# PHARMACOGNOSTIC SPECIFICATION OF *USNEA SIAMENSIS* AND QUANTITATIVE ANALYSIS OF USNIC ACID BY THIN LAYER CHROMATOGRAPHY

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Public Health Sciences
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# ลักษณะทางเภสัชเวทของฝอยลมและปริมาณวิเคราะห์กรดอุสนิคโดยวิธีทินเลเยอร์โครมาโทกราฟี

นายชยานนท์ เชาวน์วุฒิกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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| Thesis Title | PHARMACOGNOSTIC SPECIFICATION OF *USNEA SIAMENSIS* AND QUANTITATIVE ANALYSIS OF USNIC ACID BY THIN LAYER CHROMATOGRAPHY |
| By | Mr. Chayanon Chaowuttikul |
| Field of Study | Public Health Sciences |
| Thesis Advisor | Chanida Palanuvej, Ph.D. |
| Thesis Co-advisor | Associate Professor Nijsiri Ruangrungsi, Ph.D. |

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ชยานนท์ เชาวน์วุฒิกุล : ลักษณะทางเภสัชเวทของฝอยลมและปริมาณวิเคราะห์กรดอุสนิคโดยวิธีทินเลเยอร์โครมาโทกราฟี. (PHARMACOGNOSTIC SPECIFICATION OF *USNEA SIAMENSIS* AND QUANTITATIVE ANALYSIS OF USNIC ACID BY THIN LAYER CHROMATOGRAPHY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ดร. ชนิดา พลานุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. นิจศิริ เรืองรังษี, 132 หน้า.

ฝอยลม มีชื่อทางวิทยาศาสตร์ว่า *Usnea siamensis* Wainio ฝอยลมเป็นพืชสมุนไพรที่ใช้ในการรักษาของแพทย์พื้นบ้าน เนื่องจากฝอยลมยังไม่มีข้อกำหนดมาตรฐานในตำรามาตรฐานยาสมุนไพรไทย การศึกษาครั้งนี้จึงมีจุดประสงค์เพื่อจัดทำข้อกำหนดทางเภสัชเวท รวมถึงวิเคราะห์ปริมาณกรดอุสนิคในฝอยลมโดยวิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโทเมทรี และวิธีการวิเคราะห์รูปภาพทางทินเลเยอร์โครมาโทกราฟี โดยศึกษาฝอยลมจาก 15 แหล่งทั่วประเทศไทย วาดภาพลายเส้นแสดงลักษณะทั้งต้นของฝอยลม ลักษณะทางมหภาคของฝอยลม มีรูปร่างเป็นเส้น ยาวประมาณ 10-20 เซนติเมตร สีเทา-เขียว ลักษณะเด่นทางจุลภาคของฝอยลมเมื่อส่องด้วยกล้องจุลทรรศน์อิเล็กตรอน จะพบเส้นใยราบนพื้นผิวของแทลลัสและมีช่องว่างกระจายในแกนกลาง การศึกษาเอกลักษณ์ทางเคมี-ฟิสิกส์ของฝอยลม พบว่า มีสิ่งปลอมปน ปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด น้ำหนักที่หายไปเมื่อทำให้แห้ง และปริมาณน้ำ ไม่เกินร้อยละ 6.24, 0.96, 0.16, 10.80 และ 13.08 โดยน้ำหนักตามลำดับ ปริมาณสารสกัดด้วยเอทานอล และปริมาณสารสกัดด้วยน้ำ ไม่น้อยกว่าร้อยละ 5.59 และ 3.47 โดยน้ำหนักตามลำดับ การศึกษาด้วยเทคนิคของทินเลเยอร์โครมาโทกราฟี โดยใช้ตัวทำละลายโทลูอีน เอทิลอะซิเทต และกรดฟอร์มิค (139:83:8) เป็นเฟสเคลื่อนที่ ตรวจวัดภายใต้แสงอัลตราไวโอเลต (254 และ 365 นาโนเมตร) เช่นเดียวกับฉีดพ่นด้วยสารละลายกรดซัลฟิวริกร้อยละ 10 ในเมทานอล พบว่ามีค่า hRf เท่ากับ 74 การวิเคราะห์เชิงปริมาณด้วยเทคนิคทางทินเลเยอร์โครมาโทกราฟีโดยใช้ตัวทำละลายคลอโรฟอร์ม และ เมทานอล (9:1) เป็นเฟสเคลื่อนที่ วิเคราะห์ปริมาณกรดอุสนิคโดยวิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโทเมทรีโดยใช้เครื่อง CAMAG TLC Scanner ร่วมกับโปรแกรม winCATS และวิธีการวิเคราะห์รูปภาพทางทินเลเยอร์โครมาโทกราฟีโดยใช้โปรแกรม ImageJ มีช่วงวิเคราะห์แบบโพลิโนเมียล ระหว่าง 0.2-1.0 มิลลิกรัม และมีค่าสัมประสิทธิ์สหสัมพันธ์ เท่ากับ0.9981 และ 0.9994 ตามลำดับ ระดับความเที่ยงของวิธีวิเคราะห์ ประเมินจากค่าสัมประสิทธ์ของการกระจาย มีค่าระหว่างร้อยละ 10.25-19.39 และ 8.49-11.50 ตามลำดับ ค่าเฉลี่ยการคืนกลับระหว่างร้อยละ 83.77-100.45 และ 99.17-120.49 ตามลำดับ ขีดจำกัดของการตรวจพบและขีดจำกัดของการหาปริมาณมีค่า 0.06, 0.18 มิลลิกรัม และ 0.11, 0.34 มิลลิกรัม ตามลำดับ ค่าความคงทนของวิธี มีค่าสัมประสิทธ์ของการกระจายร้อยละ 0.803 และ 1.094 ตามลำดับ วิเคราะห์ปริมาณกรดอุสนิคในฝอยลม มีค่าเฉลี่ยที่ 2.33 และ 2.26 กรัม/100กรัมของพืชแห้งตามลำดับ การเปรียบเทียบปริมาณกรดอุสนิคระหว่าง 2 วิธี ถูกทดสอบโดยใช้สถิติ paired *t*-test พบว่าปริมาณกรดอุสนิคโดยทั้งสองวิธีไม่แตกต่างกันอย่างมีนัยสำคัญ ( t = 1.183, *P* = 0.256) การทดสอบหาฤทธิ์การต้านเชื้อจุลชีพโดยวิธีทีแอลซี-ไบโอออโตกราฟี แสดงขอบเขตการยับยั้งต่อเชื้อ *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis*, *Micrococcus luteus* และ *Candida albicans* ผลการศึกษาครั้งนี้สามารถจัดทำเป็นข้อกำหนดมาตรฐานของสมุนไพรฝอยลมในประเทศไทย ซึ่งจะเป็นประโยชน์ต่อการควบคุมคุณภาพวัตถุดิบ และการวิจัย พัฒนาตัวยานี้ต่อไป

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต.....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

# # 5578953953 : MAJOR PUBLIC HEALTH SCIENCES
KEYWORDS : PHARMACOGNOSTIC SPECIFICATION / *USNEA SIAMENSIS* / USNIC ACID / THIN LAYER CHROMATOGRAPHY-DENSITOMETRY / THIN LAYER CHROMATOGRAPHY IMAGE ANALYSIS

CHAYANON CHAOWUTTIKUL : PHARMACOGNOSTIC SPECIFICATION OF *USNEA SIAMENSIS* AND QUANTITATIVE ANALYSIS OF USNIC ACID BY THIN LAYER CHROMATOGRAPHY. ADVISOR : CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR : ASSOCIATE PROFESSOR NIJSIRI RUANGRUNGSI, Ph.D., 132 pp.

*Usnea siamensis* Wainio was Thai herbal drug that used to treat diseases in folk medicine. Because there was lacked of quality control in *U. siamensis* crude drug, thus this research aimed to report the pharmacognostic specification and analysis usnic acid content in *U. siamensis* by TLC-densitometry and TLC image analysis. Dried *U. siamensis* crude drugs were purchased from 15 various traditional drug stores throughout Thailand. The whole plant of *U. siamensis* was illustrated in detail. The morphological character of *U. siamensis* was fruticose lichen, up to 5 m long, grayish-green strands hanging from the branches of trees and showed mycelium on the surface of thallus and many densely distributed holes in central axis by scanning electron microscopy. The foreign matter, total ash, acid insoluble ash, loss on drying and water content should be not more than 6.24, 0.96, 0.16, 10.80 and 13.08% w/w respectively whereas ethanol soluble extractive and water soluble extractive values should be not less than 5.59 and 3.47% w/w respectively. Thin layer chromatographic fingerprint of *U. siamensis*'s ethanol extract used toluene, ethyl acetate and formic acid (139:83:8) as mobile phase, observed under 254 and 365 nm wavelength ultraviolet light and sprayed with 10% sulfuric acid in methanol showed hRf value at 74. The quantitative analysis by thin layer chromatography used chloroform and methanol (9:1) as mobile phase. The usnic acid content was analyzed by TLC-densitometry performed with winCATS software and TLC image analysis performed with ImageJ software. The regression lines of both methods were polynomial in the range of 0.2-1.0 mg/spot and correlation coefficients were 0.9981 and 0.9994 respectively. The precisions calculated by the %RSD of repeatability and intermediate precision, were between 10.25-19.39 and 8.49-11.50 %RSD respectively. The average recoveries were between 83.77-100.45 and 99.17-120.49 %recoveries respectively. LOD and LOQ were 0.06, 0.18 mg and 0.11, 0.34 mg respectively. The robustness was 0.803 and 1.094 %RSD of peak area respectively. The usnic acid contents in *U. siamensis* were 2.32 and 2.26 g/100g of dried crude drug respectively. The comparison of usnic acid contents between both methods was statistically analysed by paired *t*-test. It was found that usnic acid contents by two methods were not significantly different ($t = 1.183$, $P = 0.256$). TLC-bioautographic method showed the inhibition zone at Rf of usnic acid against *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis*, *Micrococcus luteus* and *Candida albicans*. This study provided scientific information for the quality control of *U. siamensis* including usnic acid in Thailand.

Field of Study : Public Health Sciences Student's Signature .....

Academic Year : 2013 Advisor's Signature .....

Co-advisor's Signature .....

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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| ATCC | = | American type culture collection |
| CCD | = | Charge-couple device |
| CFU | = | Colony forming unit |
| cm | = | Centimeter |
| °C | = | Degree Celsius |
| $ED_{50}$ | = | Fifty percent effective dose |
| g | = | Gram |
| GC | = | Gas chromatography |
| HPLC | = | High performance liquid chromatography |
| HPTLC | = | High performance thin layer chromatography |
| hr | = | Hour |
| $IC_{50}$ | = | Fifty percent inhibitory concentration |
| ICH | = | The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use |
| IUPAC | = | International Union of Pure and Applied Chemistry |
| kg | = | Kilogram |
| kV | = | Kilovolt |
| l | = | Liter |
| LOD | = | Limit of detection |
| LOQ | = | Limit of Quantification |
| m | = | Meter |
| mg | = | Milligram |
| mg/ml | = | Milligram per milliliter |
| min | = | Minute |
| ml | = | Milliliter |
| mm | = | Millimeter |
| nm | = | Nanometer |
| $R^2$ | = | Correlation coefficients |
| Rf | = | Retention factor |

| | | |
|---|---|---|
| RSD | = | Relative standard deviation |
| SD | = | Standard deviation |
| SEM | = | Scanning electron microscope |
| spp. | = | Species |
| TLC | = | Thin layer chromatography |
| UV | = | Ultraviolet |
| WHO | = | World Health Organization |
| µl | = | Microliter |
| µm | = | Micrometer |
| % | = | Percent |
| λ | = | Lambda |

# CHAPTER I
# INTRODUCTION

## Background and significance of the study

Since the World Health Organization recommends member countries to use folk medicines and herbal medicines in primary health care projects, herbal drugs have been popular and developmental in many countries. The herbal medicinal drugs are used as therapeutic and raw materials for the pharmaceutical industry. The quality of herbal medicinal drug still has not been shown enough researches to support the confidence of consumer. Thus, there are needs of the processes to insure the quality of herbal medicinal plant products by using modern control techniques following the World Health Organization guidelines [1].

In Thailand, the herbal medicinal products have been used for years and years. Furthermore, herbal medicinal drugs have been popular in the last few years, which these drugs are used to treat and alleviate the diseases. The herbal medicinal drugs not only are used in folk medicines but also widely used in the hospital and commercial market. Though herbal medicinal products have been used extensively in Thailand but there are insufficient scientific researches and the suitable drug used data supported acceptance of physicians and hospital staff to use herbal medicine. According to the National List of Essential Medicines 2012 (the current version), there have been a lot of new herbal medicines and remedies with the pharmacological effects to supported medical uses [2, 3].

*Usnea* spp. (Usneaceae) is a medium to large, hanging beard lichen, thallus fruticose, much branched, bushy, erect, pendent or tailing, terete with a tough central core. The whole of this lichen is reported to have medicinal value such as pain relief and fever control. In folk medicine, it is indicated for infections, dermatitis, mycosis, tuberculosis and pneumonia [4-6].

*Usnea siamensis* Wainio, in Thai is Foi-lom. It is fruticose lichen, up to 5 m long, grayish-green strands hanging from the branches of trees [6]. *U. siamensis* comprises of dibenzofuran derivative, (+)-usnic acid as a major constituent [5, 6]. Usnic acid has been shown for the biological and physiological activities such as antimicrobial, antiparasitic, antimitotic, antiproliferative, anti-inflammatory, analgesic

and antipyretic properties [7-9]. However, there are no standardization parameters to justify the quality of *U. siamensis* medicinal drug.

Thin layer chromatography (TLC) is widely used because of its easy, rapid, inexpensive and useful method for chemical identification. TLC analysis can be applied for both compound-based approach and fingerprint-based approach. Combination of TLC with TLC scanner or TLC image analysis software offers quantitative analysis of medicinal plant component.

Bioautography belongs to antimicrobial screening methods combined with TLC. This technique is useful for biological activity guided identification of potential constituents in crude drug [10, 11].

This study intends to describe the current information on the pharmacognostic specification of *U. siamensis* including usnic acid content and its antibacterial property.

**Objectives of the study**

1. To develop the standardization parameters of *Usnea siamensis* crude drug.
2. To investigate the content of usnic acid in *Usnea siamensis* crude drug by TLC image analysis using ImageJ software compared to TLC densitometry.
3. To evaluate the antimicrobial activities of usnic acid in *Usnea siamensis* crude drug by bioautography.

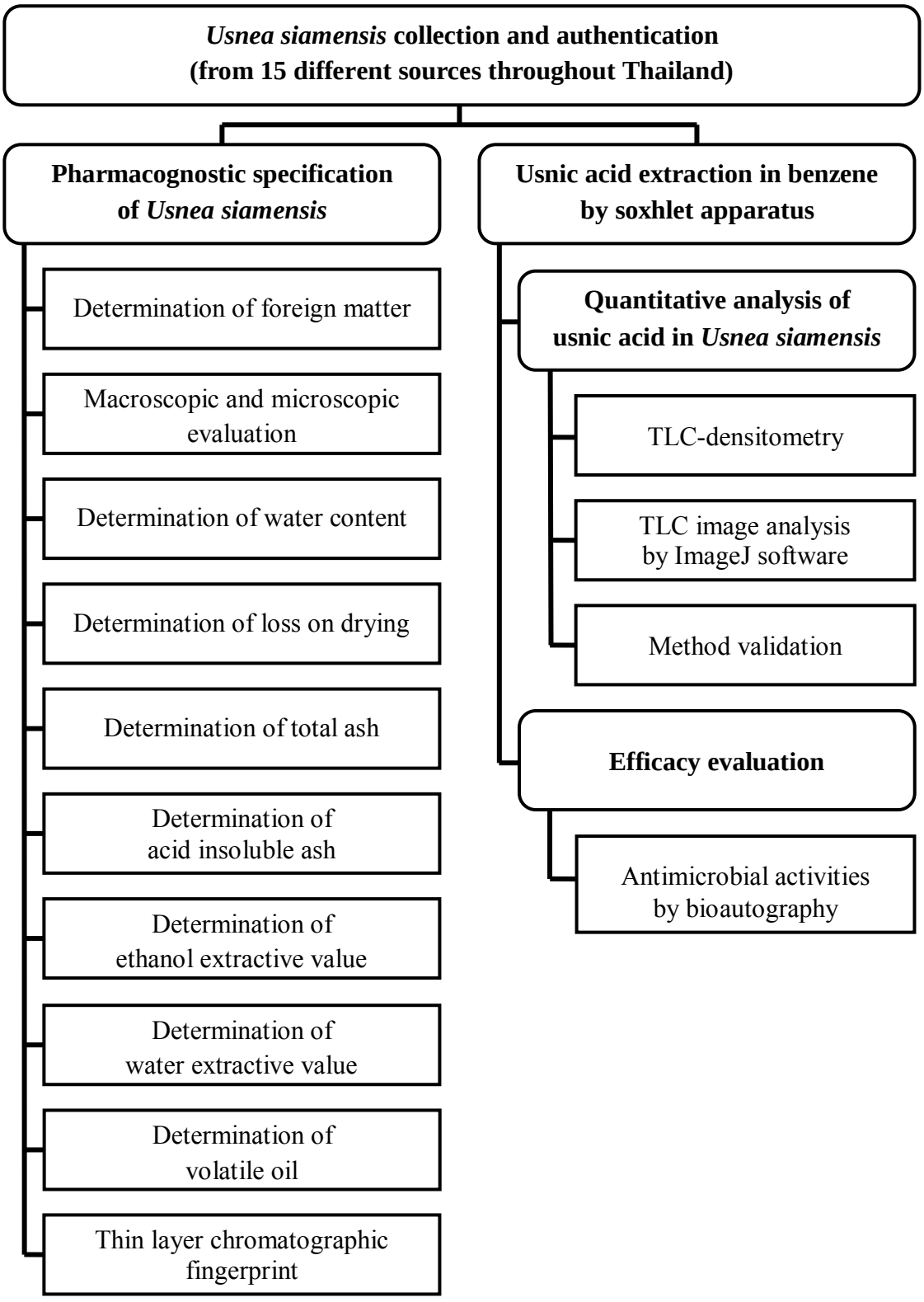



**Figure 1.** The conceptual framework

# CHAPTER II
# REVIEWED LITERATURES

## Lichen

The term "lichen" appears to have a Greek origin and was used by Theophratus in the "History of Plants" to describe the superficial growth on the bark of olive trees [6]. Lichens are symbiotic organism between fungi and algae and/or cyanobacteria. The green algae bring about photosynthesis but cannot hold moisture well whereas the fungi produce active compounds and maintain moist conditions but cannot photosynthesize [6, 12]. The lichen distribution was spread from the tropical to the polar area, approximately 18,500 lichen species found throughout the world and cover land surface about 8% [12].

Lichens are divided to three main morphological types: crustose, foliose and fruticose type. Crustose lichens are tightly attached to the substrate with their lower surface and may not be removed from it without destruction and usually found on rocks and tree bark. Foliose lichens are leaf-like, flat and only partially attached to the substrate. The thallus lobes of fruticose lichens are hair-like, strap-shaped or shrubby and the lobes may be flat or cylindrical. They always stand out from the surface of the substrate [11].

## *Usnea* spp.

*Usnea* spp. is a large worldwide genus, found about 300-600 species around the world. This species mostly grow up in mountain forests between 1,500-4,000 m, especially hang on the upper part close to the tree. [13, 14]

Botanical description: *Pendent or shrubby, filamentous, fruticose lichens; yellowish green or darkening, occasionally reddish because of pigment in the cortex; branches round or angular in cross section and characterized by a central, cartilaginous cord (axis) of supporting tissue; cortex thick, thin, or falling away; medulla thin or thick, loose and cottony (lax) or dense, white or pigmented yellow to dark red; axis slender or relatively broad, surface of branches smooth, or uneven due to various kinds of bumps or warts* [15].

In this genus, the known lichen compounds were alectorialic acid, atranorin, barbatic acid, barbactolic acid, caperatic acid, diffractaic acid, evernic acid, fumarprotocetraric acid, galbinic acid, norstictic acid, protocertraric acid, psoromic acid, salazinic acid, squamatic acid, stictic acid, stictic acid complex, thamnolic acid, usnic acid, and undetermined substances [13].

**List of *Usnea* spp. lichen in Thailand is shown below:** [16]

*Usnea abissinica* Motyka
*Usnea acanthera* Motyka
*Usnea albomaculata* Motyka
*Usnea australis* Fr.
*Usnea baileyi* (Stirt.) Zahlbr.
*Usnea bicolorata* Motyka
*Usnea bismolliuscula* Zahlbr.
*Usnea bornmuelleri* J.Steiner
*Usnea chloreoides* (Vainio) Motyka
*Usnea complanata* (Müll.Arg.) Motyka
*Usnea cristata* Motyka
*Usnea dasycera* (Nyl.) Motyka
*Usnea elata* Motyka
*Usnea endochroa* var. papillata (Vain.) Zahlbr.
*Usnea endorhodina* (Vain.) Zahlbr.
*Usnea exasperate* (Müll.Arg.) Motyka
*Usnea formula* (Stirt.) Motyka
*Usnea florida* var. subcomosa Vain.
*Usnea gigas* Motyka
*Usnea gonoides* (Stirt.) Swinscow & Krog
*Usnea haumanii* Motyka
*Usnea himantodes* Stirt.
*Usnea hossei* Vain.
*Usnea incrassate* Motyka
*Usnea leprosa* Motyka
*Usnea leucospilodea* Nyl.
*Usnea litchtenstenii* J.Steiner
*Usnea luridorufa* Stirt.
*Usnea maculate* Stirt.
*Usnea nodulosa* Swinscow & Krog
*Usnea pectinata* Taylor
*Usnea perhispidella* J.Steiner
*Usnea perplexans* Stirt.
*Usnea pulvinulata* C.W.Dodge
*Usnea roseola* Vainio
*Usnea rubicunda* Stirt.
*Usnea siamensis* Wainio
*Usnea sorediosula* Motyka
*Usnea subcristata* C.W.Dodge
*Usnea subflorida* (Zahlbr.) Motyka
*Usnea submollis* J.Steiner
*Usnea trichodeoides* Motyka
*Usnea undulate* Stirt.
*Usnea welwitschiana* Motyka

**Taxonomy**

Kingdom: Fungi

Division: Ascomycota

Class: Lecanoromycetes

Order: Lecanorales

Family: Usneaceae

Genus: Usnea

Species: *Usnea siamensis*

***Usnea siamensis* Wainio**

**Description:** Fruticose lichen, up to 5 m long, grayish-green strands hanging from the branches of trees.

**Ecology:** It is very sensitive to air pollution, especially sulfur dioxide. Under bad conditions they may grow no larger than a few millimeters, if they survive at all. Where the air is unpolluted, they can grow to 10–20 cm long.

**Distribution:** Many *Usnea* species, which are reported to be found in Thailand especially in the northern and northeastern of Thailand in mountain forest.

*Usnea siamensis* Wainio have been used in folk medicine as bitter tonic, carminative, antidiarrhea, antidysentery and anti-tumor [6]. The active constituent of *U. siamensis* was (+)-usnic acid [6, 7].

**Usnic acid**

**IUPAC:** 2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2*H*,9b*H*)-dibenzofurandione

**Molecular weight:** 344.32

**Description:** Yellow crystalline solid

**Melting point:** 204 °C

**Solubility:** practically insoluble in water but soluble in organic solvents such as, acetone, chloroform, methanol and dichloromethane [17]

**Usnic acid and distribution**

Usnic acid, a dibenzofuran derivative, is a secondary lichen metabolite. Its exits in two enantiomeric forms, (+)-usnic acids and (-)-usnic acids which differ in projection of the angular methyl group at the chiral 9b position (Figure 2.). Not only (+)- and (-)-usnic acids but also (+)- and (-)-isousnic acids occur in lichens (Figure 3.) [8, 9].

