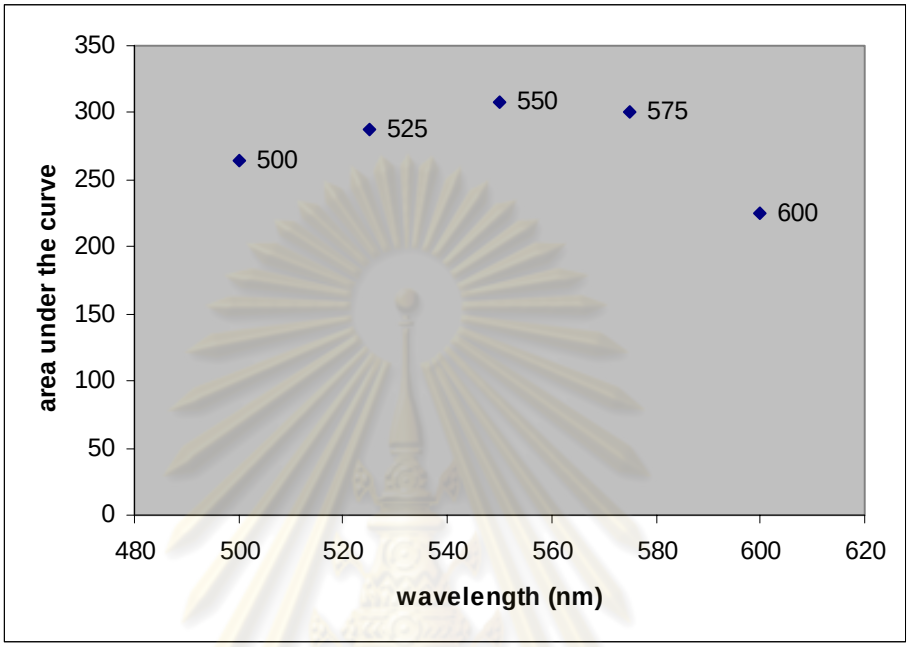


Figure 4.12 Color faded diagram in TLC chromatogram of VLFAs by using 3% phosphomolybdic acid in methanol

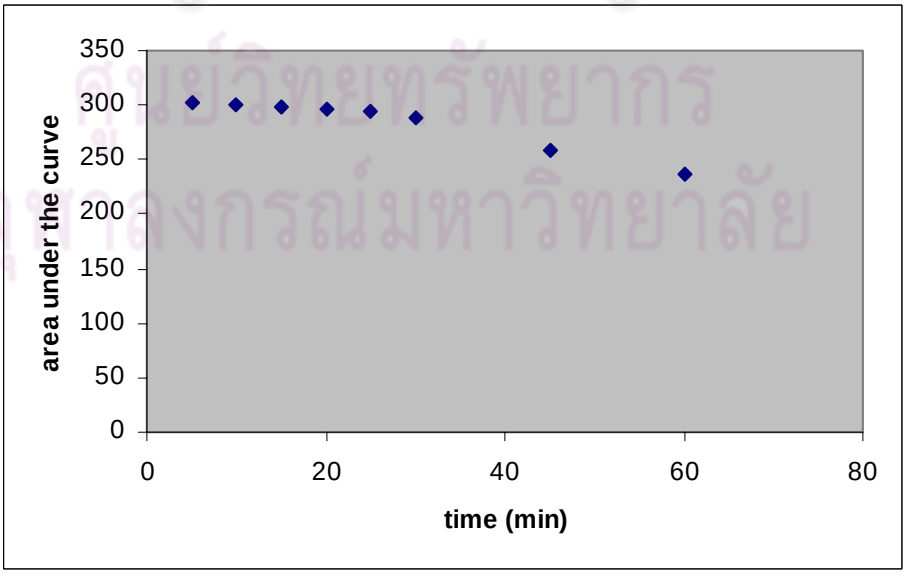


Figure 4.13 TLC spot of sample solution, VLFAs and IS in TLC-densitometric analytical system 3; spray with 3% phosphomolybdic acid in methanol