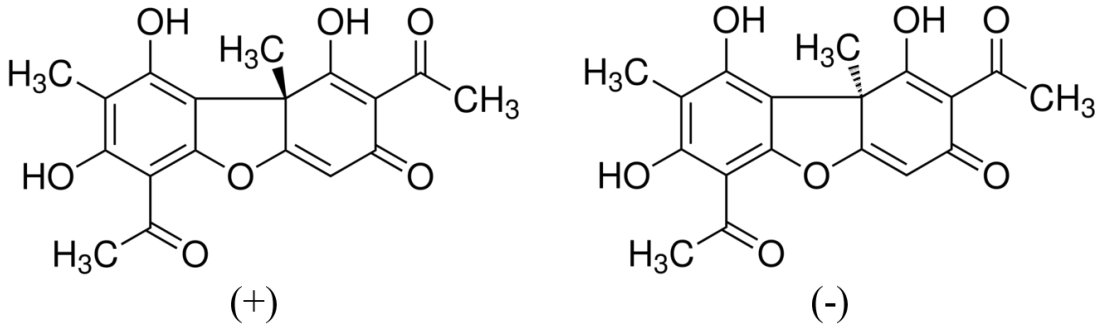



**Figure 2.** Structure of (+)- and (-)-usnic acids

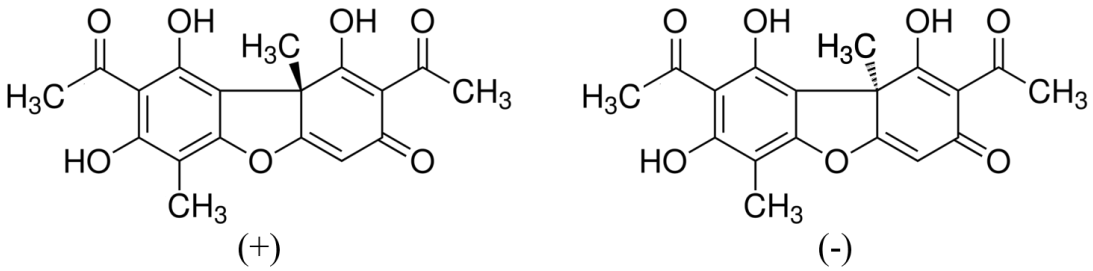



**Figure 3.** Structure of (+)- and (-)-isousnic acids

Usnic acid was found in many lichens such as *Alectoria*, *Cladonia*, *Evernia*, *Lacanora, Parmelia, Ramalina*, *Usnea* and other lichen genera. The abundant sources of usnic acid, yields of up to 6 %, have been found in *Alectoria* species. Closely related compounds of usnic acid also have been found in fungi, e.g. cercosporamide and usnic acid amide in *Cercosporidium henningsii* [8, 9].

**Medicinal use of usnic acid in lichen**

Lichens containing usnic acid have been used as crude drugs in many country. *Cladonia* species were used to treat of pulmonary tuberculosis. *Usnea* species have been used for fever control and pain relief in Africa, Asia and Europe. Chinese was used *U. longissima* in wound healing and as an expectorant. Hippocrates used *U. barbata* to treat urinary complaints. Usnic acid from extract of *U. barbata* has been used in pharmaceutical preparations and cosmetic. In Finland, *Ramalina thrausta* was used to treat wounds, athlete's foot or other skin complaints, sore throat and toothache. Lately, health food supplements containing usnic acid are used in weight reduction.

**Biological activity of usnic acid**

**Antimicrobial and antiprotozoal activities**

Both enatiomeric forms are active against gram positive bacteria and mycobacteria. Lauterwein *et al*. (1995) showed that (+)- and (-)- usnic acid have been exhibited activity against of *Enterococcus faecalis* and *E. faecium* and *Staphylococcus aureus*, including strains resistant to methicillin and mupirocin *in vitro* by using standardized assays. Both the isomers showed activity against pathogenic anaerobic gram negative bacilli, i.e. *Bacteroides* spp. and anaerobic gram positive bacteria, i.e. *Clostridium* and *Propionibacterium* species but (+)-usnic acid showed more potential against *E. faecalis* and some of the *Bacteroides* species than (-)-usnic acid [8, 9].

Ghione *et al*. (1988) examined usnic acid inhibitory activity against *Streptococcus mutans* isolated from human dental lesions. One percent of (+)-usnic acid in mouthwash was administered to human volunteers and oral bacterial flora were sampling and cultured. The study showed the selective effect of usnic acid on *S. mutans* whereas the other normal oral bacteria were not damaged. Grasso *et al*. (1989) confirmed the study using a toothpaste containing (+)-usnic acid and found the decrease in a number of *S. mutans* colony. The study also found that (+)-usnic acid was more effective against *S. mutans* than (-) enantiomer [8, 9].

Lauterwein *et al*. (1995) reported that usnic acid was active against standard strains of Staphylococci, Enterococci and anaerobic bacteria strains *in vitro*. Ten percents wet weight of usnic acid of ethoxydiglycol extracts from lichens have been shown for the preservative potential in moisturizing cream [8, 9].

Proksa *et al*. (1996) mentioned the antifungal activity of usnic acid against the plant pathogens *Penicillium frequentans* and *Verticillium albo-atrum* [8].

Wu *et al*. (1995) showed that (-)-usnic acid can inhibit *Trichomonas vaginalis*, the pathogenic protozoan, at lower concentrations than metronidazole *in vitro* [8].

**Antiviral activity**

Yamamoto *et al*. (1995) screened five lichen compounds for antiviral effect. (+)-Usnic acid isolated from *U. longissima* was indicated to be the most effective against tumour-promotor-induced Epstein–Barr virus. (+)-Usnic acid ($ED_{50}$ 1.0 µg/ml) was more active than (-)-usnic acid ($ED_{50}$ 5.0 µg/ml) [8, 9].

A clinical study in Italy used usnic acid and zinc sulphate to treat female patients infected with genital human papillomavirus as adjuvant therapy to radiosurgical treatment, the results showed patients whom receiving the Zn–usnic acid vaginal formulation, both with regard to re-epithelization of lesions and recurrence of infection over a 6 month period [8, 9].

Perry *et al*. (1999) demonstrated that (+)-usnic acid can inhibit cytopathic effects of polio type 1 and herpes simplex type 1 viruses by administration on filter paper discs which were placed on virus-infected African green monkey kidney (BS-C-1) cells [8].

**Allergology**

Allergology of usnic acid in human has been reported that adverse effects are only allergic contact dermatitis and local irritation mainly occurs in forestry workers and wood cutter whom contact with lichen [9].

**Antitumor activity**

Kupchan and Kopperman (1975) reported that usnic acid can affect against Lewis lung carcinoma [8]. Takai *et al*. (1979) tested 11 usnic acid derivatives for activity against P388 leukemia and Lewis lung carcinoma, the results showed that no increases in the survival of animal models [8, 9]. Ding *et al*. (1994) examined 6 lichen constituents to cytotoxic activity by brine shrimp lethality test, usnic acid was the most strong bio-active power out of all compounds [9].

**Antiproliferative activity**

Takai *et al*. (1979) showed that (+)-usnic acid has been exhibited moderate activity in the murine P388 leukaemia assay and *in vitro* cytotoxic activity against cultured L1210 cells [8]. Cardarelli *et al*. (1997) showed that cell counts of leukemic (K-562) and endometrial carcinoma (Ishikawa, HEC-50) cell lines have been reduced by (+)-usnic acid (50 mg/ml) when exposed to the cultures for 21 h [8, 9]. Kumar and Müller (1999) reported that (+)-usnic acid had antiproliferative activity against the human keratinocytecell line HaCaT ($IC_{50}$ 2.1mM) [8, 9].

**Anti-inflammatory, analgesic and antipyretic activities**

Vijayakumar *et al*. (2000) showed anti-inflammatory effects of oral treatment (100 mg/kg) of (+)-usnic acid in acute effect (carrageenin-induced rat paw oedema assay) and chronic effect (the cotton pellet assay) compared to ibuprofen at the same dose [8, 9]. The study of Ôtsuka *et al*. (1972) showed (+)-usnic acid activity by the cotton pellet assay in rats at an oral dose of 50 mg/kg [8].

Okuyama *et al*. (1995) demonstrated that usnic acid and diffractaic acid from *U. diffracta* methanol extract had analgesic and antipyretic activities on oral treated mice[8, 9].

**Quality control methods for herbal material** [1]

WHO has published "Quality control methods for herbal material guideline" that describes various information of analytical tests for evaluation of the quality of medicinal plant materials. The following methods facilitate to examine the quality of herbal material by using modern control techniques.

**Macroscopic and microscopic examination**

Herbal materials are categorized according to sensory, macroscopic and microscopic characteristics. An examination to determine these characteristics is the first step towards establishing the identity and the degree of purity of such materials, and should be carried out before any further tests are undertaken. Visual inspection provides the simplest and quickest means by which to establish identity, purity and possibly quality. Macroscopic identity of herbal materials is based on shape, size, color, surface characteristics, texture, fracture characteristics and appearance of the cut surface. Microscopic inspection of herbal materials is indispensable for the identification of broken or powdered materials; the specimen may have to be treated with chemical reagents.

**Determination of foreign matter**

Herbal materials should be entirely free from visible signs of contamination by moulds or insects, and other animal contamination, including animal excreta. No abnormal odour, discoloration, slime or signs of deterioration should be detected. Macroscopic examination can conveniently be employed for determining the presence of foreign matter in whole or cut plant materials. However, microscopy is indispensable for powdered materials.

Any soil, stones, sand, dust and other foreign inorganic matter must be removed before herbal materials are cut or ground for testing. Foreign matter is material consisting of any or all of the following:

— Parts of the herbal material or materials other than those named with the limits specified for the herbal material concerned;

— Any organism, part or product of an organism, other than that named in the specification and description of the herbal material concerned;

— Mineral admixtures not adhering to the herbal materials, such as soil, stones, sand and dust.

**Determination of total ash**

The total ash method is designed to measure the total amount of material remaining after ignition. This includes both "physiological ash", which is derived from the plant tissue itself, and "non-physiological" ash, which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface.

**Determination of acid-insoluble ash**

Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

**Determination of extractable matter**

This method determines the amount of active constituents extracted with solvents from a given amount of herbal material. It is employed for materials for which as yet no suitable chemical or biological assay exists.

For ethanol-soluble extractable matter, use the concentration of solvent specified in the test procedure for the herbal material concerned; for water-soluble extractable matter, use water as the solvent. Use other solvents as specified in the test procedure.

**Determination of water content**

An excess of water in herbal materials will encourage microbial growth, the presence of fungi or insects, and deterioration following hydrolysis. Limits for water content should therefore be set for every given herbal material. This is especially important for materials that absorb moisture easily or deteriorate quickly in the presence of water.

The azeotropic method gives a direct measurement of the water present in the material being examined. When the sample is distilled together with an immiscible solvent, such as toluene or xylene, the water present in the sample is absorbed by the solvent. The water and the solvent are distilled together and separated in the receiving tube on cooling. If the solvent is anhydrous, water may remain absorbed in it leading to false results. It is therefore advisable to saturate the solvent with water before use.

**Determination of loss on drying**

The test for loss on drying determines both water and volatile matter. Drying can be carried out either by heating to 100 - 105 °C or in a desiccator over phosphorus pentoxide under atmospheric or reduced pressure at room temperature for a specified period of time. The desiccation method is especially useful for materials that melt to a sticky mass at elevated temperatures.

**Determination of volatile oils**

Volatile oils are characterized by their odour, oil-like appearance and ability to volatilize at room temperature. In order to determine the volume of oil, the plant material is distilled with water and the distillate is collected in a graduated tube. The aqueous portion separates automatically and is returned to the distillation flask. If the volatile oils possess a mass density higher than or near to that of water, or are difficult to separate from the aqueous phase owing to the formation of emulsions, a solvent with a low mass density and a suitable boiling-point may be added to the measuring tube. The dissolved volatile oils will then float on top of the aqueous phase.

**Thin layer chromatography**

Thin-layer chromatography (TLC) is a fast screening method to identify and separate compounds in herbal extracts and it can separate relate to herbal species. TLC has some advantages more than other chromatographic techniques such as low cost of instrumentation, short time for analysis and easy to use [18-21].

Sample preparation for TLC method: the solution must be enough concentrated so that analysis can be detected in the applied volume. The solvent used to dissolve the sample must be proper to viscosity and volatility. Application of samples must be accurate, precise volumes and without damage the surface of TLC plate. The polarity of the solvent used for extraction should be similar to the compound mixture to be separated and analyzed [18, 22].

Stationary phase: TLC plates are coated with thin layers such as silica, alumina, cellulose, gypsum and polyamides on glass, plastic or aluminum sheet supporter. Mobile phase is a mixture of two to five different solvents selected experimental using trial and error guided by prior personal experiences and literature

reports of similar separations. Samples can be detected on TLC plate analysis under the ultraviolet light with 254 and 366 nm wavelengths [22, 23].

An important qualitative parameter, which characterizes the position of a spot on TLC plate, is the retention factor (Rf) value. It is define as: [23]

$$Rf = \frac{\text{Distance of the compound from original spot travelled to the developed spot}}{\text{Distance of the solvent from original line travelled to the developed line}}$$

TLC is frequently used as a qualitative and quantitative method. Qualitative method can be determined by the number of compounds in a mixture and identified substances. Whereas quantitative method is used for content determination of require testing substances [23].

Fingerprint is a method for the quality control of herbal samples that has been accepted by WHO. It's suitable for adulterations detection and plant species identification [24]. Chromatographic methods consist of TLC, HPLC and GC are commonly used for fingerprint. TLC is the most method used for identify and authenticate compounds in herbal medicines and its derivative for obtains a fingerprint profile [25, 26].

Quantitative analysis can be performed with data from scanning densitometry and image analysis method. Scanning densitometer is fixed wavelength to measure the difference in absorbance or fluorescence signal between a separated zone and the empty plate background. The peak area data of the unknowns are compared with data from calibration standards chromatographed on the same plate [27, 28].

A typical densitometer, which could also be used for scanning chromatogram, has the following operating characteristic:

- Reflectance or transmission modes
- Absorbance or fluorescence measurements
- Accommodates plates up to 20 x 20 cm
- Wavelength range: 190-800 nm
- Multiwavelength scanning, up to 31 channels
- Computer controlled and data processed
- Full spectra available for qualitative analysis [29].

In previous study, the charge-couple device (CCD) camera is also used in quantitative method. CCD is two-dimensional detectors containing sensors capable for imaging an area in a seconds or real time. The output from each sensor pixel on the CCD is a voltage, which is proportional to the intensity of light falling on the sensor and the exposure time. These series of voltages are digitized and transferred to a computer for storage and data processing. Coupling CCD detection with TLC, the entire chromatographic plate can be imaged in a single exposure yielding rapid quantification in shorter analysis time than of slit scanning densitometers. CCD detectors have demonstrated extremely low dark current and read noise characteristics, high sensitivity and excellent linearity. These features have made the CCD an excellent detector for many imaging applications in chemical analysis, such as fluorescence detection. The advantages of image analysis are fast data acquring and simple instrument design [27, 28, 30].

ImageJ is one of the several image analysis softwares that images from CCD camera are required for analysis [30]. ImageJ is an open source, which is developed in Java programs, that users can develop program and fix the program. It is used in many fields, for example medical researches and biological microscopy. It can be used in both Windows and Macintosh, available free download from website of the US National Institute of Mental Health. (http://rsbweb.nih.gov/ij/index.html) [31].

**Efficacy evaluation**: **Antimicrobial activities testing**

**Bioautography**

Bioautography is microbiological screening methods usually apply for the detection of antimicrobial activities. The screening can be defined as the first procedure, which is applied to analyze sample, in order to establish the presence or absence of given analytes. These screening methods give more sensitivity than any other methods. Furthermore, they are uncomplicated, inexpensive, time-saving and do not require sophisticated equipment. Bioautography screening methods are based on the biological activities such as antibacterial, antifungal, antitumor and antiprotozoae of the tested substances. This detection method can be effectively combined with layer liquid chromatography techniques, such as thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC), overpressured- layer chromatography (OPLC) and planar electrochromatography (PEC) [32].

TLC-bioautography is extensively used, compact and simple tests which can be prepared with minimum of sample in a short time. Both separation and microbial detection are performed on the same TLC plate. Generally, the method measures antibacterial properties of analyzed substances, i.e. changes in bacterial growth [32, 33].

Bioautographic methods have been divided to three techniques: contact bioautography (agar diffusion), direct bioautography and agar-overlay bioautography (immersion) [32, 33].

**Agar diffusion or contact bioautography**

In this method, place the TLC plate on the surface of inoculated agar to allow diffusion for some minutes or hours. After that, remove the TLC plate and incubated the agar layer. The inhibition zones are shown on the places, where the antimicrobial compounds were in contact with the agar layer. The problems of this method can occur in diffusion of compounds from TLC plate to the agar layer, especially in water-insoluble sample. The agar diffusion is the least-employed of all bioautographic method.

**Direct bioautography**

In this method, dip the developed TLC plate in (or spray with) a suspension of microorganisms and incubate. The microorganism enables growth directly on the TLC plate surface that covered with the broth medium. The inhibition zones appear on the area of antimicrobial compounds.

**Agar-overlay or immersion bioautography**

The developed TLC plate is dipped in or covered with agar medium containing microorganisms and then incubated for several hours. This method is a combination between contact and direct bioautography. The antimicrobial compounds are transferred from TLC plate to the agar medium by diffusion, as in a contact bioautography, while the agar layer remains onto the TLC plate surface during incubation, as in direct bioautography.

# CHAPTER III
# MATERIALS AND METHODOLOGY

## Chemicals

| | |
|---|---|
| Benzene | Merck, Darmstadt, Germany |
| Chloroform | J.T. Baker Chemical Co., Phillipsburg, USA |
| Dichloromethane | RCI Labscan Limited, Bangkok, Thailand |
| Ethanol | RCI Labscan Limited, Bangkok, Thailand |
| Ethyl acetate | Mallinckrodt® Inc., USA |
| Formic acid | RCI Labscan Limited, Bangkok, Thailand |
| Hydrochloric acid | RCI Labscan Limited, Bangkok, Thailand |
| Methanol | RCI Labscan Limited, Bangkok, Thailand |
| Mueller Hinton agar and broth | Merck, Darmstadt, Germany |
| Sabouraud Dextrose agar and broth | Merck, Darmstadt, Germany |
| Sulphuric acid | BDH Chemicals, England |
| Toluene | RCI Labscan Limited, Bangkok, Thailand |
| (+)-Usnic acid | Sigma-Aldrich, CO., St. Louis, USA |

The chemicals were analytical grade.

## Materials

| | |
|---|---|
| Filter paper No.4 | Whatman™ Paper, UK |
| Filter paper No.40 ashless | Whatman™ Paper, UK |
| TLC aluminium sheet 20 × 10 cm | Merck, Darmstadt, Germany |
| silica gel 60 $F_{254}$, 200 μm thickness | |

**Instruments and equipments**

| | |
|---|---|
| Aqua-shaker | Adolf Kühner AG, Switzerland |
| Balance readability 0.01 g (Pioneer™, PA2102) | Ohaus Corp. Pine Brook, NJ, USA |
| Balance readability 0.0001 g | SI-234, Denver Instrument, Germany |
| CAMAG Linomat 5 | CAMAG, Switzerland |
| CAMAG TLC Chamber | CAMAG, Switzerland |
| CAMAG TLC Scanner 3 | CAMAG, Switzerland |
| CAMAG TLC Visualizer | CAMAG, Switzerland |
| Digital camera (Canon PowerShot A650 IS) | Canon Marketing (Thailand) Co., LTD, Bangkok |
| Hot air oven | WTC Binder tuttlingen, Germany |
| ImageJ software (Version: 1.46r) | National Institutes of Health, USA |
| Incinerator | Carbolite, UK |
| Rotary vacuum evaporator | Büchi, Switzerland |
| Scanning electron microscope (Model JSM-5410LV) | JEOL Ltd., Tokyo, Japan |
| Sputter coater (Model SCD 040) | Balzers, Liechtenstein |
| TLC syringe | Hamilton Company, USA |
| Ultrasonic bath | Analytical Lab Science Co., LTD, Bangkok |
| Ultraviolet fluorescence analysis cabinet (Model CC-80) | Spectronics corp., USA |
| Water bath | Brinkmann, USA |
| winCATS software (Version: 1.4.6.2002) | CAMAG, Switzerland |

## Methods

### Plant materials

Dried *Usnea siamensis* crude drugs were purchased from 15 various traditional drug stores throughout Thailand as follows: Bangkok (2 stores), Buri Ram (2 stores), Chiang Rai, Khon Kaen, Krabi, Lop Buri, Nakhon Sawan, Phichit, Phrae, Ranong, Suphan Buri, Udonthani and Uttaradit. All of specimens were authenticated by Assoc. Prof. Nijsiri Ruangrungsi, Ph.D. Voucher specimens were deposited at the College of Public Health Sciences, Chulalongkorn University. *Usnea siamensis* crude drugs were pulverized for further investigation after removal of foreign matter.

### Determination of pharmacognostic specification

#### Determination of foreign matter

Weighed dried *Usnea siamensis* and spread it out in a thin layer and categorized the foreign matter into group by optical inspection or used magnifying lens (6X). Sifted the remainder of the sample through a No. 250 sieve; dust was regarded as mineral admixture. Weighed the portions of this sorted foreign matter and calculated the content of foreign matter in grams per 100 g of crude drug.

#### Morphological evaluation

Dried *Usnea siamensis* crude drug was examined for organoleptic charaters and illustrated by hand drawing in the proportion size related to the original size. The thallus was examined by scanning electron microscope. Cut the dried thallus about 0.5 cm and mounted on the stub with double sided adhesive tabs. After that, coated the specimens with gold in sputter coater then examined with scanning electron microscope at 15 kV. Selected the field, adjusted light and contrast before save to image files.

**Determination of water content**

Weighed 50 g using digital balance (readability 0.01 g) of the dried powdered *Usnea siamensis* to round bottom flask, added 200 ml of water-saturated toluene and boiled by using azeotropic apparatus (Figure 4.). When the water completely distilled, removed heat, allowed the receiving tube to be cool in room temperature and dislodged any droplets of water adhere to the receiving tube's walls. Observed and allowed the water and toluene layers to separate and read off the water's volume. Calculated water's content as the percentage of dry weight.

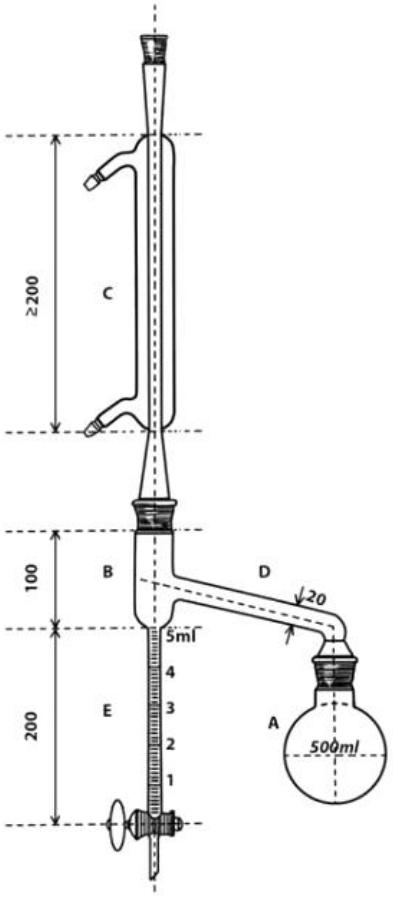



**Figure 4.** Azeotropic apparatus used for determination of water content (dimensions in mm), (A) a glass flask, (B) a cylindrical tube, (C) a reflux condenser, (D) a receiving tube, (E) a graduated receiving tube [1]

**Determination of volatile oil**

Weighed 100 g of the dried powdered *Usnea siamensis*, added 600 ml of water and distilled by Clevenger apparatus (Figure 5.). When the volatile oil completely distilled, removed heat, allowed to cool in room temperature then read off the volume of volatile oil. Calculated volatile oil's content as the percentage of dry weight.

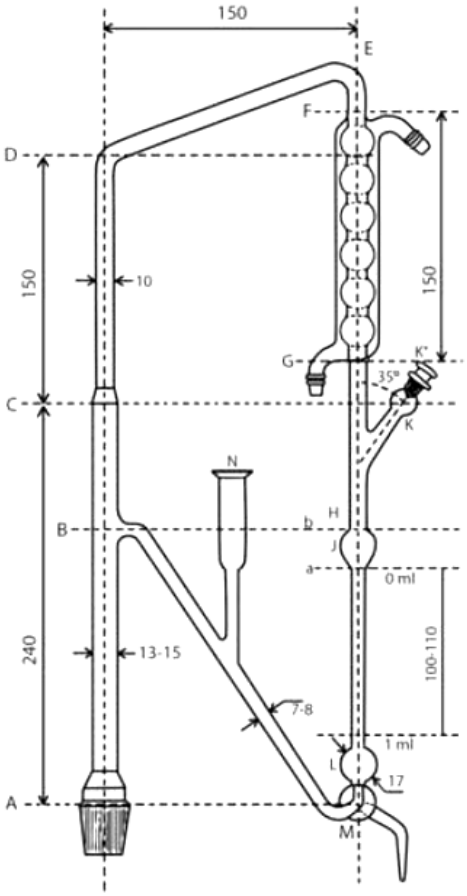



**Figure 5.** Clevenger apparatus used for determination of volatile oil (dimensions in mm), (AC) a vertical tube, (CDE) a bent tube, (FG) a bulb-condenser, (GH) a tube, (HK) a side-arm tube, (K) a tube and (K') a vented ground-glass stopper, (J) a pear-shaped bulb with a volume of 3 ml, (JL) a tube with a volume of 1 ml, (L) a bulb-like swelling with a volume of about 2 ml, (M) a three-way tap, (BM) a connecting tube, (N) a security tube [1]

**Determination of loss on drying**

Weighed exactly 3 g, using digital balance (readability 0.0001 g), of the dried powdered *Usnea siamensis* in the dried pre-weighed crucible. Dried the sample at 105 °C in an oven for 6 hours until constantly weight. Calculated the loss of weight in percentage.

**Determination of total ash**

Ignited the crucible with dried sample mentioned above in the incinerator at 500 °C by gradually increasing temperature. Cooled the crucible in a desiccator. The content of ash was weighed without delay and calculated the content of total ash in percentage.

**Determination of acid insoluble ash**

The crucible that containing the total ash from determination of total ash was added 25.0 ml of hydrochloric acid (70 g/l), covered with a watch-glass and boiled gently for 5 minutes, rinsed the watch-glass with 5 ml of hot water and added this liquid to the crucible. The insoluble matter on an ashless filter-paper No.40 was collected and washed with hot water until the filtrate was neutral, transferred the filter-paper that contained the insoluble matter to the previous crucible, dried on a hot plate and incinerated at 500 °C until ash remaining. The residue was cooled in the desiccator, weighed without delay, and calculated the content of acid-insoluble ash in percentage.

**Determination of water soluble extractive value**

Macerated exactly 5 g of the dried powdered *Usnea siamensis* with 70 ml of water for 6 hours under shaking, let standing for 18 hours, filtered rapidly, washed the marc with water, and adjusted the filtrate to 100.0 ml with water. Transferred 20 ml of the filtrate to a pre-weighed small beaker, evaporated to dryness on a water-bath, further dried at 105°C for 6 hours, cooled in the desiccator for 30 minutes, weighed without delay and calculated extractable matter's content in a percentage of dry weight.

**Determination of ethanol soluble extractive value**

Macerated 5 g of the dried powdered *Usnea siamensis* with 70 ml of 95% ethanol for 6 hours under shaking, let standing for 18 hours, filtered rapidly, washed the marc with 95% ethanol, and adjusted the filtrate to 100 ml with 95% ethanol. Transferred 20 ml of the filtrate to a pre-weighed small beaker, evaporated to dryness on a water-bath, further dried at 105 °C for 6 hours, cooled in a desiccator for 30 minutes, weighed without delay and calculated extractable matter's content in a percentage of dry weight.

**Thin layer chromatographic fingerprint**

Transferred 20 ml of the filtrate from ethanol soluble extraction mentioned above to a small beaker, evaporated to dryness on a water-bath. Dissolved the residue in 1 ml of 10% methanol in dichloromethane. Applied 3 µl of crude extract to the silica gel 60 $F_{254}$ TLC plate and allowed to dry. The TLC plate was developed in TLC chamber saturated with a solvent (toluene : ethyl acetate : formic acid, 139:83:8). After development, the plate was removed and allowed it to dry in air and observed spot on the plate under short wavelength (254 nm) and long wavelength (365 nm) ultraviolet light and sprayed the plate with 10% sulfuric acid in methanol and heated the plate at 110 °C for 10 minutes.

**Quantitative analysis of usnic acid in *Usnea siamensis***

**Preparation of usnic acid standard solutions**

The stock solution of standard usnic acid (1 mg/ml) was prepared in 10% methanol in dichloromethane. The stock solutions were appropriately diluted to obtain the concentration of 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml. These solutions were stored in refrigerator at 4 ºC.

**Preparation of benzene extracts of *Usnea siamensis***

Dried powdered *Usnea siamensis* crude drugs (20 g) were exhaustively extracted with benzene by soxhlet apparatus. The extract was filtered and evaporated to dryness under reduced pressure at ≤ 50 ºC. One milligram of the extract was dissolved in 1 ml of 10% methanol in dichloromethane for further analysis by TLC-densitometry and TLC image analysis.

**TLC-densitometry**

Three microliters of *Usnea siamensis* benzene extract and 3.0 µl of standard usnic acid solutions were applied on the silica gel 60 $F_{254}$ 20 × 10 cm TLC plate using CAMAG Linomat 5. Sample band was set at 10.0 mm while distance between bands was 8.9 mm. The TLC plate was developed using a mixture of chloroform and methanol (9:1) as mobile solvent. After development, the plate was scanned by CAMAG TLC Scanner 3 at the wavelength of 293 nm (maximum absorbance) and expressed as chromatographic peak by winCATS software. The contents of usnic acid in *Usnea siamensis* extracts and crude drugs were calculated based on the calibration curve of usnic acid prepared by plotting peak areas *versus* concentrations of usnic acid applied.

**TLC image analysis by ImageJ software**

Developed TLC plate was further observed under short wave (254 nm) ultraviolet light in ultraviolet fluorescence analysis cabinet. The photos were taken using digital camera and saved as JPEG files with C mode ISO 80. The color intensity of usnic acid band was transformed to chromatographic peak by ImageJ software. The contents of usnic acid in *Usnea siamensis* extracts and crude drugs were calculated based on the calibration curve of usnic acid prepared by plotting peak areas *versus* concentrations of usnic acid applied.

**Method validation** [34]

**Calibration range**

Regression line of peak area *vs* usnic acid concentration and correlation coefficient were determined by Excel 2007.

**Specificity**

The specificity of quantitative analysis of usnic acid in *Usnea siamensis* was determined by comparing absorption spectra of 15 sample spots to that of standard usnic acid using CAMAG TLC Scanner 3.

**Accuracy**

The accuracy of quantitative analysis of usnic acid in *Usnea siamensis* was tested by spike method. Known amounts of standard usnic acid were spiked into the extract to obtain three different levels of usnic acid (low, medium, high) in calibration range. At each level, three determinations were performed. The accuracy was determined as percent recovery by using following formula:

$$\textbf{\% Recovery} = \frac{\textbf{A}}{\textbf{B+C}} \times \textbf{100}$$

where, A = Tested amount of usnic acid in spiked sample extract

B = Amount of usnic acid spiked into sample extract

C = Tested amount of usnic acid in un-spiked sample extract

**Precision**

The precision of quantitative analysis of usnic acid in *Usnea siamensis* was determined by repeatability (intra-day) and intermediate precision (inter-day) studies. Intra-day and inter-day precision were performed by analyzing sample solution of 3 concentrations (each one in triplicate) on the same day and three different days respectively. Calculated the precision in term of %RSD of usnic acid content by following formula:

$$\textbf{\%RSD} = \frac{\textbf{SD}}{\textbf{Mean}} \times \textbf{100}$$

**Limit of detection (LOD)**

LOD was determined from the calibration curve using following formula:

$$\mathbf{LOD} = \frac{\mathbf{3.3\ (SD)}}{\mathbf{S}}$$

Where, SD = the standard deviation of y-intercept

S = the slope of calibration curve

**Limit of quantitation (LOQ)**

LOQ was determined from the calibration curve using following formula:

$$\mathbf{LOQ} = \frac{\mathbf{10\ (SD)}}{\mathbf{S}}$$

Where, SD = the standard deviation of y-intercept

S = the slope of calibration curve

**Robustness**

Mobile phase composition was selected for robustness parameter in this study. A little variation in a mixture ratio of chloroform and methanol was performed as 8.8:1.2, 8.9:1.1, 9.0:1.0, 9.1:0.9 and 9.2:0.8. The robustness was represented by %RSD of peak area of 3 µl of standard usnic acid (1 mg/ml).

$$\mathbf{\%RSD} = \frac{\mathbf{SD}}{\mathbf{Mean}} \times \mathbf{100}$$

**Efficacy evaluation**: **Antimicrobial activities testing**

**Microorganisms**

The microorganisms used in TLC-bioautography were described in table 1. They included three non-spore forming gram-positive bacteria, two spore forming gram-positive bacteria, six non-spore forming gram-negative bacteria and two fungi strains.

**Table 1.** Tested microorganisms

| **Tested microorganisms** | |
|---|---|
| Gram positive bacteria | *Micrococcus luteus* ATCC 9341[1] |
| (Non-spore forming bacteria) | *Staphylococcus aureus* ATCC 6538P[2] |
| | *Staphylococcus epidermidis* (Isolates)[3] |
| Gram positive bacteria | *Bacillus cereus* ATCC 11778[1] |
| (Spore forming bacteria) | *Bacillus subtilis* ATCC 6633[2] |
| Gram negative bacteria | *Enterobacter aerogenes* ATCC 13048[1] |
| (Non-spore forming bacteria) | *Escherichia coli* ATCC 25922[2] |
| | *Pseudomonas aeruginosa* ATCC 9027[2] |
| | *Salmonella typhi* (Isolates)[3] |
| | *Salmonella typhimurium* ATCC 13311[3] |
| | *Shigella* spp. (Isolates)[3] |
| Fungi | *Candida albicans* ATCC 10230[2] |
| | *Saccharomyces cerevisiae* ATCC 9763[2] |

**Sources:** [1]Department of Microbiology, Faculty of Science and Technology, Suan Sunandha Rajabhat University

[2]Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University

[3]Department of Microbiology, Faculty of Science, Chulalongkorn University

**Preparation of inoculums suspensions**

The bacteria and fungi strains were cultivated in Mueller Hinton agar and Sabouraud Dextrose agar respectively then incubated at 37 °C on agar media for 18-24 hrs (for bacteria) or 24-28 hrs (for fungi). Four or five well isolated colonies were suspended in 0.85% normal saline. The turbidity of bacteria and fungi suspensions were adjusted to obtain 0.5 McFarland turbidity standard (optical density 0.08-0.10 at λ 625 nm, light path 1 cm) which equivalent to $1 \times 10^8$ CFU/ml.

**Determination of zone of inhibition by TLC-bioautography**

Bioautography was done with agar-overlay technique. Three microliters of benzene extract of *Usnea siamensis* and usnic acid 1.0 mg/ml in 10% methanol in dichloromethane were separated by TLC using chloroform and methanol, 9:1. Developed TLC plates were placed on Petri dishes containing base agar, 6 ml of top agar with 100 µl of microorganism was gently covered on TLC plates then incubated for 24 hours in a humid atmosphere, allowing for growth of the microorganism. Zone of inhibition was obtained if there were any compounds effective against microorganism.

**Data analysis**

The parameters due to standardization were expressed as grand mean ± pooled SD.

The usnic acid contents between TLC-densitometry and TLC image analysis were compared by paired *t*-test statistical analysis.

# CHAPTER IV
# RESULTS

## Pharmacognostic specification of *Usnea siamensis* Wainio

| | |
|---|---|
| Common Name | Foi-Lom |
| English Names | Old Man's Beard, Beard Moss |
| Scientific Name | *Usnea siamensis* Wainio |
| Family | Usneaceae |
| Part used | Whole plant |
| Ethnomedical uses | bitter tonic, carminative, antidiarrhea, antidysentery |

*Usnea siamensis* was illustrated by hand drawing in the proportion size related to the original size (Figure 6.). Dried crude drugs were shown in Figure 7.

Scanning electron microscopy showed mycelium on the surface of thallus and many densely distributed holes in central axis (Figure 8.-10.).

The pharmacognostic constant numbers due to the quality of *Usnea siamensis* were shown in table 2. The results showed the contents of foreign matter, loss on drying, total ash, acid insoluble ash and water content should be not more than 6.238, 10.802, 0.968, 0.16 and 13.075 % by dry weight respectively whereas ethanol soluble extractive value and water soluble extractive value should be not less than 5.593 and 3.473 % by dry weight respectively.

Thin layer chromatographic fingerprint of ethanol extract of *Usnea siamensis* was shown in Figure 11.

TLC-bioautographic method showed the clear zone in five gram positive bacteria and one fungi strain. (Table 13. and Figure 14.)

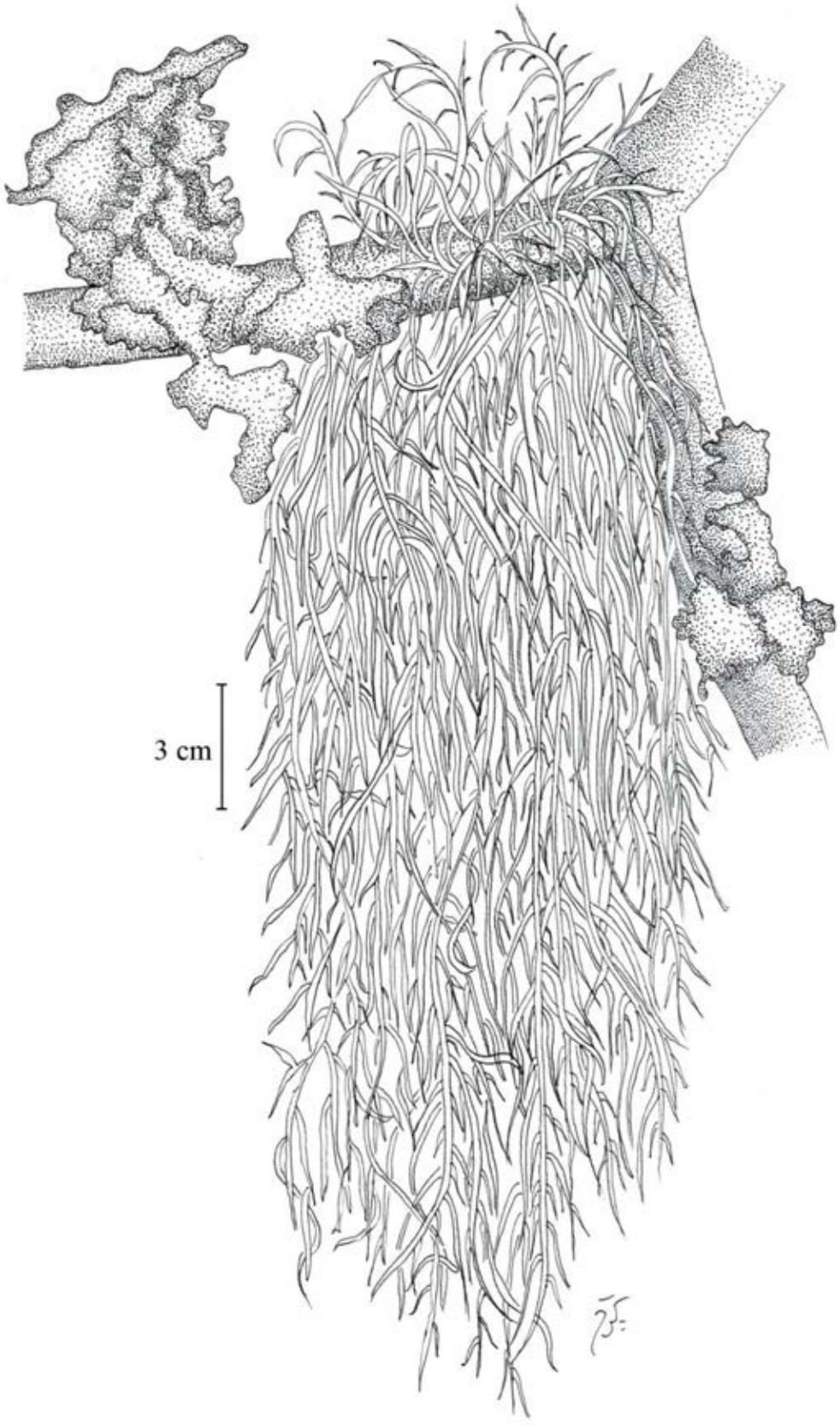



**Figure 6.** Fruticose lichen *Usnea siamensis* Wainio


**Figure 7.** Crude drug of *Usnea siamensis*

**Microscopic character**

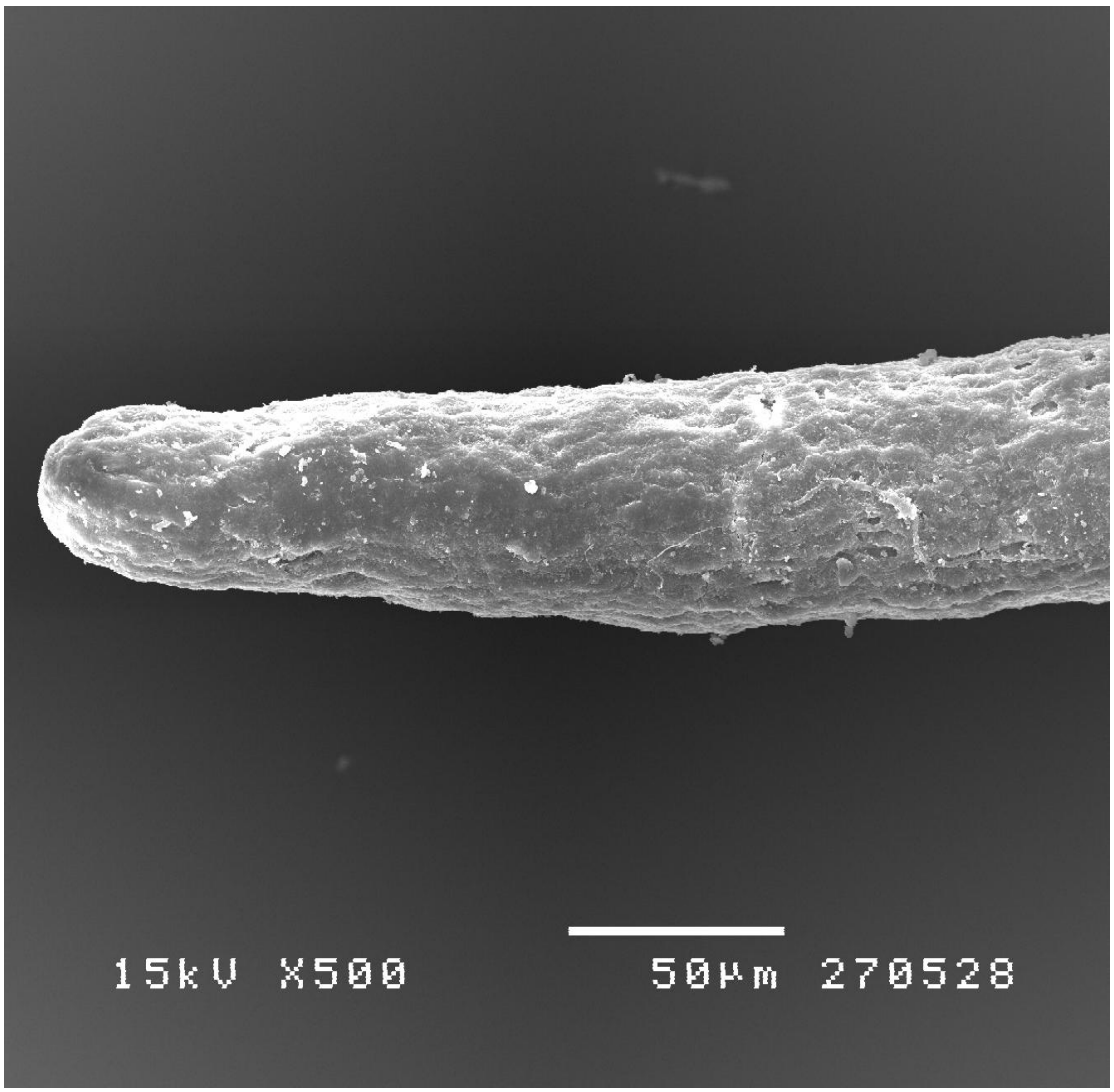



**Figure 8.** Tip of thallus branch by electron microscope

**Microscopic character**

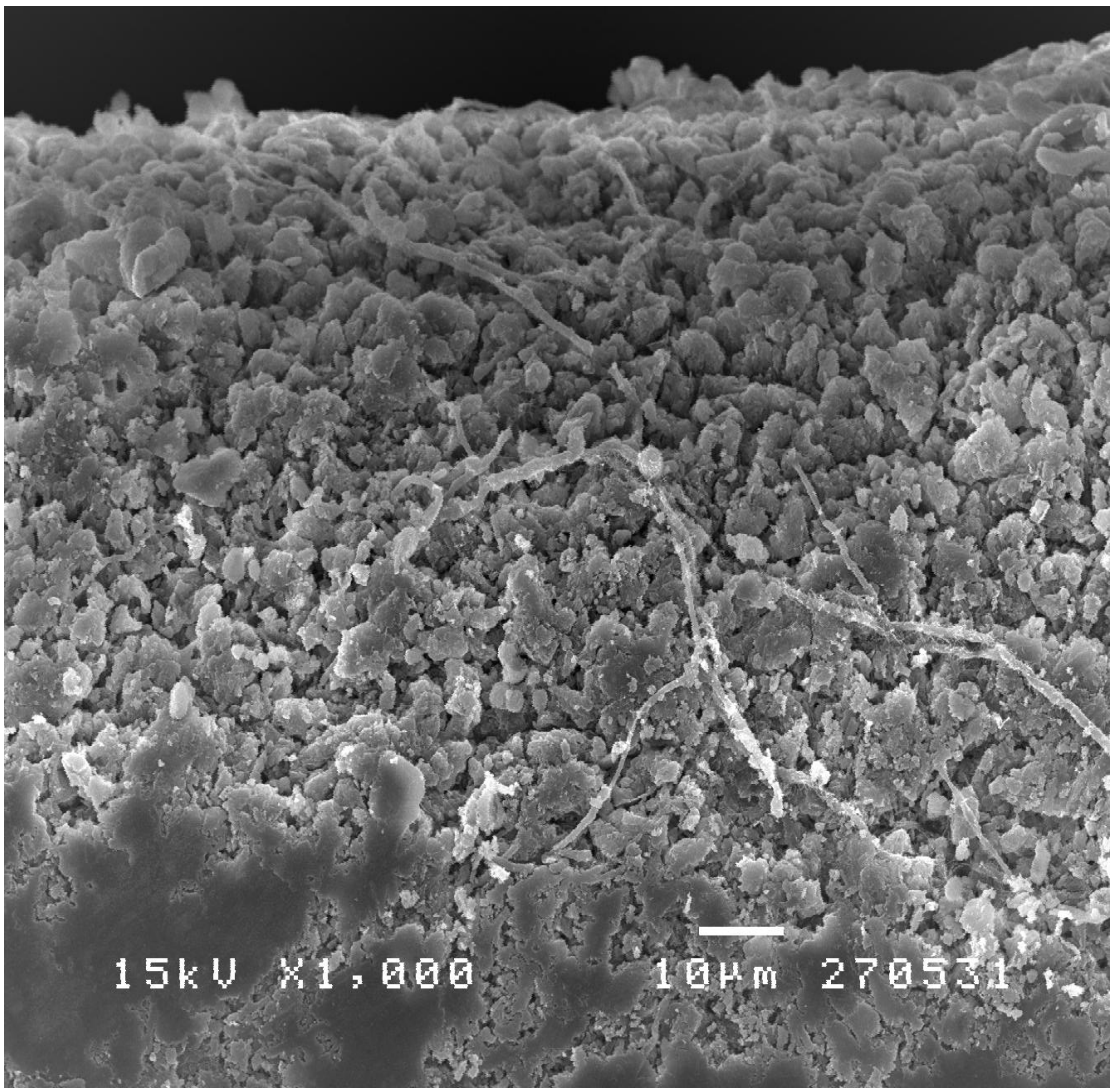



**Figure 9.** The surface of thallus branch by electron microscope

**Microscopic character**

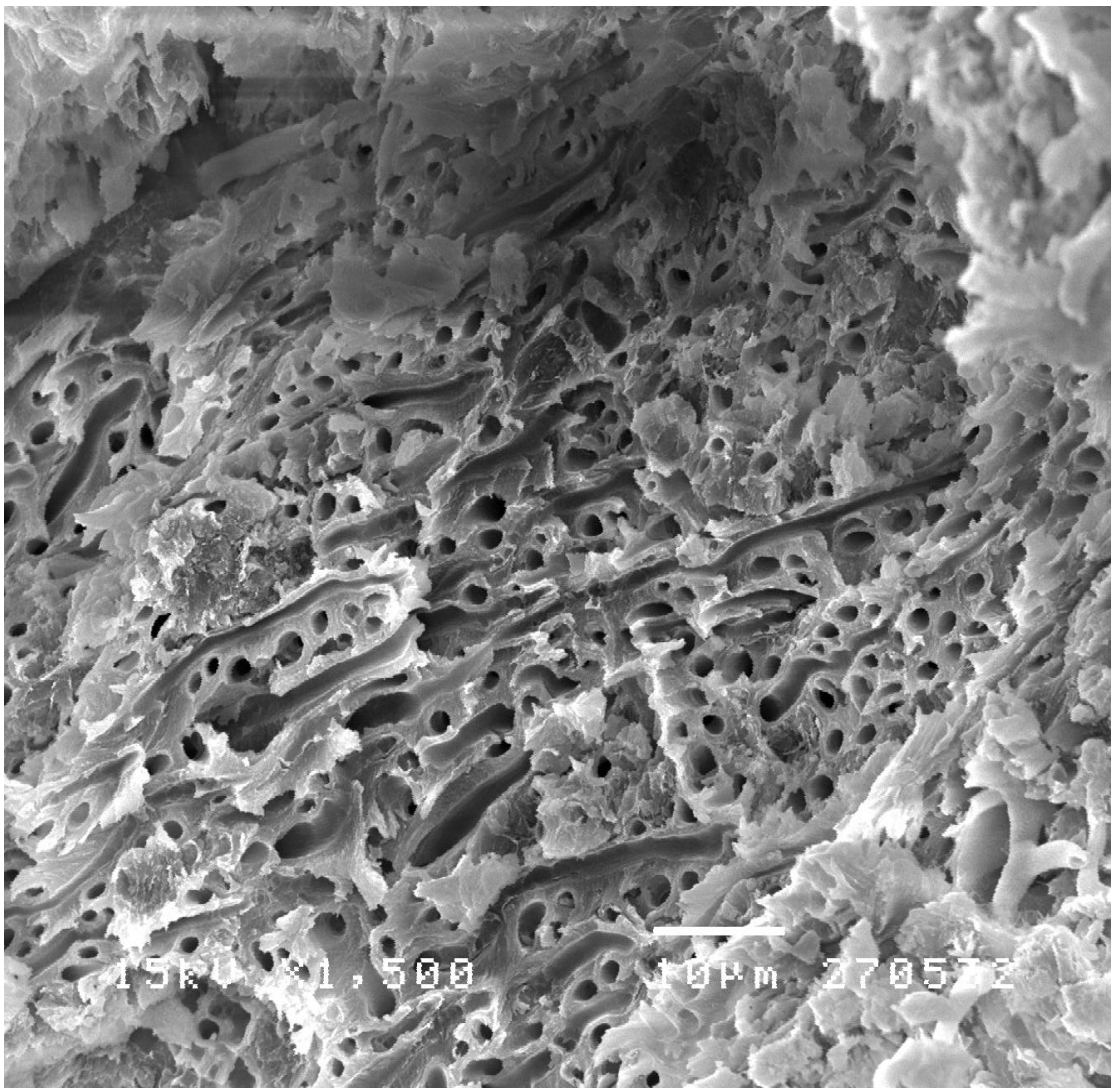



**Figure 10.** The crack of central axis thallus showed many densely distributed holes

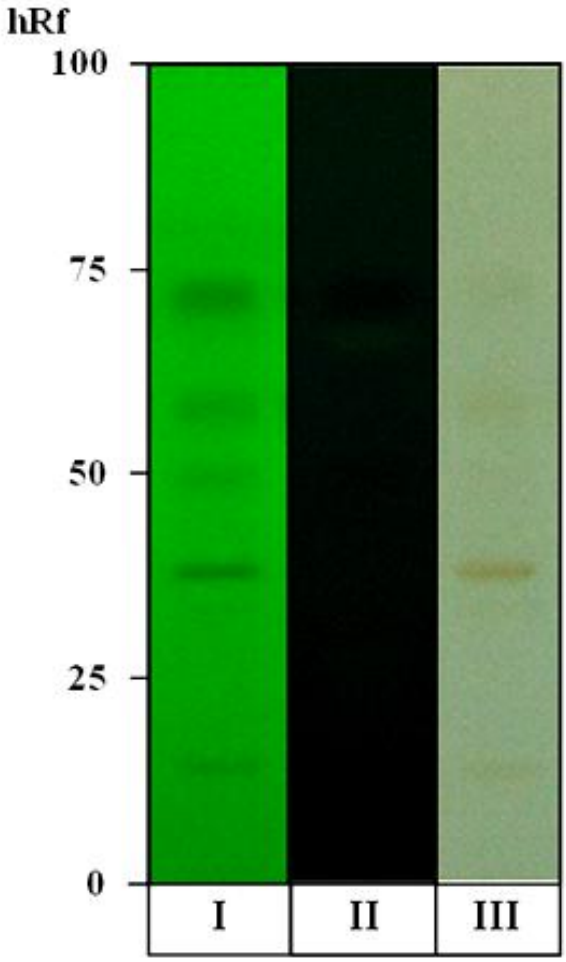



**Figure 11.** Thin layer chromatographic fingerprint of ethanol extract of *Usnea siamensis*

| | |
|---|---|
| **Solvent system** | Toluene : Ethyl acetate : Formic acid, 139:83:8 |
| **Detection** | I = detection under UV 254 nm |
| | II = detection under UV 365 nm |
| | III = detection with sulfuric acid staining reagent |