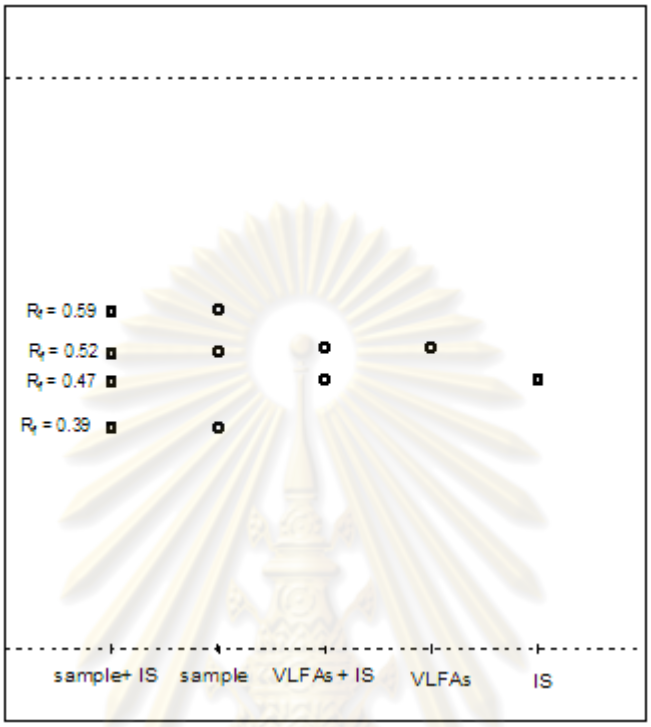
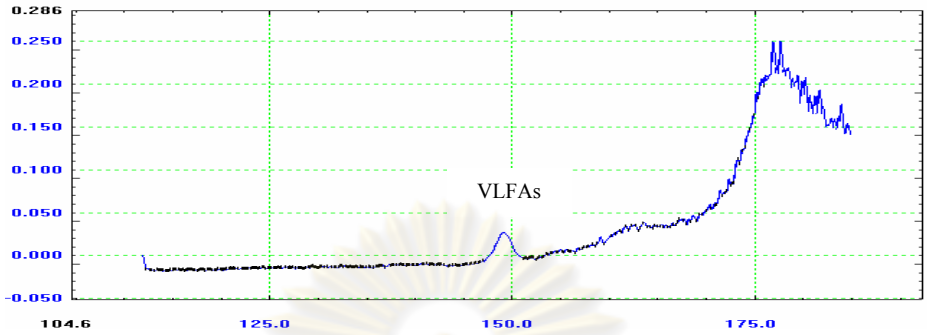
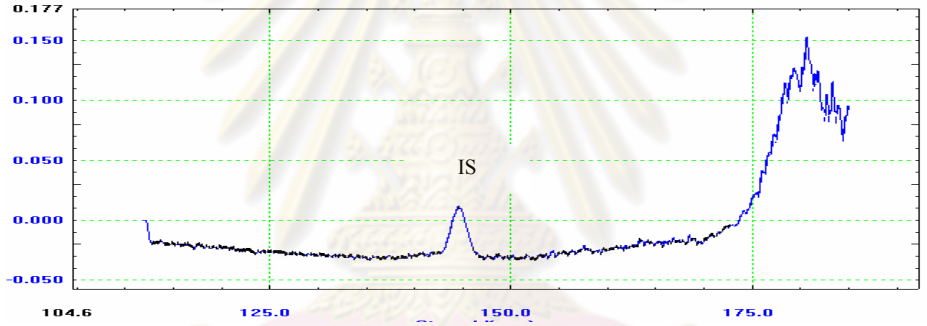


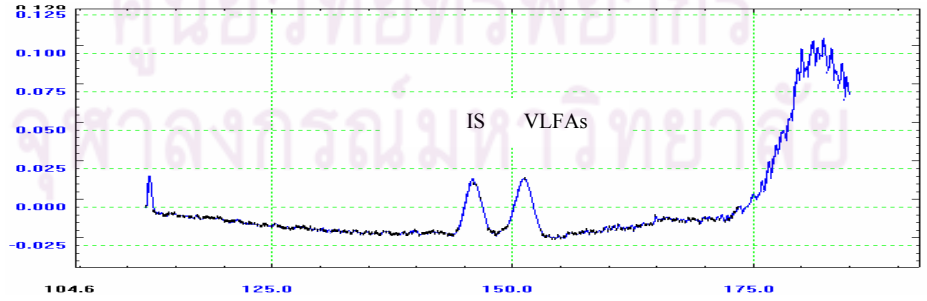
Figure 4.14 TLC densitogram of VLFAs standard **(a)**, IS **(b)**, VLFAs + IS **(c)**, CA extract **(d)** and CA extract + IS (**e**) detected by TLC- densitometer