**Table 2.** The pharmacognostic parameters of *Usnea siamensis* Wainio

| Specification (% by weight) | Mean ± SD[a] | Range (Mean ± 3SD) |
|---|---|---|
| Foreign matter | 6.238 ± 3.567 | 0.000 - 16.985 |
| Loss on drying | 10.802 ± 0.257 | 10.031 - 11.574 |
| Total ash | 0.968 ± 0.058 | 0.795 - 1.142 |
| Acid insoluble ash | 0.164 ± 0.037 | 0.055 - 0.274 |
| Ethanol soluble extractive value | 5.593 ± 0.232 | 4.898 - 6.288 |
| Water soluble extractive value | 3.473 ± 0.143 | 3.043 - 3.903 |
| Water content | 13.075 ± 1.156 | 9.607 - 16.544 |
| Volatile oil | 0 | 0 |

[a] The parameters were shown as grand mean ± pooled SD. Samples were from 15 different sources throughout Thailand. Each sample was performed in triplicate.

**Benzene extract of *Usnea siamensis***

The dried powders of *Usnea siamensis* from 15 sources were extracted with benzene by soxhlet apparatus. The percent yields of crude extracts were shown in (Table 3.). The average percent yield of *Usnea siamensis* benzene extract was 4.815 ± 0.711 g/100 g by dry weight.

**Table 3.** The percent yield of benzene extract of *Usnea siamensis* from 15 different locations in Thailand

| Source | weight of sample | weight of extractive value | % yield |
|---|---|---|---|
| 1 | 5.0092 | 0.2683 | 5.356 |
| 2 | 5.0050 | 0.2178 | 4.352 |
| 3 | 5.0061 | 0.2457 | 4.908 |
| 4 | 5.0040 | 0.2734 | 5.464 |
| 5 | 5.0076 | 0.2280 | 4.553 |
| 6 | 5.0023 | 0.2283 | 4.564 |
| 7 | 5.0027 | 0.2894 | 5.785 |
| 8 | 5.0059 | 0.1780 | 3.556 |
| 9 | 5.0086 | 0.2580 | 5.151 |
| 10 | 5.0037 | 0.2089 | 4.175 |
| 11 | 5.0098 | 0.3030 | 6.048 |
| 12 | 5.0135 | 0.2744 | 5.473 |
| 13 | 5.0034 | 0.2192 | 4.381 |
| 14 | 5.0032 | 0.1983 | 3.964 |
| 15 | 5.0045 | 0.2251 | 4.498 |
| **Average** | | | **4.815 ± 0.711** |

**TLC-densitometry**

**Calibration curve**

The calibration curve of usnic acid ranged from 0.20 - 1.00 mg was shown in Figure 12. The polynomial equation was $y = -35{,}716.25x^2 + 69{,}213.75x + 13{,}829.32$ and the correlation coefficient ($R^2$) of the curve was 0.9981.

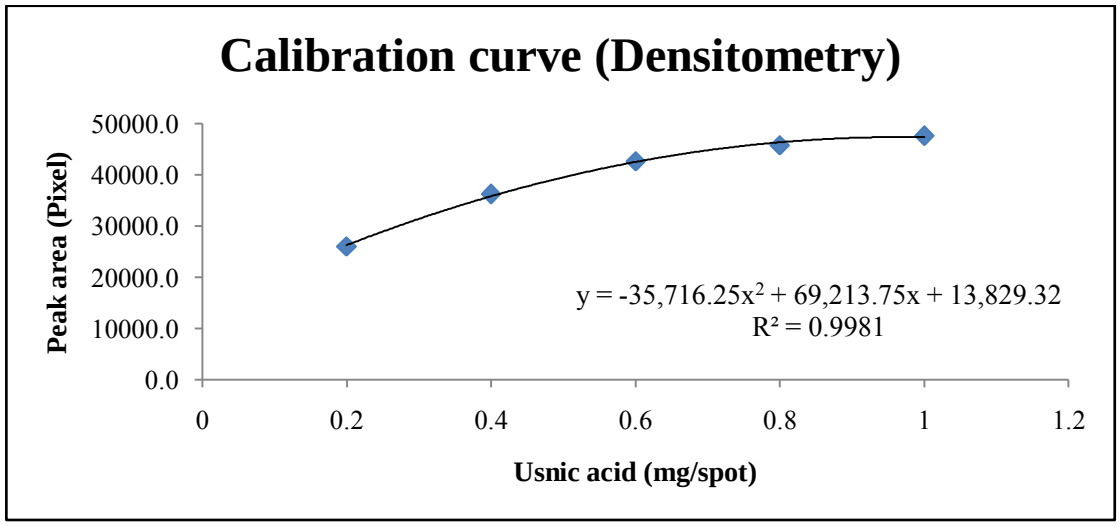



**Figure 12.** The calibration curve of usnic acid in *Usnea siamensis* by TLC-densitometric method

**Specificity**

The identity of usnic acid spots in samples was confirmed by absorption spectra overlaying with that of standard usnic acid spot on TLC plate (Figure 18). The identical peaks were obtained. The maximum absorbance of usnic acid was at the wavelength of 293 nm.

**Accuracy**

The recovery assay was used to validate the accuracy of usnic acid quantitation by TLC-densitometric method. Three concentrations (0.3, 0.5, 0.7 mg) of standard usnic acid were spiked into the sample matrix. The tested amount of usnic acid was compared to the theoretical one. The accuracy of usnic acid quantitative analysis in *Usnea siamensis* were between 83.77 - 100.45 % recoveries as shown in table 4.

**Table 4.** Accuracy of quantitation of usnic acid in *Usnea siamensis* by TLC-densitometry (n = 3)

| Usnic acid added (mg) | Usnic acid found (mg) | % Recovery |
|---|---|---|
| 0.0 | 0.440 | - |
| 0.3 | 0.747 | 100.45 |
| 0.5 | 0.837 | 88.63 |
| 0.7 | 0.958 | 83.77 |
| **Average** | | **90.95 ± 8.58** |

**Precision**

The precision of usnic acid quantitation by TLC-densitometric method was determined 4 concentrations × 3 replicates at same and different days of experiments. The values were shown as %RSD which meant the error of the method. The repeatability and intermediate precision were between 4.062 - 13.803 %RSD and 17.288 - 20.770 %RSD respectively (Table 5.).

**Table 5.** Repeatability and intermediate precision of quantitation of usnic acid in *Usnea siamensis* by TLC-densitometry (n = 3)

| Amount added (mg) | Repeatability | | Intermediate precision | |
|---|---|---|---|---|
| | Amount detection | %RSD | Amount detection | %RSD |
| 0.00 | 0.444 ± 0.048 | 10.700 | 0.390 ± 0.079 | 20.127 |
| 0.30 | 0.747 ± 0.030 | 4.062 | 0.637 ± 0.110 | 17.288 |
| 0.50 | 0.837 ± 0.115 | 13.803 | 0.746 ± 0.145 | 19.374 |
| 0.70 | 0.958 ± 0.119 | 12.445 | 0.803 ± 0.167 | 20.770 |
| **Average** | **10.253 ± 4.318** | | **19.390 ± 1.513** | |

**Limit of Detection (LOD) and Limit of Quantitation (LOQ)**

In this study, LOD and LOQ of TLC-densitometric method were calculated by the y-intercept standard deviation and the slope of calibration curve. The y-intercept standard deviation of the calibration curve was 1330.047. The slope of the calibration curve was 72920.354. The LOD and LOQ for TLC-densitometry were 0.060 and 0.182 mg/spot respectively.

**Robustness**

The robustness of usnic acid quantitation in *Usnea siamensis* by TLC-densitometric method was determined in five mobile phase ratios. The result of robustness was 0.803 %RSD of peak area (Table 6.).

**Table 6.** Robustness of quantitation of usnic acid in *Usnea siamensis* by TLC-densitometry (n=5)

| **Mobile phase ratio (v/v)** | | **Usnic acid peak area** |
|---|---|---|
| **Chloroform** | **Methanol** | |
| 8.8 | 1.2 | 42647.0 |
| 8.9 | 1.1 | 42323.9 |
| 9.0 | 1.0 | 42617.2 |
| 9.1 | 0.9 | 41802.0 |
| 9.2 | 0.8 | 42306.7 |
| | **Mean** | **42339.4** |
| | **SD** | **339.8** |
| | **%RSD** | **0.803** |

**The usnic acid content in *Usnea siamensis***

The amount of usnic acid in the benzene extract were done in triplicate and evaluated by calibration curve. The values were calculated and shown as grams of usnic acid per 100 grams of dried *Usnea siamensis* (Table 7.). The average content of usnic acid in 100 g of dried *Usnea siamensis* crude drug was 2.326 ± 0.285 g.

**Table 7.** The amount of usnic acid in *Usnea siamensis* in percent by weight (TLC-densitometry)

| Source | Usnic acid in the benzene extract * (mg/mg) | | Yield of the benzene extract (g/100 g of dried crude drug) | Usnic acid in *Usnea siamensis* * (g/100 g of dried crude drug) | |
|---|---|---|---|---|---|
| | Mean | SD | | Mean | SD |
| 1 | 0.4480 | 0.0406 | 5.3561 | 2.3995 | 0.2174 |
| 2 | 0.4557 | 0.0248 | 4.3516 | 1.9829 | 0.1080 |
| 3 | 0.6287 | 0.0525 | 4.9080 | 3.0855 | 0.2579 |
| 4 | 0.3457 | 0.0618 | 5.4636 | 1.8886 | 0.3379 |
| 5 | 0.5270 | 0.0632 | 4.5531 | 2.3995 | 0.2879 |
| 6 | 0.4250 | 0.0397 | 4.5639 | 1.9397 | 0.1811 |
| 7 | 0.3043 | 0.0398 | 5.7849 | 1.7605 | 0.2303 |
| 8 | 0.6343 | 0.0401 | 3.5558 | 2.2556 | 0.1425 |
| 9 | 0.6940 | 0.0465 | 5.1511 | 3.5749 | 0.2396 |
| 10 | 0.6943 | 0.0406 | 4.1749 | 2.8988 | 0.1696 |
| 11 | 0.3270 | 0.0590 | 6.0481 | 1.9777 | 0.3569 |
| 12 | 0.4967 | 0.0761 | 5.4732 | 2.7184 | 0.4166 |
| 13 | 0.5230 | 0.0515 | 4.3810 | 2.2913 | 0.2257 |
| 14 | 0.4377 | 0.0456 | 3.9635 | 1.7347 | 0.1809 |
| 15 | 0.4433 | 0.0160 | 4.4980 | 1.9941 | 0.0720 |
| | **Average** | | | **2.3267 ± 0.2853** | |

* Samples were from 15 different sources throughout Thailand. Each sample was performed in triplicate.

**TLC image analysis by ImageJ software**

**Calibration curve**

The calibration curve of usnic acid ranged from 0.20 - 1.00 mg was shown in Figure 13. The polynomial equation was $y = -10{,}066.98x^2 + 40{,}347.33x - 1{,}440.65$ and the correlation coefficient ($R^2$) of the curve was 0.9994.

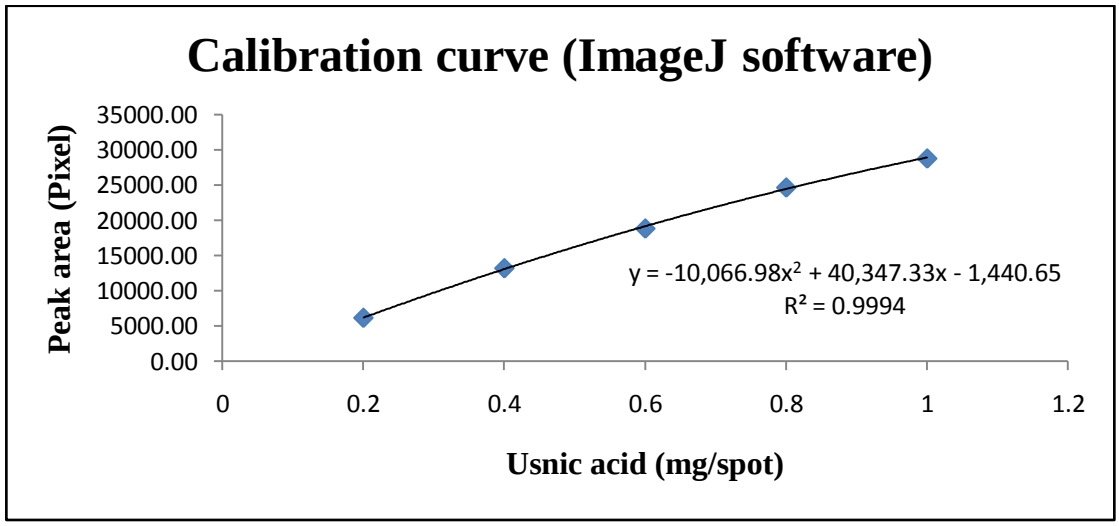



**Figure 13.** The calibration curve of usnic acid in *Usnea siamensis* by TLC image analysis method

**Accuracy**

The recovery assay was used to validate the accuracy of usnic acid quantitation by TLC image analysis method. Three concentrations (0.3, 0.5, 0.7 mg) of standard usnic acid were spiked into the sample matrix. The tested amount of usnic acid was compared to the theoretical one. The accuracy of usnic acid quantitative analysis in *Usnea siamensis* were between 99.17 - 120.49 % recoveries as shown in table 8.

**Table 8.** Accuracy of quantitation of usnic acid in *Usnea siamensis* by TLC image analysis (n = 3)

| Usnic acid added (mg) | Usnic acid found (mg) | % Recovery |
|---|---|---|
| 0.00 | 0.266 | - |
| 0.30 | 0.682 | 120.49 |
| 0.50 | 0.850 | 110.97 |
| 0.70 | 0.958 | 99.17 |
| **Average** | | **110.21 ± 10.68** |

**Precision**

The precision of usnic acid quantitation by TLC image analysis method was determined 4 concentrations × 3 replicates at same and different days of experiments. The values were shown as %RSD which meant the error of the method. The repeatability and intermediate precision were between 1.586 - 24.511 %RSD and 6.431 - 18.203 %RSD respectively (Table 9.).

**Table 9.** Repeatability and intermediate precision of quantitation of usnic acid in *Usnea siamensis* by TLC image analysis (n = 3)

| Amount added (mg) | Repeatability | | Intermediate precision | |
|---|---|---|---|---|
| | Amount detection | %RSD | Amount detection | %RSD |
| 0.00 | 0.266 ± 0.065 | 24.511 | 0.266 ± 0.048 | 18.203 |
| 0.30 | 0.578 ± 0.009 | 1.586 | 0.662 ± 0.043 | 6.431 |
| 0.50 | 0.850 ± 0.040 | 4.690 | 0.817 ± 0.079 | 9.613 |
| 0.70 | 0.958 ± 0.030 | 3.178 | 0.884 ± 0.104 | 11.756 |
| **Average** | **8.491 ± 10.755** | | **11.501 ± 4.975** | |

**Limit of Detection and Limit of Quantitation**

In this study, LOD and LOQ of TLC image analysis method were calculated by the y-intercept standard deviation and the slope of calibration curve. The y-intercept standard deviation of the calibration curve was 1545.695. The slope of the calibration curve was 46086.449. The LOD and LOQ for TLC image analysis were 0.110 and 0.335 mg/spot respectively.

**Robustness**

The robustness of usnic acid quantitation in *Usnea siamensis* by TLC image analysis method was determined in five mobile phase ratios. The result of robustness was 1.094 %RSD of peak area (Table 10.).

**Table 10.** Robustness of quantitation of usnic acid in *Usnea siamensis* by TLC image analysis (n=5)

| **Mobile phase ratio (v/v)** | | **Usnic acid peak area** |
|---|---|---|
| **Chloroform** | **Methanol** | |
| 8.8 | 1.2 | 19383.8 |
| 8.9 | 1.1 | 19707.0 |
| 9.0 | 1.0 | 19732.4 |
| 9.1 | 0.9 | 19368.3 |
| 9.2 | 0.8 | 19834.0 |
| | **Mean** | **19605.1** |
| | **SD** | **214.5** |
| | **%RSD** | **1.094** |

**The usnic acid content in *Usnea siamensis***

The amount of usnic acid in the benzene extract were done in triplicate and evaluated by calibration curve. The values were calculated and shown as grams of usnic acid per 100 grams of dried *Usnea siamensis* (Table 11.). The average content of usnic acid in 100 g of dried *Usnea siamensis* crude drug was 2.261 ± 0.248 g.

**Table 11.** The amount of usnic acid in *Usnea siamensis* in percent by weight (TLC image analysis)

| Source | Usnic acid in the benzene extract * (mg/mg) | | Yield of the benzene extract (g/100 g of dried crude drug) | Usnic acid in *Usnea siamensis* * (g/100 g of dried crude drug) | |
|---|---|---|---|---|---|
| | Mean | SD | | Mean | SD |
| 1 | 0.5220 | 0.0072 | 5.3561 | 2.7959 | 0.0386 |
| 2 | 0.4533 | 0.0112 | 4.3516 | 1.9727 | 0.0485 |
| 3 | 0.5533 | 0.0289 | 4.9080 | 2.7158 | 0.1419 |
| 4 | 0.3483 | 0.0038 | 5.4636 | 1.9032 | 0.0207 |
| 5 | 0.4763 | 0.0255 | 4.5531 | 2.1688 | 0.1163 |
| 6 | 0.3673 | 0.0247 | 4.5639 | 1.6765 | 0.1128 |
| 7 | 0.2987 | 0.0258 | 5.7849 | 1.7278 | 0.1492 |
| 8 | 0.5790 | 0.0305 | 3.5558 | 2.0588 | 0.1085 |
| 9 | 0.6470 | 0.0723 | 5.1511 | 3.3328 | 0.3726 |
| 10 | 0.6880 | 0.0658 | 4.1749 | 2.8723 | 0.2749 |
| 11 | 0.3900 | 0.0173 | 6.0481 | 2.3588 | 0.1049 |
| 12 | 0.4677 | 0.0192 | 5.4732 | 2.5596 | 0.1052 |
| 13 | 0.5043 | 0.0559 | 4.3810 | 2.2095 | 0.2449 |
| 14 | 0.4223 | 0.0146 | 3.9635 | 1.6739 | 0.0578 |
| 15 | 0.4210 | 0.0244 | 4.4980 | 1.8937 | 0.1099 |
| | | **Average** | | **2.2613 ± 0.2487** | |

* Samples were from 15 different sources throughout Thailand. Each sample was performed in triplicate.

**The comparison of usnic acid content between TLC-densitometry and TLC image analysis**

The comparison of usnic acid content between TLC-densitometry and TLC image analysis (Table 12.) were statistically tested using paired *t*-test. It was found that the usnic acid content by two methods were not significantly different ( t = 1.183, *P* = 0.256).

**Table 12.** The comparison of usnic acid contents between TLC-densitometry and TLC image analysis

| Source | % Usnic acid content | |
|---|---|---|
| | **TLC-densitometry** | **TLC image analysis** |
| 1 | 2.3995 | 2.7959 |
| 2 | 1.9829 | 1.9727 |
| 3 | 3.0855 | 2.7158 |
| 4 | 1.8886 | 1.9032 |
| 5 | 2.3995 | 2.1688 |
| 6 | 1.9397 | 1.6765 |
| 7 | 1.7605 | 1.7278 |
| 8 | 2.2556 | 2.0588 |
| 9 | 3.5749 | 3.3328 |
| 10 | 2.8988 | 2.8723 |
| 11 | 1.9777 | 2.3588 |
| 12 | 2.7184 | 2.5596 |
| 13 | 2.2913 | 2.2095 |
| 14 | 1.7347 | 1.6739 |
| 15 | 1.9941 | 1.8937 |

**TLC-bioautography**

TLC-bioautographic method showed the clear zone at Rf of usnic acid against *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis*, *Micrococcus luteus* and *Candida albicans* (Table 13. and Figure 14.).

**Table 13.** Antimicrobial activity of benzene extract of *U. siamensis* and usnic acid by overlay bioautography

| Tested bacteria | Inhibition zone[a] | |
|---|---|---|
| | **Benzene extract of *U. siamensis*** | **Usnic acid** |
| *Bacillus cereus* ATCC 11778 | + | + |
| *Bacillus subtilis* ATCC 6633 | + | + |
| *Staphylococcus aureus* ATCC 6538P | + | + |
| *Staphylococcus epidermidis* (Isolates) | + | + |
| *Micrococcus luteus* ATCC 9341 | + | + |
| *Salmonella typhi* (Isolates) | - | - |
| *Salmonella typhimurium* ATCC 13311 | - | - |
| *Shigella* spp. (Isolates) | - | - |
| *Pseudomonas aeruginosa* ATCC 9027 | - | - |
| *Enterobacter aerogenes* ATCC 13048 | - | - |
| *Escherichia coli* ATCC 25922 | - | - |
| *Candida albicans* ATCC 10230 | + | + |
| *Saccharomyces cerevisiae* ATCC 9763 | - | - |

[a] Inhibition zone expressed as (+) and (-)

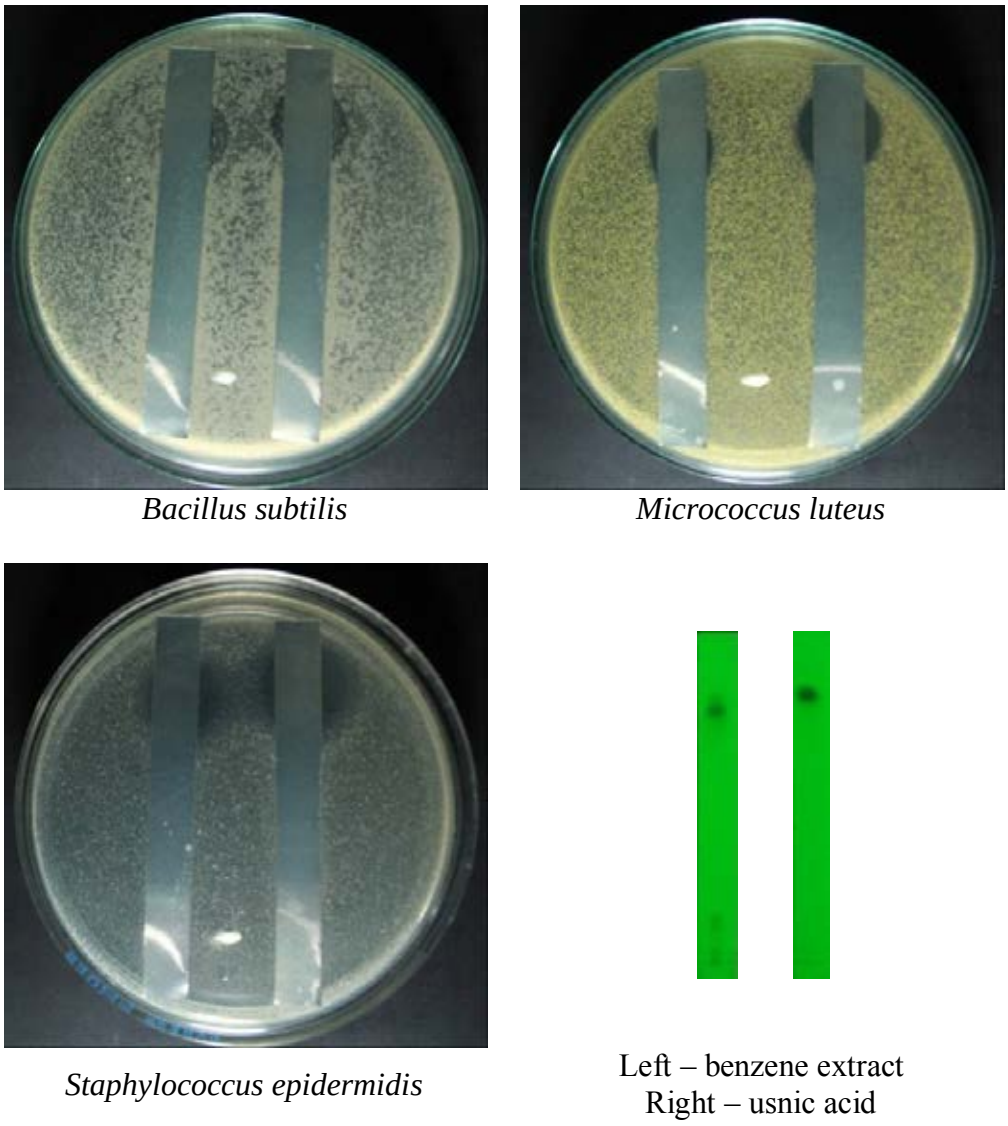



**Figure 14.** Zone of inhibition by bioautography, left - benzene extract, right - usnic acid

# CHAPTER V
# DISCUSSION AND CONCLUSION

Nowadays, the herbal medicines are used extensively around the world in modern medicine and folk medicine. World Health Organization published "Quality control methods for herbal material" guideline that describes various analytical methods for evaluation of the quality of medicinal plant materials. Quality control of medicinal plant materials is important for safety and efficiency of herbal medicines as well as increasing the confidence of hospital staff and consumer in medicinal plant use [1]. The standardization of Thai herbal medicines is lacked but the demand of drugs from herb is increased. This research showed the data of pharmacognostic specification and usnic acid content in *Usnea siamensis* which used in folk medicines as carminative, bitter tonic and anti-tumor [6].

The crude drug authentication was determined in anatomical and histological structures of herbal materials that different in each species and part used. The morphological evaluation of *U. siamensis* by scanning electron microscopy showed mycelium on the surface of thallus (Figure 9.) and many densely distributed holes in central axis (Figure 10.) that were also found in other *Usnea* species reported in the previous study (Figure 15.-16.) [35].

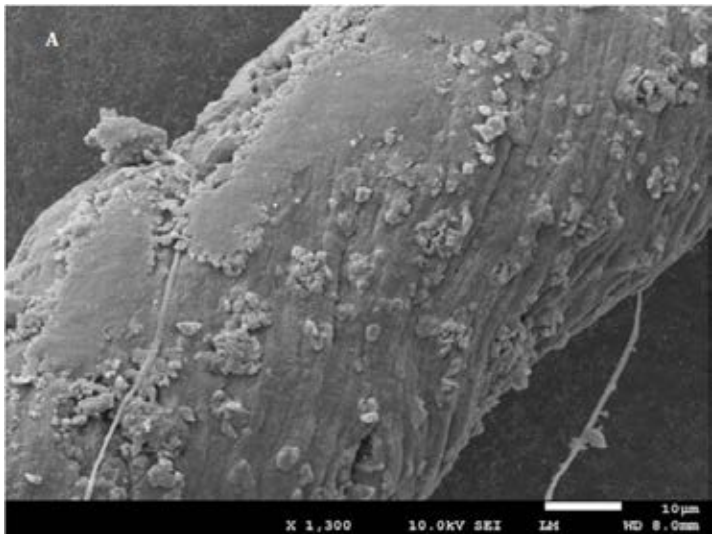



**Figure 15.** Vertical helical arrangement of mycelium on the surface of *Usnea longissima* Ach.

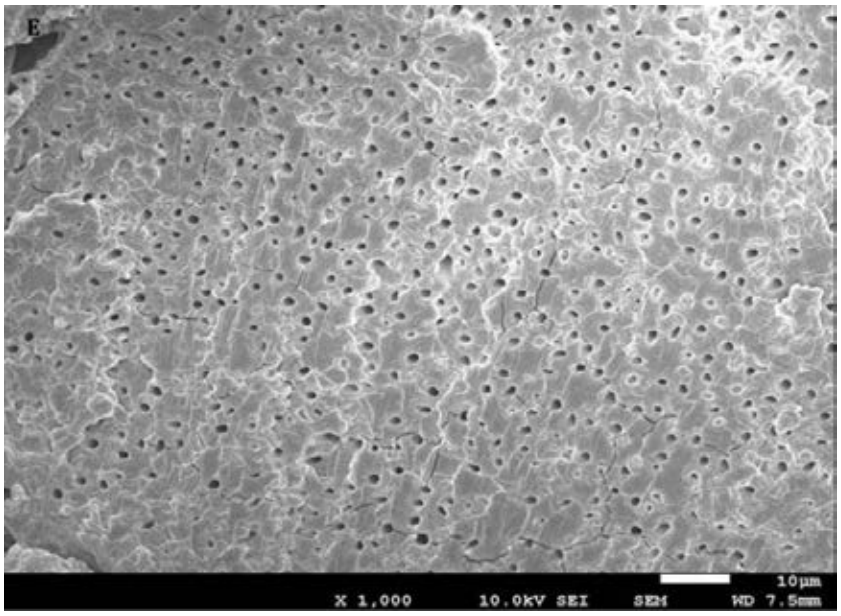



**Figure 16.** Cross-section of the central axis of *Usnea longissima* Ach.

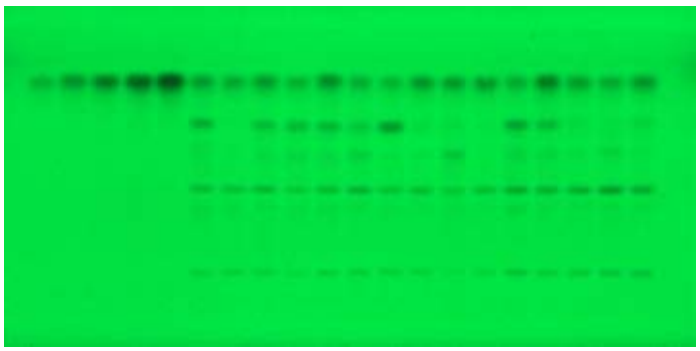
Furthermore, the other important parameters to indicate the characteristics of herbal materials are the physico-chemical parameters. These parameters are mainly used for quality and purity of crude drug.

The methods normally apply to obtain the qualitative and quantitative data about the purity and standard of crude drug include the determination of various parameters. The physico-chemical specification of this crude drug was shown in Table 2. Foreign matter indicated that the crude drug should be not contaminated by insect parts, animal excreta or soil etc. Loss on drying directs to lose in weight both water and volatile matter in crude drugs while water content directs to measure water in crude drug. An excess of water in herbal materials will encourage microbial growth, the presence of fungi or insects [1].

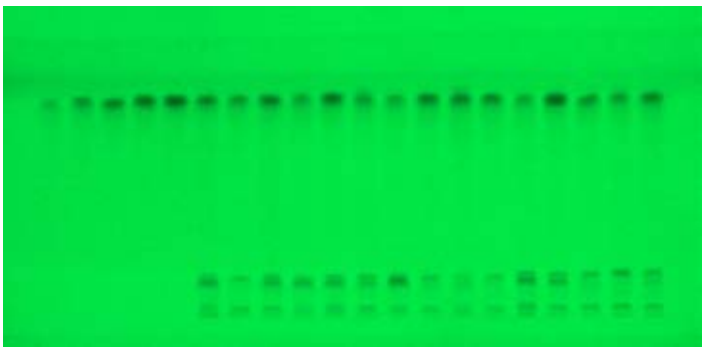
The ash parameters of crude drug determine the total amount of substances remaining after incineration. The ashes include "physiological ash" which comes from the plant tissue itself, and "non-physiological ash", which is the residue of the extraneous matter sticking to the crude drug surface. The high ash value is referred to contamination, adulteration and substitution in crude drug. In this study, less amounts of total ash and acid insoluble ash show that the earthy and inorganic matters less in *U. siamensis*. The extractive values show the amount of constituents in crude drug. Besides quantity information, this parameter can indicate fundamental character of

crude drug constituent based on polarity strength of solvent used. The constituents in crude drug can be extracted with polar, medium polar or non-polar solvents. The results from this study showed that ethanol soluble extractive value yielded more than water soluble extractive value. Therefore, more constituents in *U. siamensis* are non-polar to medium polar than polar compounds.

Thin layer chromatographic fingerprint was suitable for adulteration detection and plant species identification. The mobile phase (toluene : ethyl acetate : formic acid, 139:83:8) was suitable to separate chemical compounds and obtain TLC chromatogram capable to be chemical fingerprint in standardization of crude drug. On the other hand, chloroform : methanol (9:1) was suitable mobile phase for usnic acid quantitation because this mobile phase could separate usnic acid to be distinct from another bands (Figure 17.).



(A)



(B)

**Figure 17.** The chromatograms of usnic acid and *U. siamensis* from 15 various traditional drug stores detected at 254 nm UV light (A) toluene : ethyl acetate : formic acid, 139:83:8 as mobile phase, (B) chloroform : methanol, 9:1 as mobile phase

TLC-densitometric method measured the difference in absorbance or fluorescence signal between a compound band and surrounding plate background. The advantage of TLC-densitometry is high accuracy, low operating cost (compared to other instrumental methods e.g. HPLC), minimum sample clean up, using a small sample and less analysis time. Whereas, TLC image analysis used CCD camera to capture the image of TLC chromatogram and interpret the intensity of color of compound band and contrast background to chromatographic peak by ImageJ software. The advantage of TLC image analysis is low cost, simple equipment need, quick and easy experiment and available free software.

Although the calibration curves of usnic acid by both methods were polynomial, the correlation coefficients were shown to be more than 0.99 in range of 0.2-1.0 mg/spot. The methods were demonstrated their validity for usnic acid quantitation in *U. siamensis*. Absorption spectrum of standard usnic acid in this study showed the maximum absorbance at the wavelength of 293 nm which in accordance with the previous study that densitometric analysis of usnic acid could be detect at 295 nm [6]. The absorption spectra of usnic acid in 15 sample extracts and standard usnic acid were identical which represented the method specificity. The recovery of usnic acid by TLC-densitometry was between 83.77 - 100.45 % and by TLC image analysis was between 99.17 - 120.49 %. Recovery assay showed that these methods were accurate. Determination of usnic acid content in *U. siamensis* repeatedly within and between set of experiments by both methods revealed acceptable precisions. LOD and LOQ of TLC densitometry were 0.060 and 0.182 mg/spot respectively and those of TLC image analysis were 0.110 and 0.335 mg/spot respectively. The robustness of TLC densitometry was 0.803 %RSD of peak area and TLC image analysis was 1.094 %RSD of peak area. Although TLC image analysis could detect less amount of usnic acid than TLC densitometry and seemed to be less robust, the results of usnic acid content obtained by both methods were not statistically significant different. Therefore, TLC image analysis can be used instead of TLC densitometry in small laboratory with limited budget.

The previous studies showed that usnic acid could inhibit some microorganisms but not *Candida albicans* [8, 9]. TLC-bioautography in this study showed the inhibition zones by usnic acid against *Bacillus cereus, Bacillus subtilis,*

*Staphylococcus aureus, Staphylococcus epidermidis*, *Micrococcus luteus* and slight inhibition zone against *Candida albicans*.

In summary, this research provided the pharmacognostic specification and usnic acid content of *U. siamensis* which could be used as parameter guidance for quality control and standardization of *U. siamensis* crude drug. The *in vitro* antimicrobial efficacy of *U. siamensis* regarding usnic acid was revealed.

## REFERENCES


[1] World Health Organization. Quality control methods for medicinal plant materials. Geneva: World Health Organization, 2011.

[2] Satyapan, N., Patarakitvanit, S., Temboonkiet, S., Vudhironarit, T., and Tankanitlert, T. Herbal Medicine: Affecting Factors and Prevalence of Use Among Thai Population In Bangkok. Journal of The Medical Association of Thailand 93 (2010): 139-144.

[3] Chotchoungchatchai, S., Saralamp, P., Jenjittikul, T., Pornsiripongse, S., and Prathanturarug, S. Medicinal plants used with Thai Traditional Medicine in modern healthcare services: A case study in Kabchoeng Hospital, Surin Province, Thailand. Journal of Ethnopharmacology 141 (2012): 193-205.

[4] Marcano, V., Rodriguez-Alcocer, V., and Morales Méndez, A. Occurrence of usnic acid in *Usnea lavis* Nylander (lichenized ascomycetes) from the Venezuelan Andes. Journal of Ethnopharmacology 66 (1999): 343-346.

[5] Choudhary, M.I., Azizuddin, Jalil, S., and Atta-ur-Rahman. Bioactive phenolic compounds from a medicinal lichen, *Usnea longissima*. Phytochemistry 66 (2005): 2346-2350.

[6] Kamkaen, N. Study on the constituents of *Usnea siamensis* Wainio. Master's Thesis, Department of Pharmacognosy, Graduate School, Chulalongkorn University, 1994.

[7] Pramyothin, P., Janthasoot, W., Pongnimitprasert, N., Phrukudom, S., and Ruangrungsi, N. Hepatotoxic effect of (+)usnic acid frome *Usnea siamensis* Wainio in rats, isolated rat hepatocytes and isolated rat liver mitochondria. Journal of Ethnopharmacology 90 (2004): 381-387.

[8] Ingólfsdóttir, K. Molecules of Interest Usnic acid. Phytochemistry 61 (2002): 729-736.

[9] Cocchietto, M., Skert, N., Nimis, P.L., and Sava, G. A review on usnic acid, an interesting natural compound. Naturwissenschaften 89 (2002): 137-146.

[10] Liu, H. Extraction and Isolation of Compounds from Herbal Medicines. In Liu W. (ed.), Traditional herbal medicine research methods, pp.117-132. Singapore : A John Wiley & Sons, Inc., Publication, 2011.

[11] Elix, J.A., and Stocker-Wörgötter, E. Biochemistry and secondary methabolites. In Nash T.H. (ed.), Lichen Biology, pp.119-121. Cambridge : Cambridge University Press, 2008.

[12] Shrestha, G., and Clair, L.L.S. Lichens: a promising source of antibiotic and anticancer drugs. Phytochemistry Reviews 12 (2013): 229-244.

[13] Lin, C.-K. The Lichen Genus *Usnea* at Meifeng, Central Taiwan. Collection and Research 20 (2007): 1-7.

[14] Truong, C., Bungartz, F., and Clerc, P. The lichen genus *Usnea* (Parmeliaceae) in the tropical Andes and the Galapagos: species with a red-orange cortical or subcortical pigmentation. The Bryologist 113 (2011): 477-503.

[15] Government of Nova Scotia, Canada. Identifying lichen of Nova Scotia: a reference guidebook [Online]. 2013. Available from : http://www.gov.ns.ca/nse/protectedareas/docs/identifying.nova.scotia.lichens.pdf [2013, September 6]

[16] Ramkhamhaeng, University. Lichen Research Unit. Checklist of lichens in Thailand [Online]. 2006. Available from : http://joomlas.ru.ac.th/lichen/English/herbarium/checklist/checklistUV.html [2013, May 6]

[17] O'Neil, M.J., Heckeiman, P.E., Koch, C.B., and Roman, K.J. The MERCK Index, An encyclopedia of chemicals, drugs, and biologicals. 14. Whitehouse Station, New York, USA: Merck Research Laboratories Division of MERCK, 2006.

[18] Cimpan, G. Plant Extracts: TLC Analysis. In Cazes J. (ed.), Encyclopedia of chromatography, pp.1821-1823. New York : CRC Press, 2010.

[19] Tistaert, C., Dejaegher, B., and Heyden, Y.V. Chromatographic separation techniques and data handling methods for herbal fingerprints: A review. Analytica Chimica Acta 690 (2011): 148-161.

[20] Hess, A.V.I. Digitally Enhanced Thin-Layer Chromatography: An Inexpensive, New Technique for Qualitative and Quantitative Analysis. Journal of Chemical Education 84 (2007): 842-847.

[21] Soponar, F., Mot, A.C., and Sârbu, C. Quantitative determination of some food dyes using digital processing of images obtained by thin-layer chromatography. Journal of Chromatography A 1188 (2008): 295-300.

[22] Sherma, J. Thin Layer Chromatography. In Cazes J. (ed.), Ewing's Analytical Instrumentation Handbook, pp.995-1014. New York : Marcel Dekker, 2005.

[23] Shewiyo, D.H. *et al*. HPTLC methods to assay active ingredients in pharmaceutical formulations: A review of the method development and validation steps. Journal of Pharmaceutical and Biomedical Analysis 66 (2012): 11-23.

[24] Cieśla, Ł. Review Article: Biological Fingerprinting of Herbal Samples by Means of Liquid Chromatography. Chromatography Research International 2012 (2011): 1-9.

[25] Hajimehdipoor, H., *et al*. Fingerprint Study of *Thymus* spp. by TLC. Journal of Medicicnal Plant 8 (2009): 19-24.

[26] Rafi, M., Rohaeti, E., Miftahudin, A., and Darusman, L.K. Differentiation of *Curcuma longa*, *Curcuma xanthorrhiza* and *Zingiber cassumunar* by thin layer chromatography fingerprint analysis. Indonesian Journal of Chemistry 11 (2011): 71-74.

[27] Zhang, L., and Lin, X. Quantitative evaluation of thin-layer chromatography with image background estimation based on charge-coupled device imaging. Journal of Chromatography A 1109 (2006): 273-278.

[28] Sherma, J. Optical Quantification or Densitometry in TLC. In Cazes J. (ed.), Encyclopedia of chromatography, pp.1640-1647. New York : CRC Press, 2010.

[29] Miller, J. M. Chromatography Concepts and Contrasts. Second Edition. New Jersey, USA: A John Wiley & Sons, Inc., Publication, 2005.

[30] Hoeltz, M., Noll, J., and Dottori, H.A. Photometric procedure for quantitative analysis of Aflatoxin B1 in peanuts by thin-layer chromatography using charge coupled device detector. Química Nova 33 (2010): 43-47.

[31] Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9 (2012): 671-675.

[32] Choma, I. M., and Grzelak, E. M. Review Bioautography detection in thin-layer chromatography. Journal of Chromatography A 1218 (2011): 2684–2691.

[33] Marston, A. Review Thin-layer chromatography with biological detection in phytochemistry. Journal of Chromatography A 1218 (2011): 2676–2683.

[34] The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Q2(R1). Geneva: ICH, 2005.

[35] He, Y., Tang, H., and Zhang, Z. Ultramicromorphological observation of *Usnea longissima* Ach. African Journal of Biotechnology 11 (2012): 7102-7108.

# APPENDIX

**Table 14.** Foreign matter of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crud drugs (g) | Weight of foreign matter (g) | Foreign matter content (% by weight) |
|---|---|---|---|---|
| 1 | Phichit | 439.28 | 36.04 | 8.204 |
| 2 | Bangkok 1 | 192.57 | 7.32 | 3.801 |
| 3 | Bangkok 2 | 946.61 | 37.52 | 3.964 |
| 4 | Buri Ram 1 | 558.38 | 10.87 | 1.947 |
| 5 | Buri Ram 2 | 387.66 | 7.85 | 2.025 |
| 6 | Ranong | 394.41 | 52.37 | 13.278 |
| 7 | Suphan Buri | 451.49 | 43.16 | 9.559 |
| 8 | Phrae | 431.38 | 16.88 | 3.913 |
| 9 | Nakhon Sawan | 488.45 | 57.98 | 11.87 |
| 10 | Udon Thani | 490.56 | 22.77 | 4.642 |
| 11 | Khon Kaen | 451.55 | 22.96 | 5.085 |
| 12 | Krabi | 464.37 | 31.71 | 6.829 |
| 13 | Lop Buri | 444.53 | 17.2 | 3.869 |
| 14 | Chiang Rai | 456.38 | 47.57 | 10.423 |
| 15 | Uttaradit | 512.58 | 24.75 | 4.829 |
| | | | Mean | 6.283 |
| | | | SD | 3.567 |

**Table 15.** Loss on drying of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crucible and crude drug | | | loss on drying | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight of crucible (g) | Total weight (g) | Weight of crude drug (g) | Weight after drying (g) | Loss of weight (g) | Amount (% by weight) | | |
| 1 | Phichit | 28.5056 | 31.5130 | 31.5130 | 31.1701 | 0.3429 | 11.402 | | |
| | | 28.3837 | 31.3869 | 31.3869 | 31.0472 | 0.3397 | 11.311 | | |
| | | 29.1704 | 32.1768 | 32.1768 | 31.8476 | 0.3292 | 10.950 | 11.221 | 0.239 |
| 2 | Bangkok 1 | 28.8309 | 31.8329 | 31.8329 | 31.5596 | 0.2733 | 9.104 | | |
| | | 30.3891 | 33.3936 | 33.3936 | 33.1189 | 0.2747 | 9.143 | | |
| | | 29.4964 | 32.5002 | 32.5002 | 32.2299 | 0.2703 | 8.999 | 9.082 | 0.075 |
| 3 | Bangkok 2 | 26.9670 | 29.9731 | 29.9731 | 29.6824 | 0.2907 | 9.670 | | |
| | | 29.1661 | 32.1705 | 32.1705 | 31.8808 | 0.2897 | 9.643 | | |
| | | 27.7657 | 30.7709 | 30.7709 | 30.4813 | 0.2896 | 9.637 | 9.650 | 0.018 |
| 4 | Buri Ram 1 | 29.3326 | 32.3354 | 32.3354 | 32.0429 | 0.2925 | 9.741 | | |
| | | 28.8170 | 31.8202 | 31.8202 | 31.5267 | 0.2935 | 9.773 | | |
| | | 29.6990 | 32.7013 | 32.7013 | 32.3894 | 0.3119 | 10.389 | 9.968 | 0.365 |
| 5 | Buri Ram 2 | 28.4940 | 31.4963 | 31.4963 | 31.1863 | 0.3100 | 10.325 | | |
| | | 29.1324 | 32.1374 | 32.1374 | 31.8467 | 0.2907 | 9.674 | | |
| | | 32.8945 | 35.8960 | 35.8960 | 35.5882 | 0.3078 | 10.255 | 10.085 | 0.358 |
| 6 | Ranong | 28.5014 | 31.5072 | 31.5072 | 31.1622 | 0.3450 | 11.478 | | |
| | | 29.0009 | 32.0050 | 32.0050 | 31.6712 | 0.3338 | 11.111 | | |
| | | 29.3315 | 32.3370 | 32.3370 | 31.9868 | 0.3502 | 11.652 | 11.414 | 0.276 |