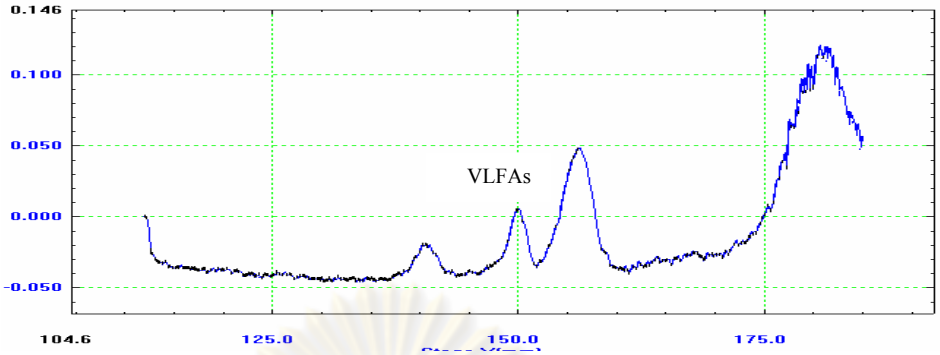
(a)
VLFAs standard



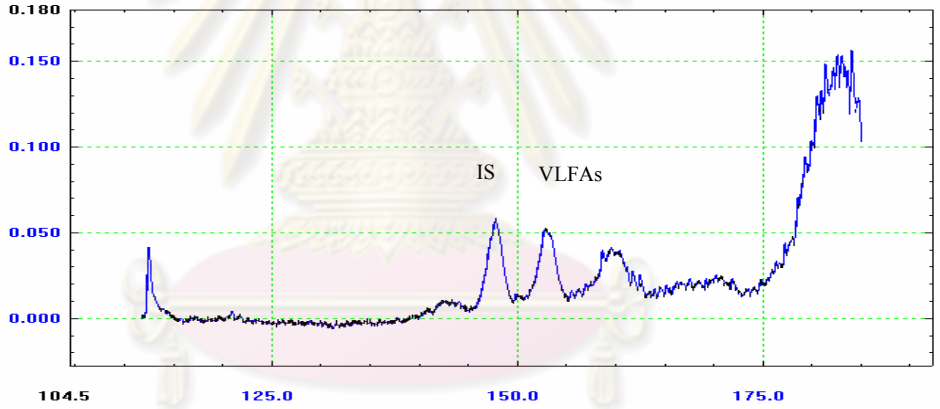
(b)
IS



(c)
VLFAs + IS



(d)
Sample solution



(e)
Sample solution + IS

Figure 4.15 Calibration curve for analytical VLFAs in TLC-densitometric analytical method