**Table 15. (Continue)** Loss on drying of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crucible and crude drug | | | loss on drying | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight of crucible (g) | Total weight (g) | Weight of crude drug (g) | Weight after drying (g) | Loss of weight (g) | Amount (% by weight) | | |
| 7 | Suphan Buri | 28.5495 | 31.5554 | 3.0059 | 31.2524 | 0.3030 | 10.080 | | |
| | | 30.1268 | 33.1329 | 3.0061 | 32.8492 | 0.2837 | 9.437 | | |
| | | 29.0685 | 32.0731 | 3.0046 | 31.7692 | 0.3039 | 10.114 | 9.877 | 0.381 |
| 8 | Phrae | 29.7348 | 32.7408 | 3.0060 | 32.3891 | 0.3517 | 11.700 | | |
| | | 29.3234 | 32.3283 | 3.0049 | 31.9793 | 0.3490 | 11.614 | | |
| | | 28.5845 | 31.5886 | 3.0041 | 31.2403 | 0.3483 | 11.594 | 11.636 | 0.056 |
| 9 | Nakhon Sawan | 29.3122 | 32.3202 | 3.0080 | 31.9960 | 0.3242 | 10.778 | | |
| | | 29.4556 | 32.4625 | 3.0069 | 32.1415 | 0.3210 | 10.675 | | |
| | | 30.9492 | 33.9506 | 3.0014 | 33.6306 | 0.3200 | 10.662 | 10.705 | 0.064 |
| 10 | Udon Thani | 29.9139 | 32.9158 | 3.0019 | 32.5923 | 0.3235 | 10.777 | | |
| | | 29.3159 | 32.3176 | 3.0017 | 31.9908 | 0.3268 | 10.887 | | |
| | | 29.1482 | 32.1500 | 3.0018 | 31.8033 | 0.3467 | 11.550 | 11.071 | 0.418 |
| 11 | Khon Kaen | 29.5456 | 32.5505 | 3.0049 | 32.2208 | 0.3297 | 10.972 | | |
| | | 30.6270 | 33.6294 | 3.0024 | 33.3199 | 0.3095 | 10.308 | | |
| | | 28.0491 | 31.0536 | 3.0045 | 30.7231 | 0.3305 | 11.000 | 10.760 | 0.392 |
| 12 | Krabi | 32.5321 | 35.5356 | 3.0035 | 35.1933 | 0.3423 | 11.397 | | |
| | | 33.8438 | 36.8504 | 3.0066 | 36.5212 | 0.3292 | 10.949 | | |
| | | 28.5632 | 31.5663 | 3.0031 | 31.2185 | 0.3478 | 11.581 | 11.309 | 0.325 |

**Table 15. (Continue)** Loss on drying of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crucible and crude drug | | | loss on drying | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight of crucible (g) | Total weight (g) | Weight of crude drug (g) | Weight after drying (g) | Loss of weight (g) | Amount (% by weight) | | |
| 13 | Lop Buri | 29.1080 | 32.1133 | 3.0053 | 31.7494 | 0.3639 | 12.109 | | |
| | | 27.9002 | 30.9071 | 3.0069 | 30.5447 | 0.3624 | 12.052 | | |
| | | 29.3666 | 32.3697 | 3.0031 | 32.0053 | 0.3644 | 12.134 | 12.098 | 0.042 |
| 14 | Chiang Rai | 32.4482 | 35.4530 | 3.0048 | 35.1009 | 0.3521 | 11.718 | | |
| | | 28.7069 | 31.7134 | 3.0065 | 31.3626 | 0.3508 | 11.668 | | |
| | | 28.1745 | 31.1766 | 3.0021 | 30.8241 | 0.3525 | 11.742 | 11.709 | 0.038 |
| 15 | Uttaradit | 30.6054 | 33.6113 | 3.0059 | 33.2658 | 0.3455 | 11.494 | | |
| | | 29.4612 | 32.4686 | 3.0074 | 32.1247 | 0.3439 | 11.435 | | |
| | | 28.4245 | 31.4269 | 3.0024 | 31.0843 | 0.3426 | 11.411 | 11.447 | 0.043 |
| | | | | | | | Grand mean | | 10.802 |
| | | | | | | | Pooled SD | | 0.257 |

**Table 16.** Total ash of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crucible and crude drug | | | Total ash | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight of crucible (g) | Total weight (g) | Weight of crude drug (g) | Weight of crucible and ash (g) | Weight of ash (g) | Amount (% by weight) | | |
| 1 | Phichit | 28.5056 | 31.5130 | 31.5130 | 28.5328 | 0.0272 | 0.904 | | |
| | | 28.3837 | 31.3869 | 31.3869 | 28.4108 | 0.0271 | 0.902 | | |
| | | 29.1704 | 32.1768 | 32.1768 | 29.2021 | 0.0317 | 1.054 | 0.954 | 0.087 |
| 2 | Bangkok 1 | 28.8309 | 31.8329 | 31.8329 | 28.8656 | 0.0347 | 1.156 | | |
| | | 30.3891 | 33.3936 | 33.3936 | 30.4213 | 0.0322 | 1.072 | | |
| | | 29.4964 | 32.5002 | 32.5002 | 29.5337 | 0.0373 | 1.242 | 1.156 | 0.085 |
| 3 | Bangkok 2 | 26.9670 | 29.9731 | 29.9731 | 27.0016 | 0.0346 | 1.151 | | |
| | | 29.1661 | 32.1705 | 32.1705 | 29.1994 | 0.0333 | 1.108 | | |
| | | 27.7657 | 30.7709 | 30.7709 | 27.7964 | 0.0307 | 1.022 | 1.094 | 0.066 |
| 4 | Buri Ram 1 | 29.3326 | 32.3354 | 32.3354 | 29.3722 | 0.0396 | 1.319 | | |
| | | 28.8170 | 31.8202 | 31.8202 | 28.8558 | 0.0388 | 1.292 | | |
| | | 29.6990 | 32.7013 | 32.7013 | 29.7344 | 0.0354 | 1.179 | 1.263 | 0.074 |
| 5 | Buri Ram 2 | 28.4940 | 31.4963 | 31.4963 | 28.5318 | 0.0378 | 1.259 | | |
| | | 29.1324 | 32.1374 | 32.1374 | 29.1733 | 0.0409 | 1.361 | | |
| | | 32.8945 | 35.8960 | 35.8960 | 32.9316 | 0.0371 | 1.236 | 1.285 | 0.067 |
| 6 | Ranong | 28.5014 | 31.5072 | 31.5072 | 28.5389 | 0.0375 | 1.248 | | |
| | | 29.0009 | 32.0050 | 32.0050 | 29.0360 | 0.0351 | 1.168 | | |
| | | 29.3315 | 32.3370 | 32.3370 | 29.3657 | 0.0342 | 1.138 | 1.185 | 0.057 |

**Table 16. (Continue)** Total ash of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crucible and crude drug | | | Total ash | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight of crucible (g) | Total weight (g) | Weight of crude drug (g) | Weight of crucible and ash (g) | Weight of ash (g) | Amount (% by weight) | | |
| 7 | Suphan Buri | 28.5495 | 31.5554 | 3.0059 | 28.5748 | 0.0253 | 0.842 | | |
| | | 30.1268 | 33.1329 | 3.0061 | 30.1534 | 0.0266 | 0.885 | | |
| | | 29.0685 | 32.0731 | 3.0046 | 29.0914 | 0.0229 | 0.762 | 0.830 | 0.062 |
| 8 | Phrae | 29.7348 | 32.7408 | 3.0060 | 29.7624 | 0.0276 | 0.918 | | |
| | | 29.3234 | 32.3283 | 3.0049 | 29.3499 | 0.0265 | 0.882 | | |
| | | 28.5845 | 31.5886 | 3.0041 | 28.613 | 0.0285 | 0.949 | 0.916 | 0.033 |
| 9 | Nakhon Sawan | 29.3122 | 32.3202 | 3.0080 | 29.3398 | 0.0276 | 0.918 | | |
| | | 29.4556 | 32.4625 | 3.0069 | 29.4847 | 0.0291 | 0.968 | | |
| | | 30.9492 | 33.9506 | 3.0014 | 30.9767 | 0.0275 | 0.916 | 0.934 | 0.029 |
| 10 | Udon Thani | 29.9139 | 32.9158 | 3.0019 | 29.9432 | 0.0293 | 0.976 | | |
| | | 29.3159 | 32.3176 | 3.0017 | 29.3448 | 0.0289 | 0.963 | | |
| | | 29.1482 | 32.1500 | 3.0018 | 29.1744 | 0.0262 | 0.873 | 0.937 | 0.056 |
| 11 | Khon Kaen | 29.5456 | 32.5505 | 3.0049 | 29.5759 | 0.0303 | 1.008 | | |
| | | 30.6270 | 33.6294 | 3.0024 | 30.6596 | 0.0326 | 1.086 | | |
| | | 28.0491 | 31.0536 | 3.0045 | 28.0795 | 0.0304 | 1.012 | 1.035 | 0.044 |
| 12 | Krabi | 32.5321 | 35.5356 | 3.0035 | 32.5572 | 0.0251 | 0.836 | | |
| | | 33.8438 | 36.8504 | 3.0066 | 33.8696 | 0.0258 | 0.858 | | |
| | | 28.5632 | 31.5663 | 3.0031 | 28.5862 | 0.0230 | 0.766 | 0.820 | 0.048 |

**Table 16. (Continue)** Total ash of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crucible and crude drug | | | Total ash | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight of crucible (g) | Total weight (g) | Weight of crude drug (g) | Weight of crucible and ash (g) | Weight of ash (g) | Amount (% by weight) | | |
| 13 | Lop Buri | 29.1080 | 32.1133 | 3.0053 | 29.1292 | 0.0212 | 0.705 | | |
| | | 27.9002 | 30.9071 | 3.0069 | 27.9226 | 0.0224 | 0.745 | | |
| | | 29.3666 | 32.3697 | 3.0031 | 29.3893 | 0.0227 | 0.756 | 0.735 | 0.027 |
| 14 | Chiang Rai | 32.4482 | 35.4530 | 3.0048 | 32.4683 | 0.0201 | 0.669 | | |
| | | 28.7069 | 31.7134 | 3.0065 | 28.7286 | 0.0217 | 0.722 | | |
| | | 28.1745 | 31.1766 | 3.0021 | 28.195 | 0.0205 | 0.683 | 0.691 | 0.027 |
| 15 | Uttaradit | 30.6054 | 33.6113 | 3.0059 | 30.6243 | 0.0189 | 0.629 | | |
| | | 29.4612 | 32.4686 | 3.0074 | 29.4825 | 0.0213 | 0.708 | | |
| | | 28.4245 | 31.4269 | 3.0024 | 28.4465 | 0.0220 | 0.733 | 0.690 | 0.054 |
| | | | | | | | Grand mean | | 0.968 |
| | | | | | | | Pooled SD | | 0.058 |

**Table 17.** Acid insoluble ash of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crucible and crude drug | | | Acid insoluble ash | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight of crucible (g) | Total weight (g) | Weight of crude drug (g) | Weight of crucible and ash (g) | Weight of ash (g) | Amount (% by weight) | | |
| 1 | Phichit | 28.5056 | 31.5130 | 31.5130 | 28.5097 | 0.0041 | 0.136 | | |
| | | 28.3837 | 31.3869 | 31.3869 | 28.3866 | 0.0029 | 0.097 | | |
| | | 29.1704 | 32.1768 | 32.1768 | 29.1765 | 0.0061 | 0.203 | 0.145 | 0.054 |
| 2 | Bangkok 1 | 28.8309 | 31.8329 | 31.8329 | 28.8356 | 0.0047 | 0.157 | | |
| | | 30.3891 | 33.3936 | 33.3936 | 30.3940 | 0.0049 | 0.163 | | |
| | | 29.4964 | 32.5002 | 32.5002 | 29.5023 | 0.0059 | 0.196 | 0.172 | 0.021 |
| 3 | Bangkok 2 | 26.9670 | 29.9731 | 29.9731 | 26.9744 | 0.0074 | 0.246 | | |
| | | 29.1661 | 32.1705 | 32.1705 | 29.1722 | 0.0061 | 0.203 | | |
| | | 27.7657 | 30.7709 | 30.7709 | 27.7701 | 0.0044 | 0.146 | 0.199 | 0.050 |
| 4 | Buri Ram 1 | 29.3326 | 32.3354 | 32.3354 | 29.3381 | 0.0055 | 0.183 | | |
| | | 28.8170 | 31.8202 | 31.8202 | 28.8230 | 0.0060 | 0.200 | | |
| | | 29.6990 | 32.7013 | 32.7013 | 29.7039 | 0.0049 | 0.163 | 0.182 | 0.018 |
| 5 | Buri Ram 2 | 28.4940 | 31.4963 | 31.4963 | 28.4988 | 0.0048 | 0.160 | | |
| | | 29.1324 | 32.1374 | 32.1374 | 29.1391 | 0.0067 | 0.223 | | |
| | | 32.8945 | 35.8960 | 35.8960 | 32.8995 | 0.0050 | 0.167 | 0.183 | 0.035 |
| 6 | Ranong | 28.5014 | 31.5072 | 31.5072 | 28.5083 | 0.0069 | 0.230 | | |
| | | 29.0009 | 32.0050 | 32.0050 | 29.0071 | 0.0062 | 0.206 | | |
| | | 29.3315 | 32.3370 | 32.3370 | 29.3377 | 0.0062 | 0.206 | 0.214 | 0.013 |

**Table 17. (Continue)** Acid insoluble ash of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crucible and crude drug | | | Acid insoluble ash | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight of crucible (g) | Total weight (g) | Weight of crude drug (g) | Weight of crucible and ash (g) | Weight of ash (g) | Amount (% by weight) | | |
| 7 | Suphan Buri | 28.5495 | 31.5554 | 3.0059 | 28.5546 | 0.0051 | 0.170 | | |
| | | 30.1268 | 33.1329 | 3.0061 | 30.1325 | 0.0057 | 0.190 | | |
| | | 29.0685 | 32.0731 | 3.0046 | 29.0729 | 0.0044 | 0.146 | 0.169 | 0.022 |
| 8 | Phrae | 29.7348 | 32.7408 | 3.0060 | 29.7394 | 0.0046 | 0.153 | | |
| | | 29.3234 | 32.3283 | 3.0049 | 29.3273 | 0.0039 | 0.130 | | |
| | | 28.5845 | 31.5886 | 3.0041 | 28.5888 | 0.0043 | 0.143 | 0.142 | 0.012 |
| 9 | Nakhon Sawan | 29.3122 | 32.3202 | 3.0080 | 29.3203 | 0.0081 | 0.269 | | |
| | | 29.4556 | 32.4625 | 3.0069 | 29.4612 | 0.0056 | 0.186 | | |
| | | 30.9492 | 33.9506 | 3.0014 | 30.9548 | 0.0056 | 0.187 | 0.214 | 0.048 |
| 10 | Udon Thani | 29.9139 | 32.9158 | 3.0019 | 29.917 | 0.0031 | 0.103 | | |
| | | 29.3159 | 32.3176 | 3.0017 | 29.3203 | 0.0044 | 0.147 | | |
| | | 29.1482 | 32.1500 | 3.0018 | 29.1538 | 0.0056 | 0.187 | 0.145 | 0.042 |
| 11 | Khon Kaen | 29.5456 | 32.5505 | 3.0049 | 29.5525 | 0.0069 | 0.230 | | |
| | | 30.6270 | 33.6294 | 3.0024 | 30.6325 | 0.0055 | 0.183 | | |
| | | 28.0491 | 31.0536 | 3.0045 | 28.0550 | 0.0059 | 0.196 | 0.203 | 0.024 |
| 12 | Krabi | 32.5321 | 35.5356 | 3.0035 | 32.5365 | 0.0044 | 0.146 | | |
| | | 33.8438 | 36.8504 | 3.0066 | 33.8487 | 0.0049 | 0.163 | | |
| | | 28.5632 | 31.5663 | 3.0031 | 28.5676 | 0.0044 | 0.147 | 0.152 | 0.010 |

**Table 17. (Continue)** Acid insoluble ash of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crucible and crude drug | | | Acid insoluble ash | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight of crucible (g) | Total weight (g) | Weight of crude drug (g) | Weight of crucible and ash (g) | Weight of ash (g) | Amount (% by weight) | | |
| 13 | Lop Buri | 29.1080 | 32.1133 | 3.0053 | 29.1118 | 0.0038 | 0.126 | | |
| | | 27.9002 | 30.9071 | 3.0069 | 27.9028 | 0.0026 | 0.086 | | |
| | | 29.3666 | 32.3697 | 3.0031 | 29.3738 | 0.0072 | 0.240 | 0.151 | 0.080 |
| 14 | Chiang Rai | 32.4482 | 35.4530 | 3.0048 | 32.4511 | 0.0029 | 0.097 | | |
| | | 28.7069 | 31.7134 | 3.0065 | 28.711 | 0.0041 | 0.136 | | |
| | | 28.1745 | 31.1766 | 3.0021 | 28.1771 | 0.0026 | 0.087 | 0.106 | 0.026 |
| 15 | Uttaradit | 30.6054 | 33.6113 | 3.0059 | 30.6084 | 0.0030 | 0.100 | | |
| | | 29.4612 | 32.4686 | 3.0074 | 29.4638 | 0.0026 | 0.086 | | |
| | | 28.4245 | 31.4269 | 3.0024 | 28.4267 | 0.0022 | 0.073 | 0.087 | 0.013 |
| | | | | | | | Grand mean | | 0.164 |
| | | | | | | | Pooled SD | | 0.037 |

**Table 18.** Ethanol soluble extractive of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of beaker (g) | Weight of crude drug (g) | Weight of extractable matter and beaker (g) | Weight of extractable matter (g) | Amount (% by weight) | Mean | SD |
|---|---|---|---|---|---|---|---|---|
| | | 29.6024 | 5.0068 | 29.6697 | 0.0673 | 6.721 | | |
| 1 | Phichit | 29.0159 | 5.0090 | 29.0837 | 0.0678 | 6.768 | | |
| | | 30.3226 | 5.0068 | 30.3878 | 0.0652 | 6.511 | 6.667 | 0.137 |
| | | 31.0879 | 5.0012 | 31.1499 | 0.0620 | 6.199 | | |
| 2 | Bangkok 1 | 29.9015 | 5.0035 | 29.9618 | 0.0603 | 6.026 | | |
| | | 30.4931 | 5.0084 | 30.5531 | 0.0600 | 5.990 | 6.071 | 0.112 |
| | | 29.3625 | 5.0024 | 29.4190 | 0.0565 | 5.647 | | |
| 3 | Bangkok 2 | 29.9983 | 5.0008 | 30.0545 | 0.0562 | 5.619 | | |
| | | 29.3620 | 5.0075 | 29.4153 | 0.0533 | 5.322 | 5.529 | 0.180 |
| | | 30.0638 | 5.0016 | 30.1270 | 0.0632 | 6.318 | | |
| 4 | Buri Ram 1 | 30.0583 | 5.0038 | 30.1228 | 0.0645 | 6.445 | | |
| | | 29.3980 | 5.0047 | 29.4608 | 0.0628 | 6.274 | 6.346 | 0.089 |
| | | 28.9819 | 5.0062 | 29.0494 | 0.0675 | 6.742 | | |
| 5 | Buri Ram 2 | 30.4636 | 5.0020 | 30.5257 | 0.0621 | 6.208 | | |
| | | 30.0037 | 5.0083 | 30.0625 | 0.0588 | 5.870 | 6.273 | 0.439 |
| | | 31.1465 | 5.0090 | 31.1998 | 0.0533 | 5.320 | | |
| 6 | Ranong | 29.9541 | 5.0066 | 30.0049 | 0.0508 | 5.073 | | |
| | | 29.8292 | 5.0098 | 29.8820 | 0.0528 | 5.270 | 5.221 | 0.131 |

**Table 18. (Continue)** Ethanol soluble extractive of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of beaker (g) | Weight of crude drug (g) | Weight of extractable matter and beaker (g) | Weight of extractable matter (g) | Amount (% by weight) | Mean | SD |
|---|---|---|---|---|---|---|---|---|
| 7 | Suphan Buri | 29.9313 | 5.0046 | 29.9957 | 0.0644 | 6.434 | | |
| | | 29.1282 | 5.0097 | 29.2002 | 0.0720 | 7.186 | | |
| | | 29.7062 | 5.0086 | 29.7686 | 0.0624 | 6.229 | 6.616 | 0.504 |
| 8 | Phrae | 31.1939 | 5.0098 | 31.2344 | 0.0405 | 4.042 | | |
| | | 29.7625 | 5.0063 | 29.8014 | 0.0389 | 3.883 | | |
| | | 28.9414 | 5.0074 | 28.9814 | 0.0400 | 3.994 | 3.973 | 0.082 |
| 9 | Nakhon Sawan | 28.9804 | 5.0079 | 29.0471 | 0.0667 | 6.659 | | |
| | | 29.9991 | 5.0050 | 30.0625 | 0.0634 | 6.334 | | |
| | | 29.8170 | 5.0020 | 29.8797 | 0.0627 | 6.267 | 6.420 | 0.210 |
| 10 | Udon Thani | 29.3541 | 5.0094 | 29.3970 | 0.0429 | 4.282 | | |
| | | 29.1253 | 5.0050 | 29.1665 | 0.0412 | 4.116 | | |
| | | 29.9967 | 5.0017 | 30.0372 | 0.0405 | 4.049 | 4.149 | 0.120 |
| 11 | Khon Kaen | 30.4535 | 5.0052 | 30.5146 | 0.0611 | 6.104 | | |
| | | 29.8957 | 5.0065 | 29.9567 | 0.0610 | 6.092 | | |
| | | 30.0522 | 5.0053 | 30.1148 | 0.0626 | 6.253 | 6.150 | 0.090 |
| 12 | Krabi | 31.1338 | 5.0003 | 31.1806 | 0.0468 | 4.680 | | |
| | | 29.6973 | 5.0015 | 29.7446 | 0.0473 | 4.729 | | |
| | | 28.9367 | 5.0042 | 28.9848 | 0.0481 | 4.806 | 4.738 | 0.064 |

**Table 18. (Continue)** Ethanol soluble extractive of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of beaker (g) | Weight of crude drug (g) | Weight of extractable matter and beaker (g) | Weight of extractable matter (g) | Amount (% by weight) | Mean | SD |
|---|---|---|---|---|---|---|---|---|
| | | 30.4728 | 5.0090 | 30.5189 | 0.0461 | 4.602 | | |
| 13 | Lop Buri | 29.9412 | 5.0061 | 29.9954 | 0.0542 | 5.413 | | |
| | | 29.3804 | 5.0078 | 29.4320 | 0.0516 | 5.152 | 5.056 | 0.414 |
| | | 29.3624 | 5.0030 | 29.4157 | 0.0533 | 5.327 | | |
| 14 | Chiang Rai | 30.0738 | 5.0038 | 30.1282 | 0.0544 | 5.436 | | |
| | | 32.4144 | 5.0033 | 32.4678 | 0.0534 | 5.336 | 5.366 | 0.060 |
| | | 30.2924 | 5.0027 | 30.3471 | 0.0547 | 5.467 | | |
| 15 | Uttaradit | 32.8047 | 5.0036 | 32.8570 | 0.0523 | 5.226 | | |
| | | 29.5472 | 5.0069 | 29.5998 | 0.0526 | 5.253 | 5.315 | 0.132 |
| | | | | | | Grand mean | | 5.593 |
| | | | | | | Pooled SD | | 0.232 |

**Table 19.** Water soluble extractive of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of beaker (g) | Weight of crude drug (g) | Weight of extractable matter and beaker (g) | Weight of extractable matter (g) | Amount (% by weight) | Mean | SD |
|---|---|---|---|---|---|---|---|---|
| | | 30.0869 | 5.0058 | 30.1258 | 0.0389 | 3.885 | | |
| 1 | Phichit | 29.0140 | 5.0054 | 29.0518 | 0.0378 | 3.776 | | |
| | | 30.3370 | 5.0053 | 30.3751 | 0.0381 | 3.806 | 3.822 | 0.057 |
| | | 32.2625 | 5.0079 | 32.3072 | 0.0447 | 4.463 | | |
| 2 | Bangkok 1 | 32.0325 | 5.0073 | 32.0758 | 0.0433 | 4.324 | | |
| | | 29.4318 | 5.0030 | 29.4747 | 0.0429 | 4.287 | 4.358 | 0.093 |
| | | 31.2793 | 5.0101 | 31.3218 | 0.0425 | 4.241 | | |
| 3 | Bangkok 2 | 29.7010 | 5.0044 | 29.7376 | 0.0366 | 3.657 | | |
| | | 30.4898 | 5.0025 | 30.5317 | 0.0419 | 4.188 | 4.029 | 0.323 |
| | | 31.5256 | 5.0033 | 31.5650 | 0.0394 | 3.937 | | |
| 4 | Buri Ram 1 | 31.1582 | 5.0057 | 31.1934 | 0.0352 | 3.516 | | |
| | | 29.9168 | 5.0075 | 29.9531 | 0.0363 | 3.625 | 3.693 | 0.219 |
| | | 29.6063 | 5.0006 | 29.6471 | 0.0408 | 4.080 | | |
| 5 | Buri Ram 2 | 29.5152 | 5.0049 | 29.5555 | 0.0403 | 4.026 | | |
| | | 30.3226 | 5.0011 | 30.3665 | 0.0439 | 4.389 | 4.165 | 0.196 |
| | | 32.4072 | 5.0026 | 32.4327 | 0.0255 | 2.549 | | |
| 6 | Ranong | 29.9148 | 5.0054 | 29.9368 | 0.0220 | 2.198 | | |
| | | 29.9161 | 5.0061 | 29.9385 | 0.0224 | 2.237 | 2.328 | 0.192 |