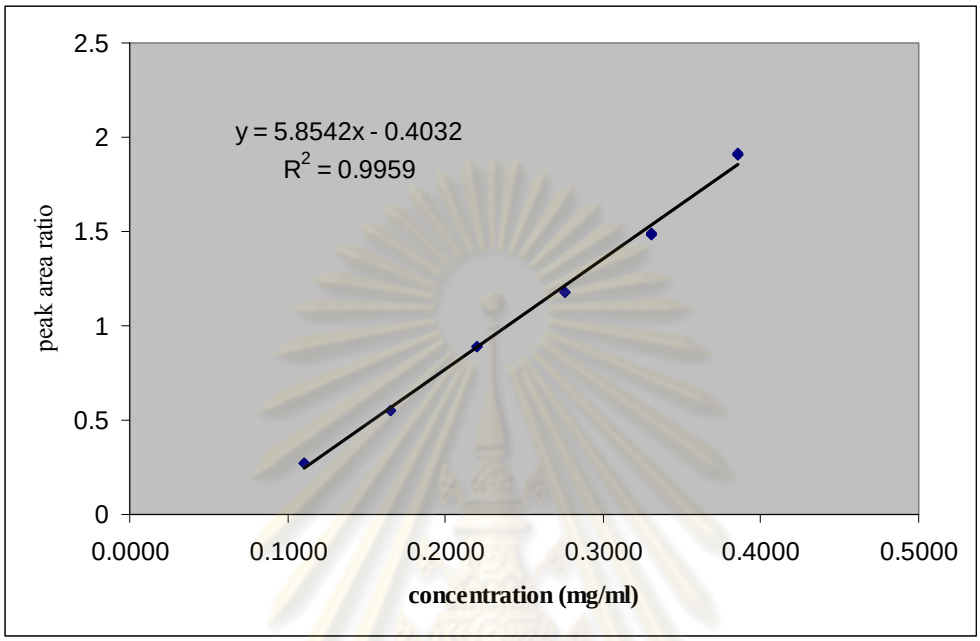


Figure 4.16 Linearity and range test of TLC-densitometric analytical methods

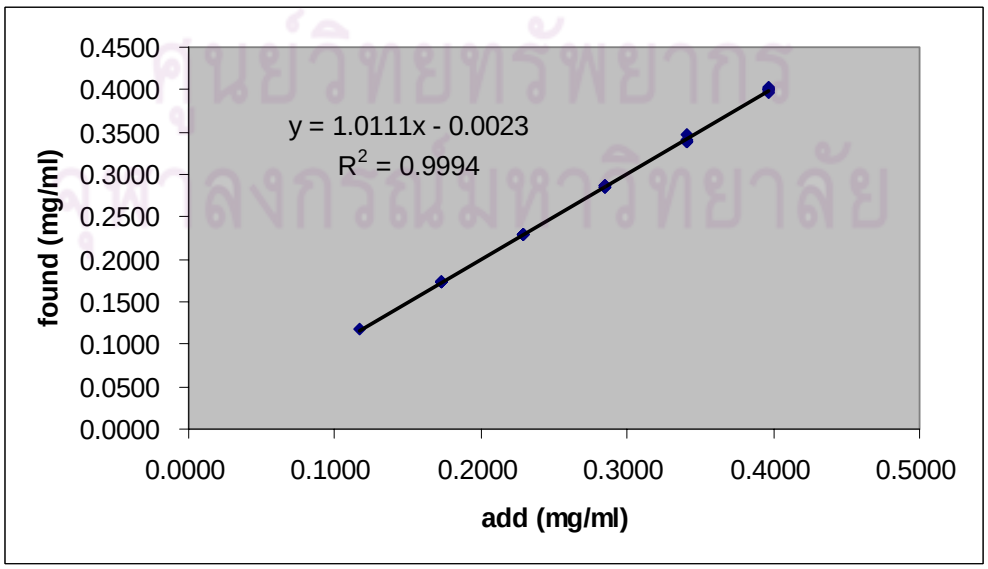


Figure 4.17 Quantitation limit (QL) of VLFAs in TLC-densitometric analytical method (0.10-0.38 mg/ml)

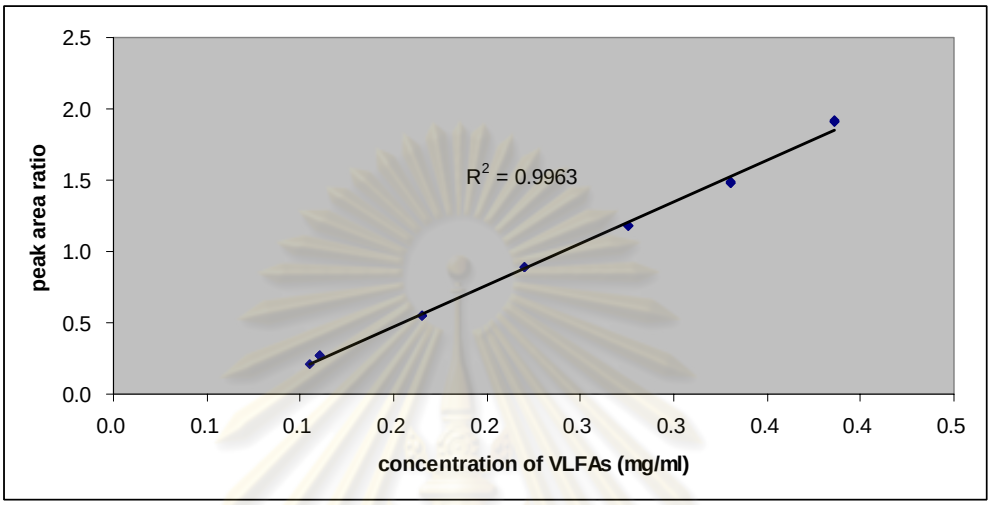


Figure 4.18 Stability of VLFAs in solution under the air condition room, cooling condition (at 4°C) and the ambient condition