**Table 19. (Continue)** Water soluble extractive of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of beaker (g) | Weight of crude drug (g) | Weight of extractable matter and beaker (g) | Weight of extractable matter (g) | Amount (% by weight) | Mean | SD |
|---|---|---|---|---|---|---|---|---|
| 7 | Suphan Buri | 30.0960 | 5.0073 | 30.1487 | 0.0527 | 5.262 | | |
| | | 29.3812 | 5.0073 | 29.4319 | 0.0507 | 5.063 | | |
| | | 30.0335 | 5.0065 | 30.0837 | 0.0502 | 5.013 | 5.113 | 0.132 |
| 8 | Phrae | 29.1678 | 5.0028 | 29.1878 | 0.0200 | 1.999 | | |
| | | 30.0310 | 5.0081 | 30.0515 | 0.0205 | 2.047 | | |
| | | 32.8040 | 5.0066 | 32.8223 | 0.0183 | 1.828 | 1.958 | 0.115 |
| 9 | Nakhon Sawan | 32.5983 | 5.0085 | 32.6485 | 0.0502 | 5.011 | | |
| | | 29.0137 | 5.0034 | 29.0657 | 0.0520 | 5.196 | | |
| | | 29.9304 | 5.0073 | 29.9800 | 0.0496 | 4.953 | 5.054 | 0.127 |
| 10 | Udon Thani | 32.4288 | 5.0014 | 32.4493 | 0.0205 | 2.049 | | |
| | | 30.3377 | 5.0020 | 30.3578 | 0.0201 | 2.009 | | |
| | | 29.3683 | 5.0077 | 29.3885 | 0.0202 | 2.017 | 2.025 | 0.021 |
| 11 | Khon Kaen | 29.9544 | 5.0067 | 29.9921 | 0.0377 | 3.765 | | |
| | | 29.5483 | 5.0088 | 29.5857 | 0.0374 | 3.733 | | |
| | | 32.2921 | 5.0002 | 32.3274 | 0.0353 | 3.530 | 3.676 | 0.128 |
| 12 | Krabi | 29.1809 | 5.0009 | 29.2030 | 0.0221 | 2.210 | | |
| | | 29.9351 | 5.0060 | 29.9573 | 0.0222 | 2.217 | | |
| | | 32.8150 | 5.0075 | 32.8366 | 0.0216 | 2.157 | 2.195 | 0.033 |

**Table 19. (Continue)** Water soluble extractive of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of beaker (g) | Weight of crude drug (g) | Weight of extractable matter and beaker (g) | Weight of extractable matter (g) | Amount (% by weight) | Mean | SD |
|---|---|---|---|---|---|---|---|---|
| | | 29.6036 | 5.0055 | 29.6340 | 0.0304 | 3.037 | | |
| 13 | Lop Buri | 29.3732 | 5.0055 | 29.4028 | 0.0296 | 2.957 | | |
| | | 29.3844 | 5.0084 | 29.4147 | 0.0303 | 3.025 | 3.006 | 0.043 |
| | | 29.9483 | 5.0039 | 29.9792 | 0.0309 | 3.088 | | |
| 14 | Chiang Rai | 29.5469 | 5.0020 | 29.5780 | 0.0311 | 3.109 | | |
| | | 29.1608 | 5.0049 | 29.1913 | 0.0305 | 3.047 | 3.081 | 0.031 |
| | | 32.8157 | 5.0030 | 32.8520 | 0.0363 | 3.628 | | |
| 15 | Uttaradit | 32.4217 | 5.0088 | 32.4576 | 0.0359 | 3.584 | | |
| | | 30.4807 | 5.0096 | 30.5165 | 0.0358 | 3.573 | 3.595 | 0.029 |
| | | | | | | Grand mean | | 3.473 |
| | | | | | | Pooled SD | | 0.143 |

**Table 20.** Water content of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crude drug (g) | Water content (ml) | Amount (% by weight) | Mean | SD |
|---|---|---|---|---|---|---|
| | | 30.03 | 4.20 | 13.986 | | |
| 1 | Phichit | 30.06 | 4.30 | 14.305 | | |
| | | 30.07 | 3.90 | 12.970 | 13.753 | 0.697 |
| | | 30.02 | 3.70 | 12.325 | | |
| 2 | Bangkok 1 | 30.01 | 3.20 | 10.663 | | |
| | | 30.02 | 3.30 | 10.993 | 11.327 | 0.880 |
| | | 30.02 | 3.40 | 11.326 | | |
| 3 | Bangkok 2 | 30.08 | 4.50 | 14.960 | | |
| | | 30.08 | 3.50 | 11.636 | 12.641 | 2.015 |
| | | 30.03 | 3.80 | 12.654 | | |
| 4 | Buri Ram 1 | 30.01 | 3.20 | 10.663 | | |
| | | 30.01 | 3.50 | 11.663 | 11.660 | 0.995 |
| | | 30.01 | 4.00 | 13.329 | | |
| 5 | Buri Ram 2 | 30.02 | 3.20 | 10.660 | | |
| | | 30.01 | 3.50 | 11.663 | 11.884 | 1.348 |
| | | 30.01 | 4.50 | 14.995 | | |
| 6 | Ranong | 30.04 | 4.00 | 13.316 | | |
| | | 30.04 | 4.10 | 13.648 | 13.986 | 0.889 |

**Table 20. (Continue)** Water content of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crude drug (g) | Water content (ml) | Amount (% by weight) | Mean | SD |
|---|---|---|---|---|---|---|
| | | 30.04 | 3.60 | 11.984 | | |
| 7 | Suphan Buri | 30.02 | 3.00 | 9.993 | | |
| | | 30.03 | 3.90 | 12.987 | 11.655 | 1.524 |
| | | 30.01 | 4.40 | 14.662 | | |
| 8 | Phrae | 30.03 | 4.20 | 13.986 | | |
| | | 30.03 | 4.80 | 15.984 | 14.877 | 1.016 |
| | | 30.01 | 3.60 | 11.996 | | |
| 9 | Nakhon Sawan | 30.02 | 4.80 | 15.989 | | |
| | | 30.01 | 4.20 | 13.995 | 13.994 | 1.997 |
| | | 30.01 | 3.70 | 12.329 | | |
| 10 | Udon Thani | 30.01 | 4.00 | 13.329 | | |
| | | 30.03 | 4.20 | 13.986 | 13.215 | 0.834 |
| | | 30.03 | 3.60 | 11.988 | | |
| 11 | Khon Kaen | 30.01 | 3.30 | 10.996 | | |
| | | 30.02 | 3.40 | 11.326 | 11.437 | 0.505 |
| | | 30.02 | 4.20 | 13.991 | | |
| 12 | Krabi | 30.03 | 4.50 | 14.985 | | |
| | | 30.01 | 4.10 | 13.662 | 14.213 | 0.689 |

**Table 20. (Continue)** Water content of *Usnea siamensis* from 15 various traditional drug stores throughout Thailand

| No. | Sources | Weight of crude drug (g) | Water content (ml) | Amount (% by weight) | Mean | SD |
|---|---|---|---|---|---|---|
| | | 30.03 | 4.50 | 14.985 | | |
| 13 | Lop Buri | 30.05 | 4.00 | 13.311 | | |
| | | 30.08 | 4.60 | 15.293 | 14.530 | 1.066 |
| | | 30.03 | 4.50 | 14.985 | | |
| 14 | Chiang Rai | 30.06 | 4.00 | 13.307 | | |
| | | 30.02 | 4.40 | 14.657 | 14.316 | 0.890 |
| | | 30.06 | 3.80 | 12.641 | | |
| 15 | Uttaradit | 30.07 | 4.00 | 13.302 | | |
| | | 30.04 | 3.60 | 11.984 | 12.643 | 0.659 |
| | | | | Grand mean | | 13.075 |
| | | | | Pooled SD | | 1.156 |