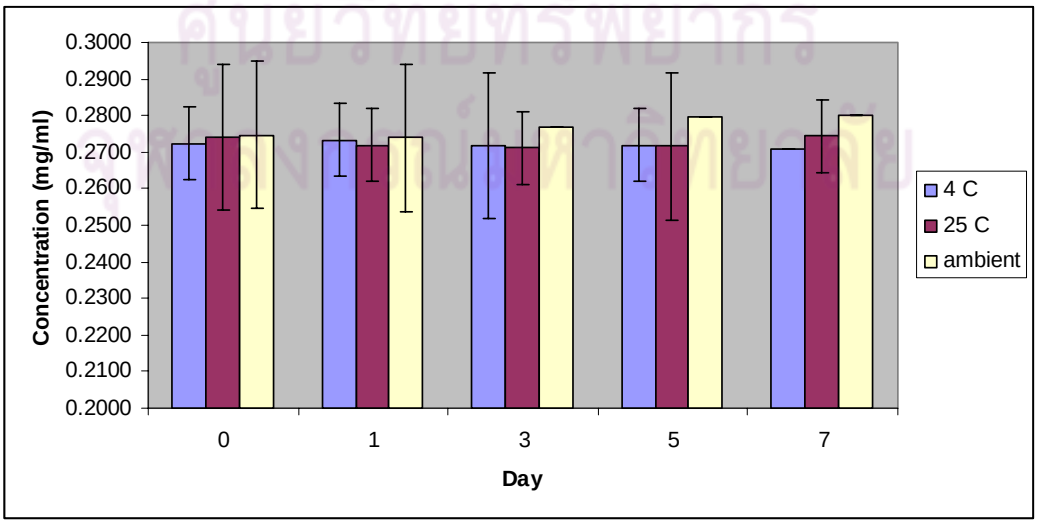


Figure 4.19 Percentage contents of VLFAs in whole plant of CA by varied polarity of solvent

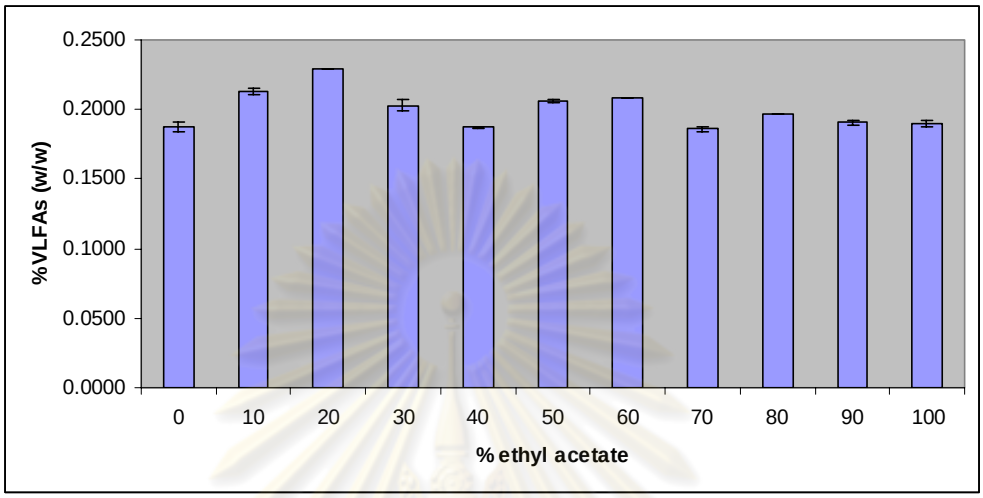


Figure 4.20 Percentage contents of VLFAs in whole plant of CA by reflux extraction at various times

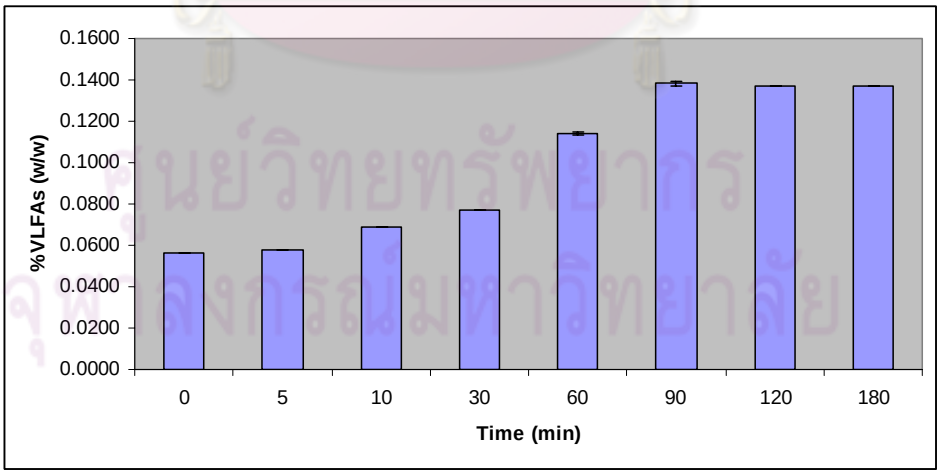


Figure 4.21 The effect of ultrasonic extraction time and power on percentage contents of VLFAs

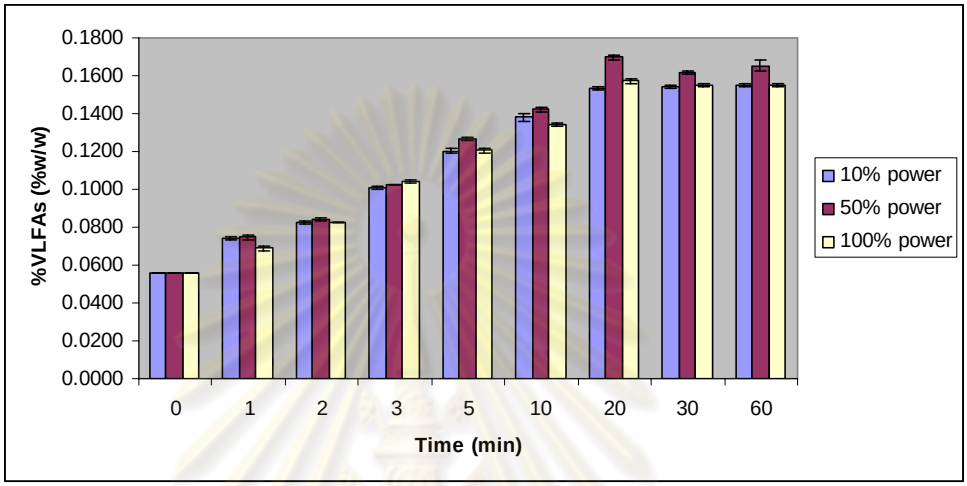


Figure 4.22 The stability of VLFAs during UAE, values expressed as concentration of VLFAs

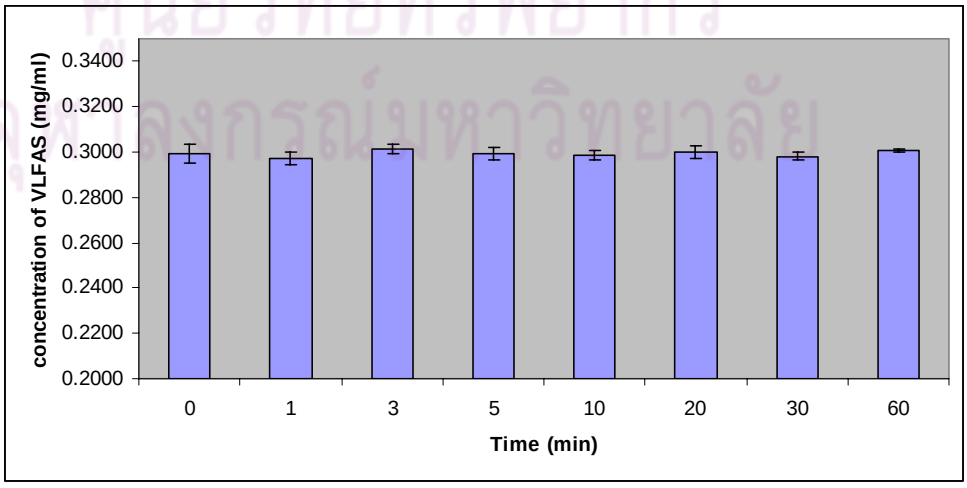


Figure 4.23 Contents of VLFAs in whole plant of various CA from Thailand Institute of Scientific and Technological Research

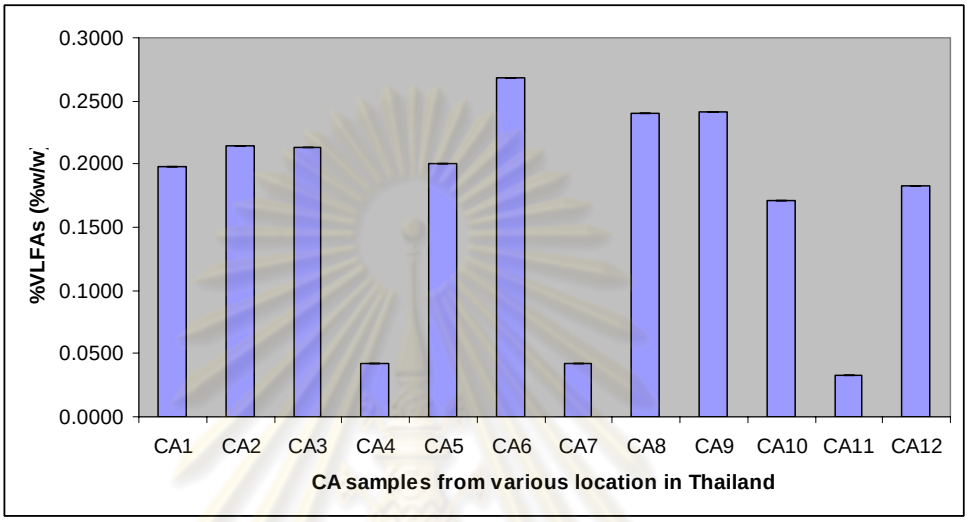
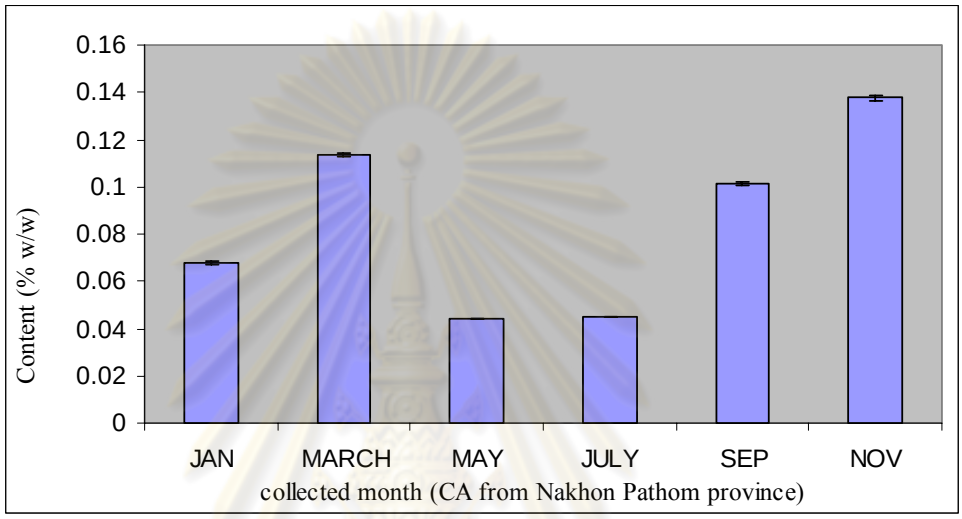
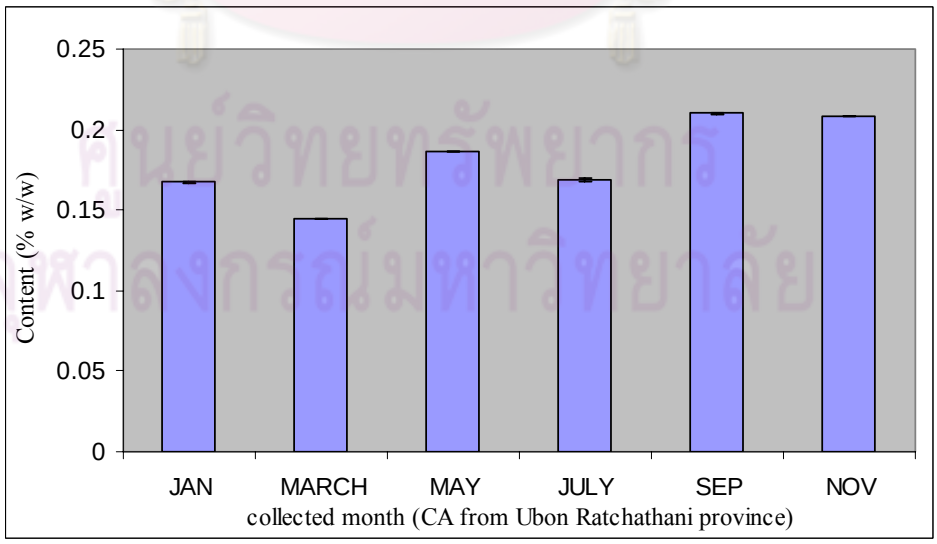


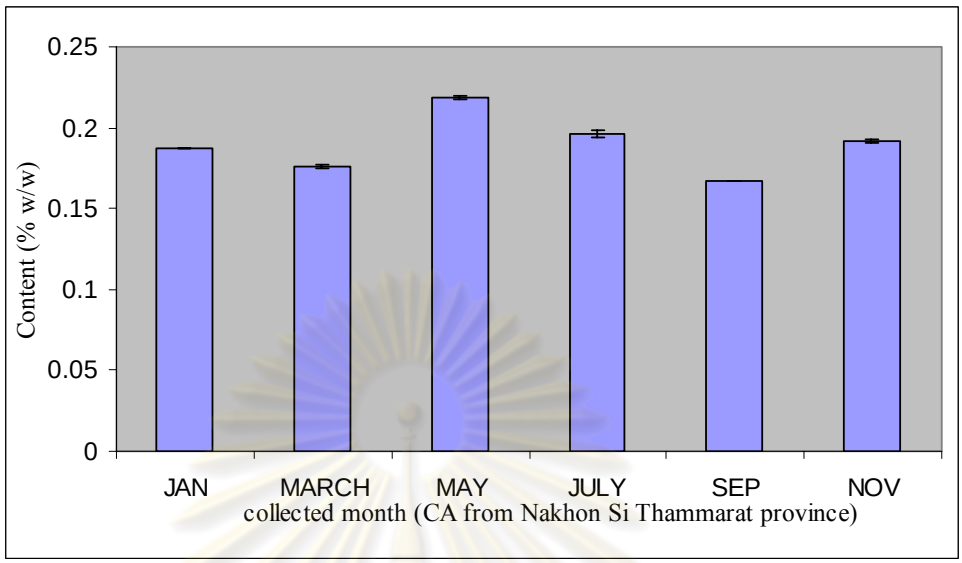
Figure 4.24 Contents of in whole plant of CA from the commercial crop province in annual period Nakhon Pathom **(a)**, Ubon Ratchathani **(b)**, Nakhon Si Thammarat province **(c)** and compared three provinces **(d)**



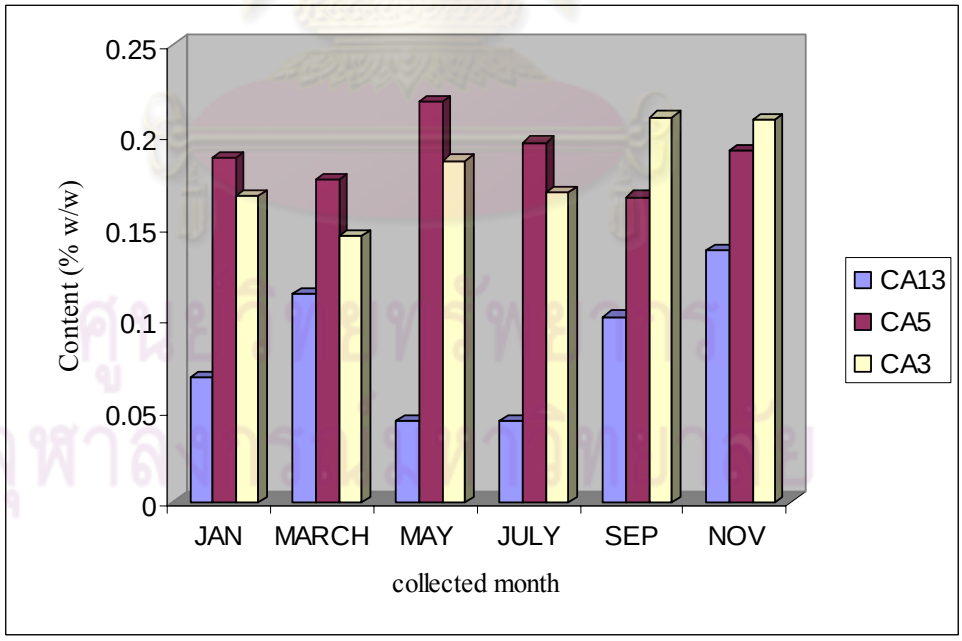
(a)



(b)


0.25
0.2
0.15
0.1
0.05
0
Content (% w/w)
JAN
MARCH
MAY
JULY
SEP
NOV
collected month (CA from Nakhon Si Thammarat province)

(c)


0.25
0.2
0.15
0.1
0.05
0
Content (% w/w)
JAN
MARCH
MAY
JULY
SEP
NOV
collected month
CA13
CA5
CA3

(d)

VITAE

Miss Pornpak Sirathanarun was born on June 1, 1979 in Bangkok, Thailand. She has received her Bachelor of Pharmaceutical Sciences in 2002 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.