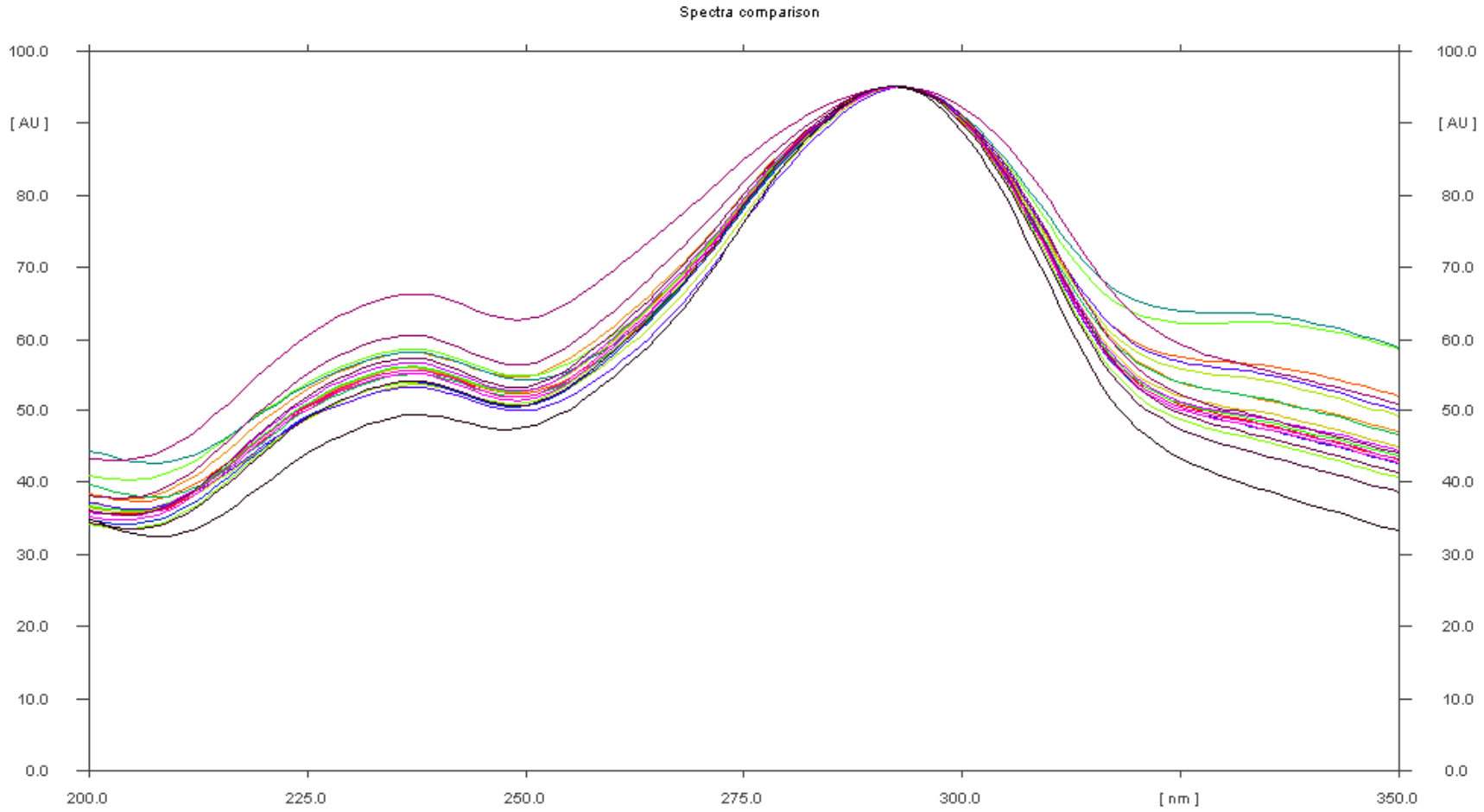



**Figure 18.** 3D TLC-densitometric chromatogram of maximum wavelength of usnic acid

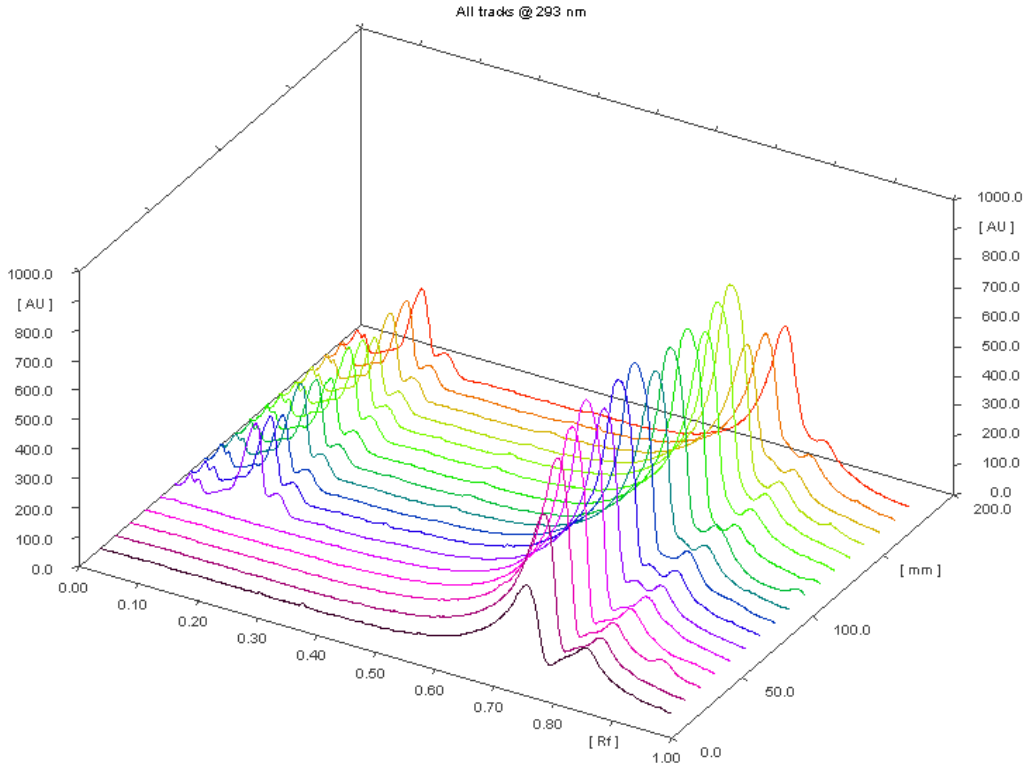



**Figure 19.** 3D TLC-densitometic chromatogram (Accuracy)

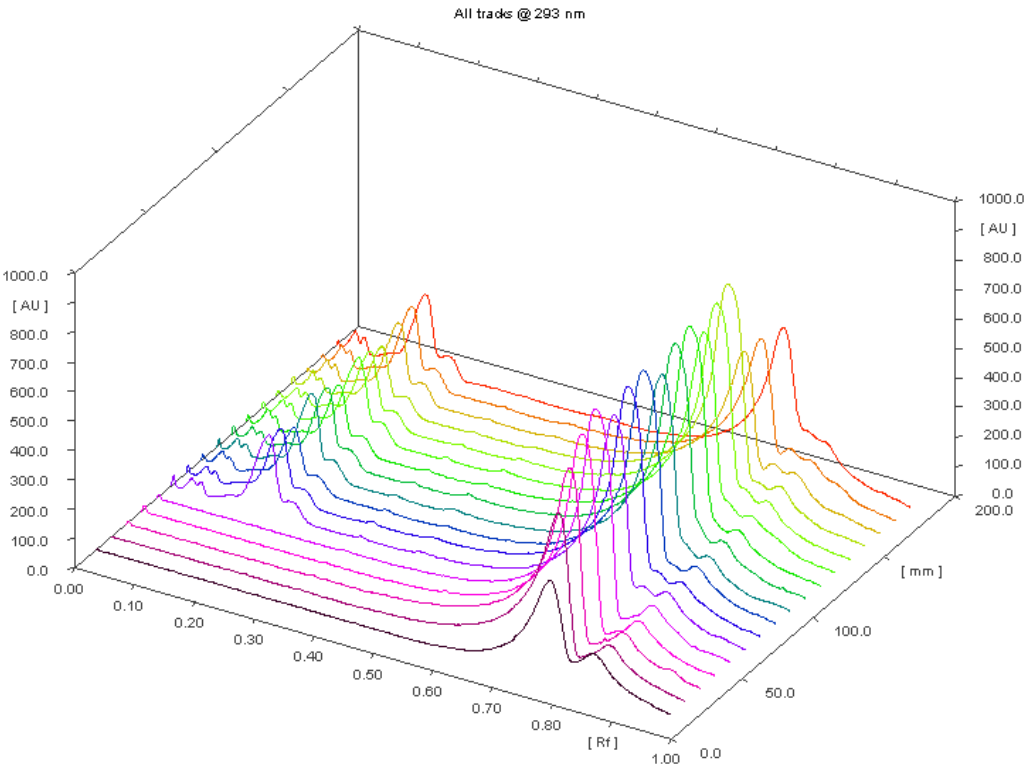



**Figure 20.** 3D TLC-densitometic chromatogram (Precision No.1)

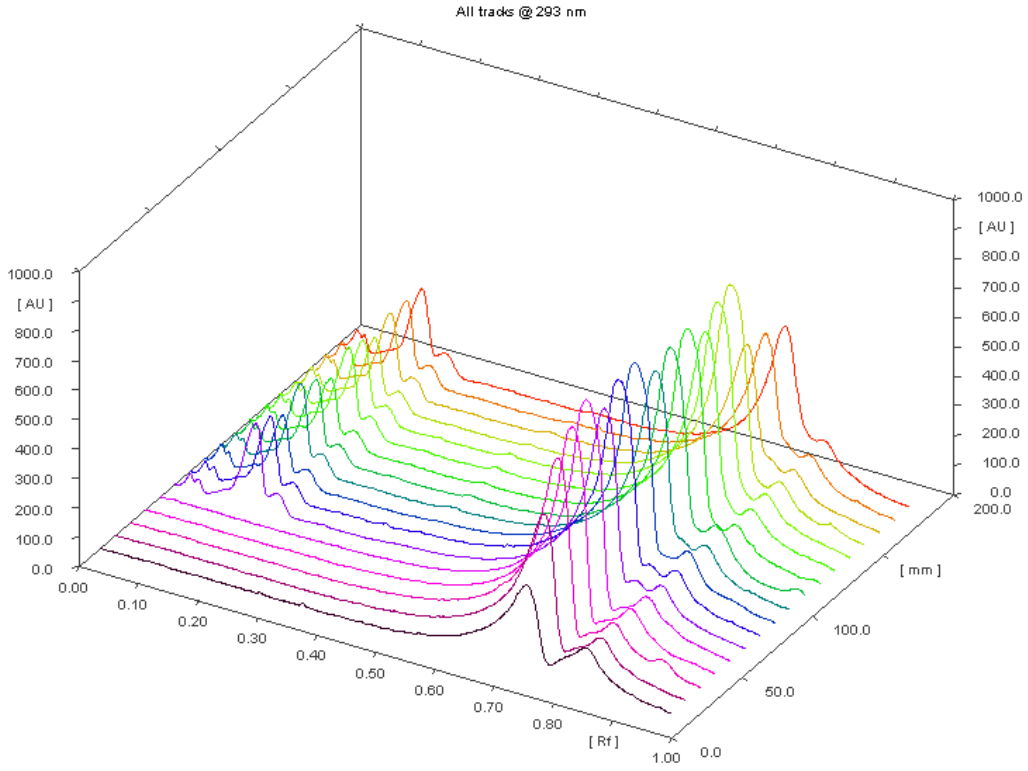



**Figure 21.** 3D TLC-densitometric chromatogram (Precision No.2)

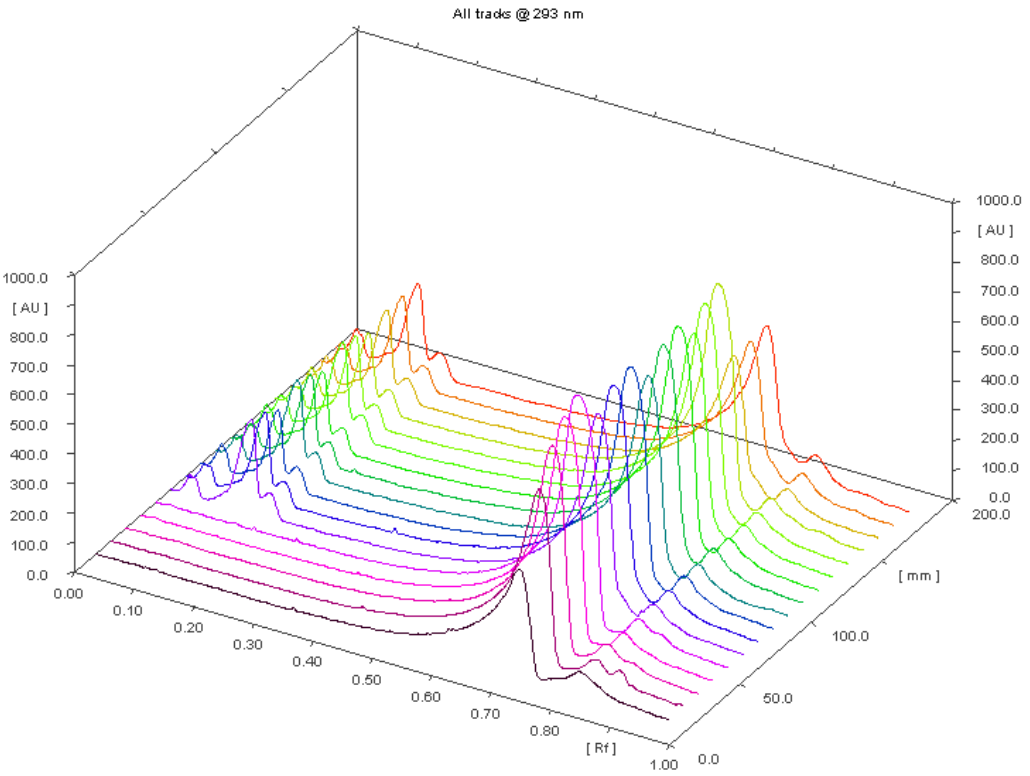



**Figure 22.** 3D TLC-densitometric chromatogram (Precision No.3)

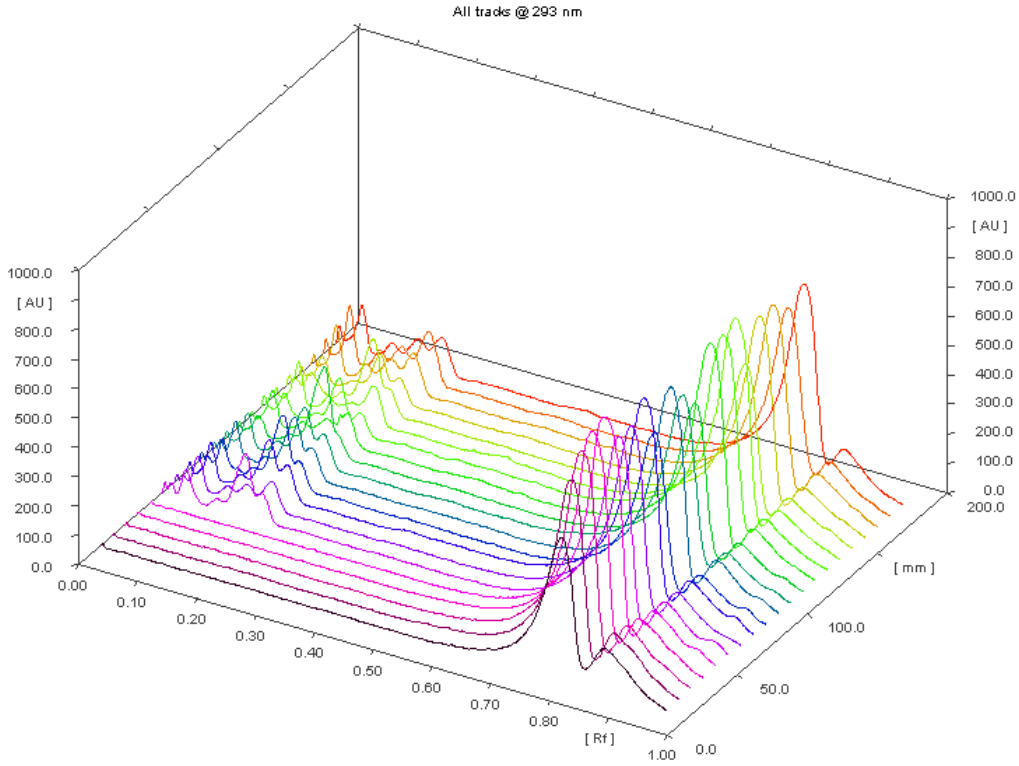



**Figure 23.** 3D TLC-densitometric chromatogram (Sample Plate 1)

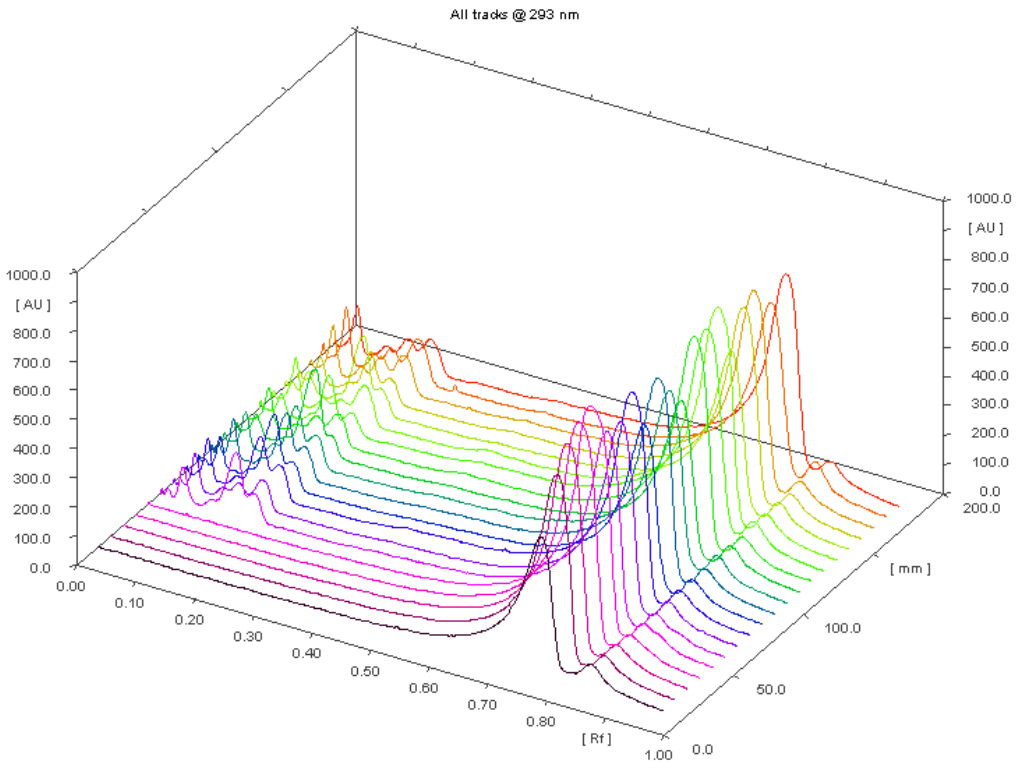



**Figure 24.** 3D TLC-densitometric chromatogram (Sample Plate 2)

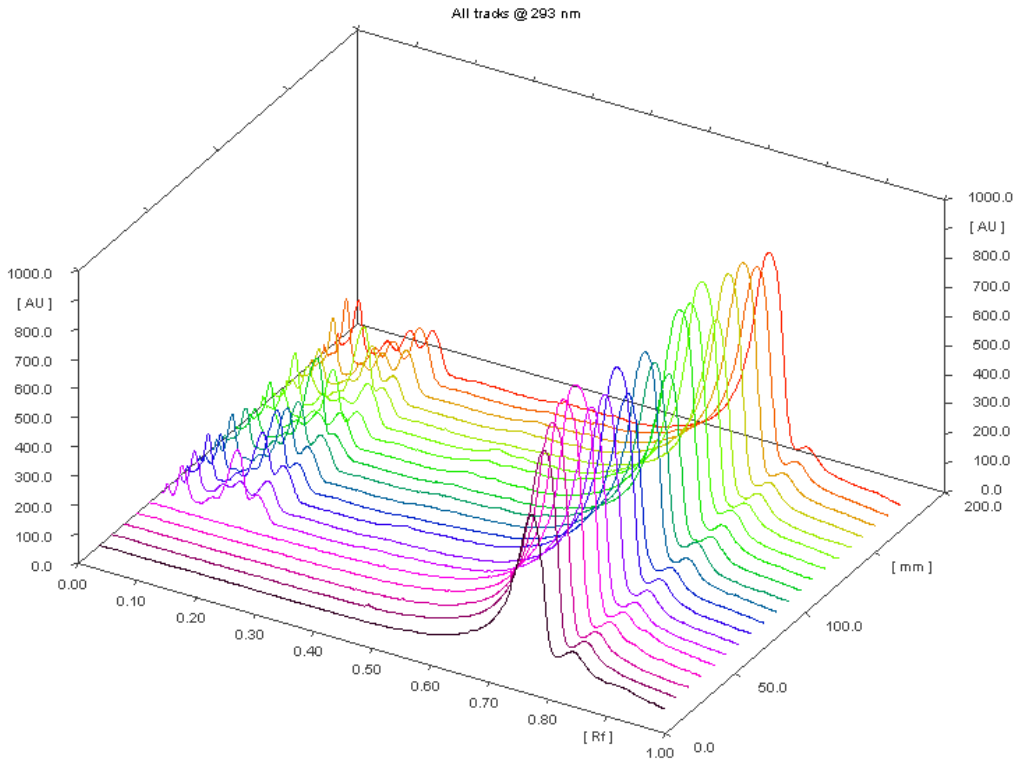



**Figure 25.** 3D TLC-densitometric chromatogram (Sample Plate 3)

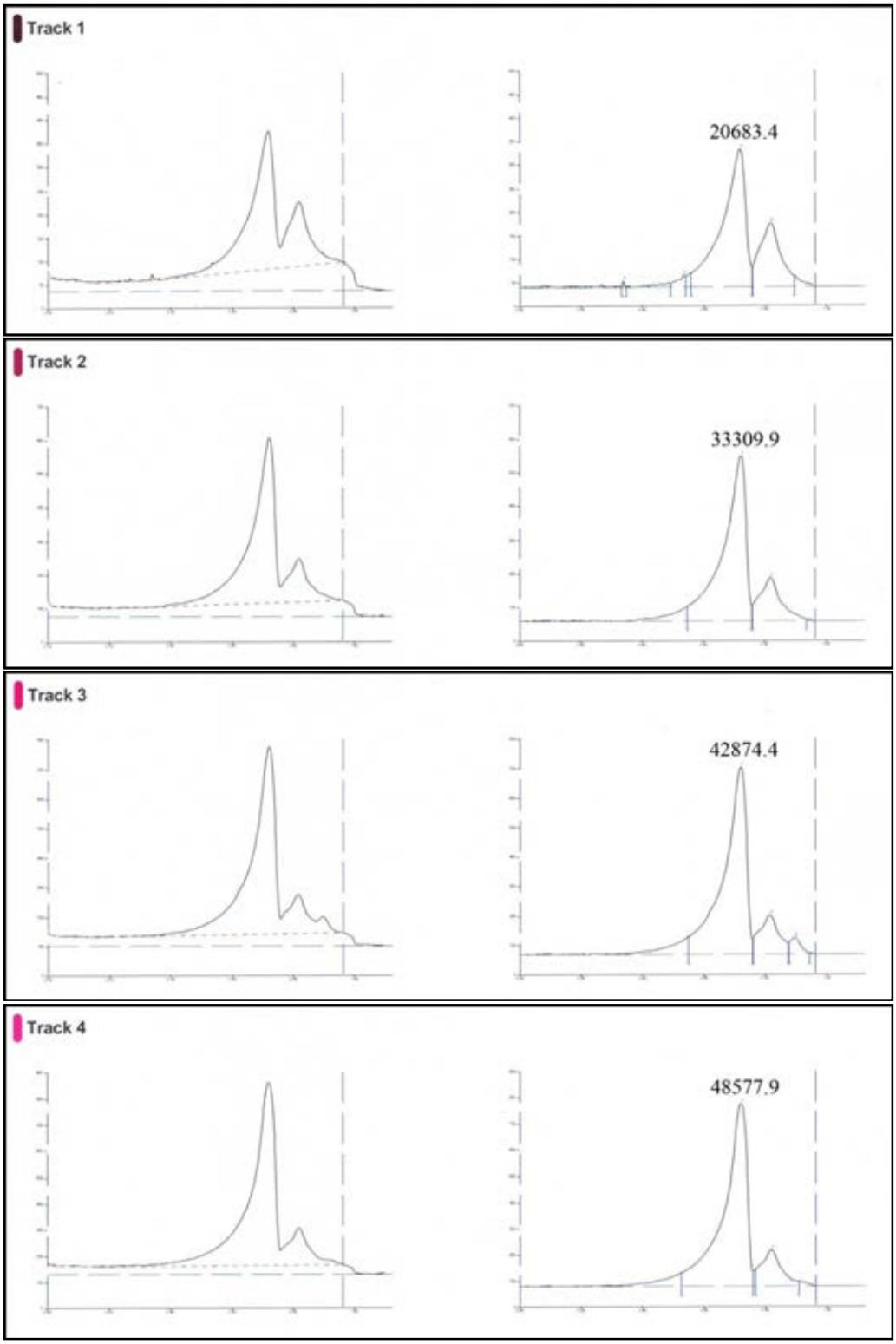



**Figure 26.** TLC-densitometric chromatogram (Accuracy); Track 1 was usnic acid 0.2 mg/ml, Track 2 was usnic acid 0.4 mg/ml, Track 3 was usnic acid 0.6 mg/ml, Track 4 was usnic acid 0.8 mg/ml

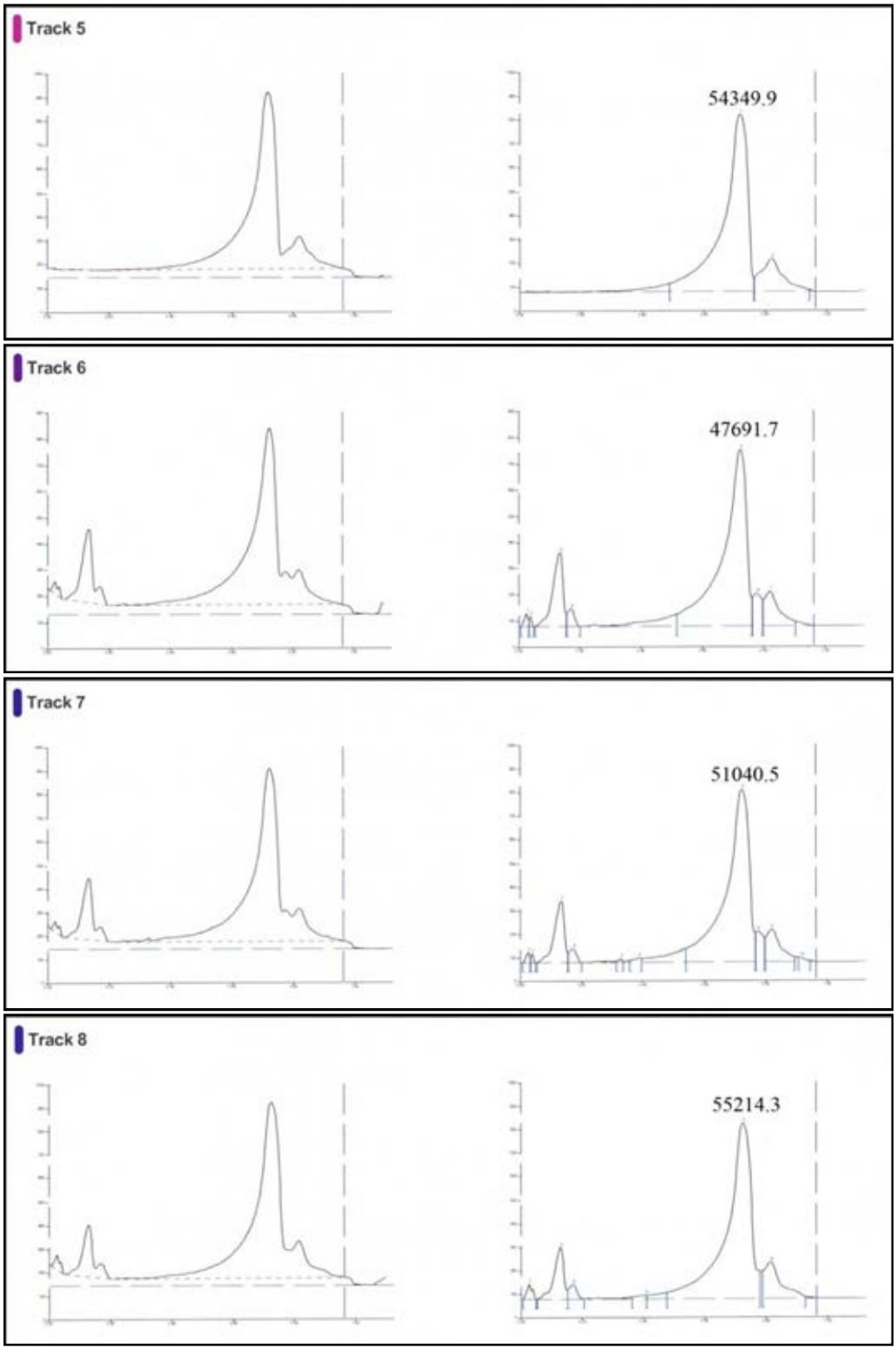



**Figure 26. (Continue)** TLC-densitometric chromatogram (Accuracy); Track 5 was usnic acid 1.0 mg/ml, Track 6 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml (first time), Track 7 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml (first time), Track 8 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml (first time)

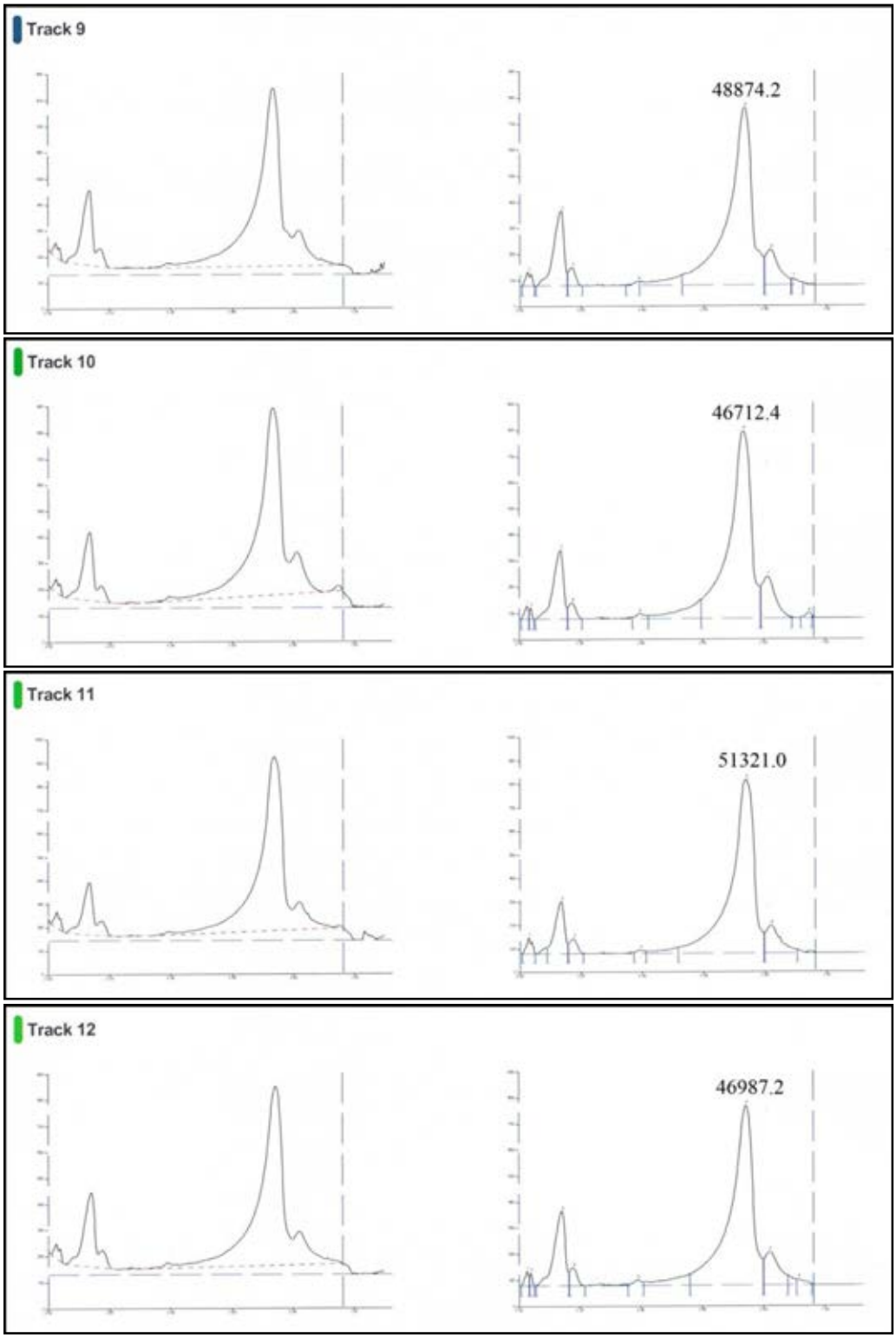



**Figure 26. (Continue)** TLC-densitometric chromatogram (Accuracy); Track 9 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml, Track 10 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml, Track 11 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml, Track 12 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml

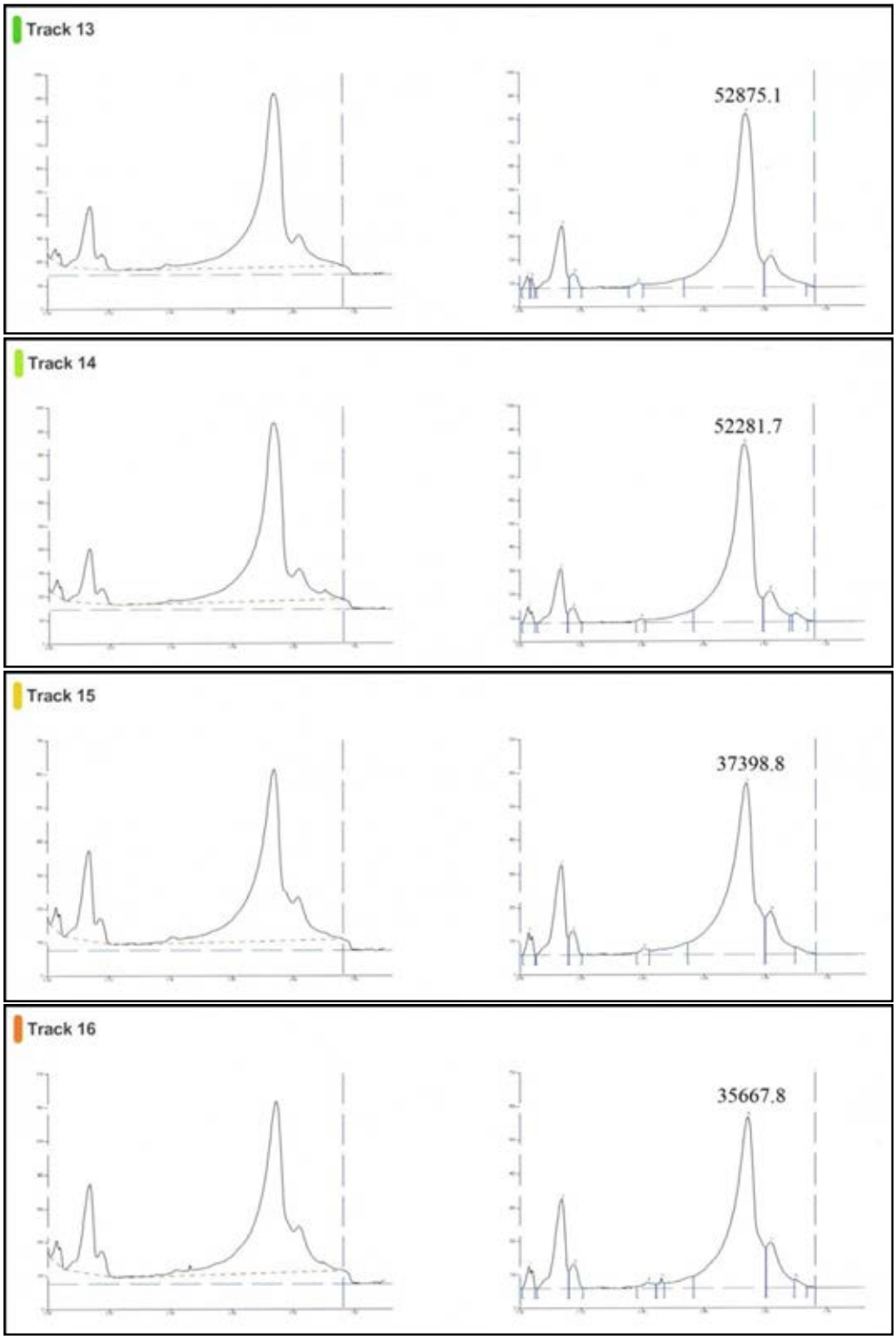



**Figure 26. (Continue)** TLC-densitometric chromatogram (Accuracy); Track 13 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml, Track 14 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml, Track 15 was sample 1.0 mg/ml, Track 16 was sample 1.0 mg/ml

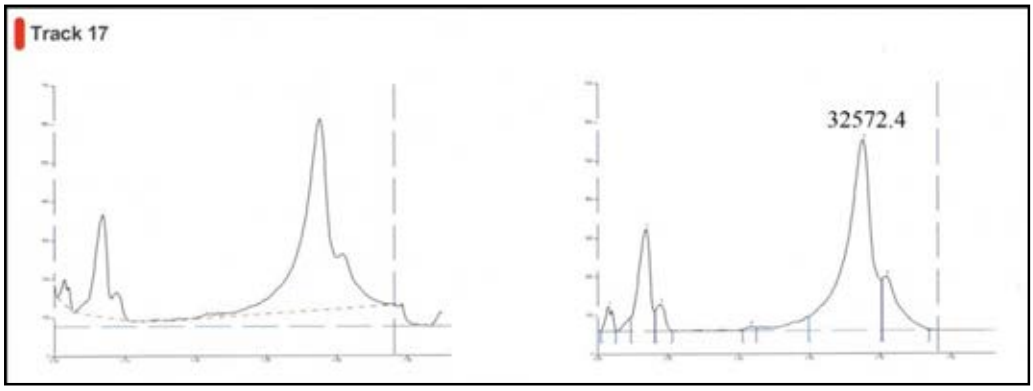



**Figure 26. (Continue)** TLC-densitometric chromatogram (Accuracy); Track 17 was sample 1.0 mg/ml

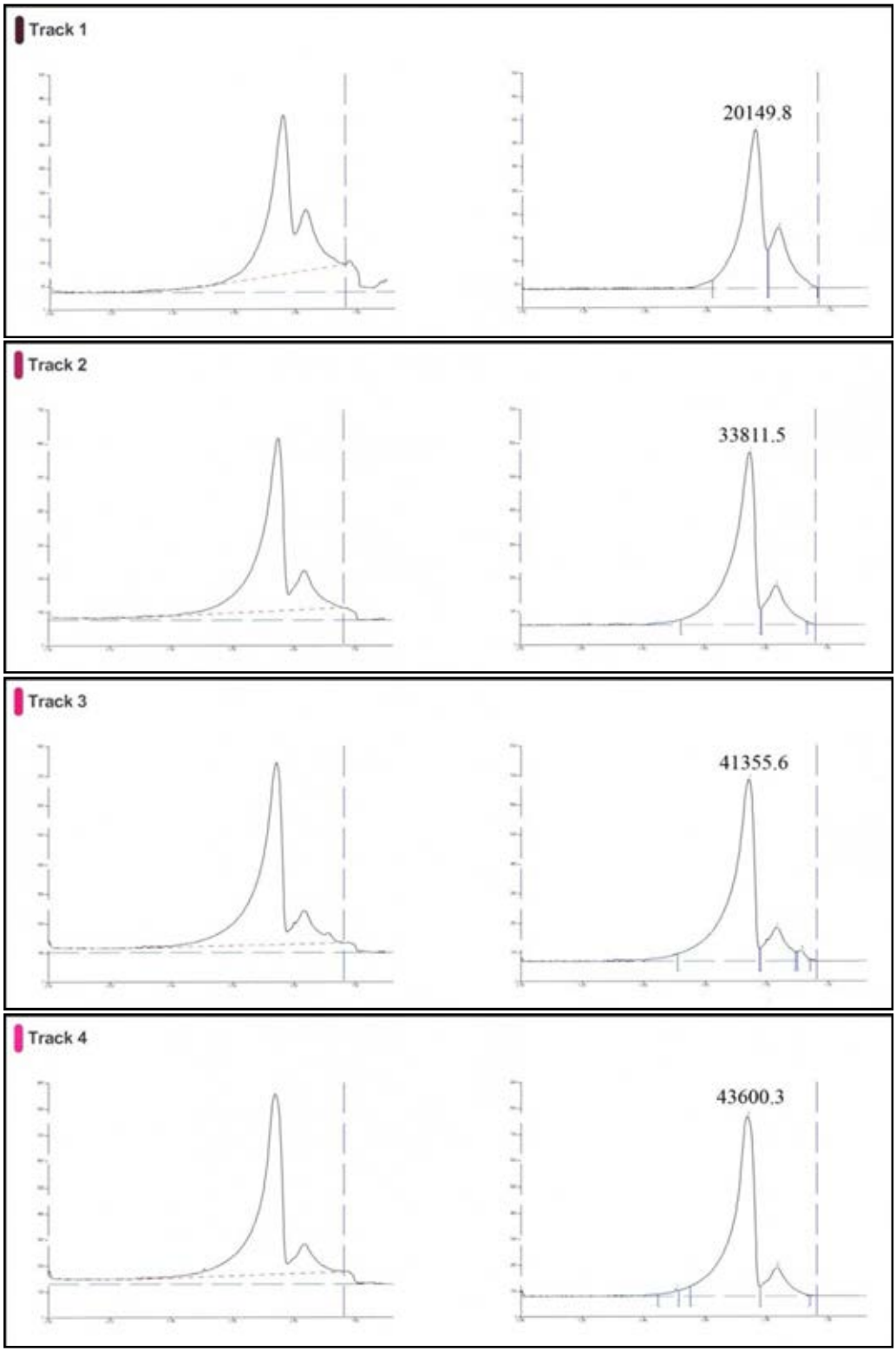



**Figure 27.** TLC-densitometric chromatogram (Precision No.1); Track 1 was usnic acid 0.2 mg/ml, Track 2 was usnic acid 0.4 mg/ml, Track 3 was usnic acid 0.6 mg/ml, Track 4 was usnic acid 0.8 mg/ml

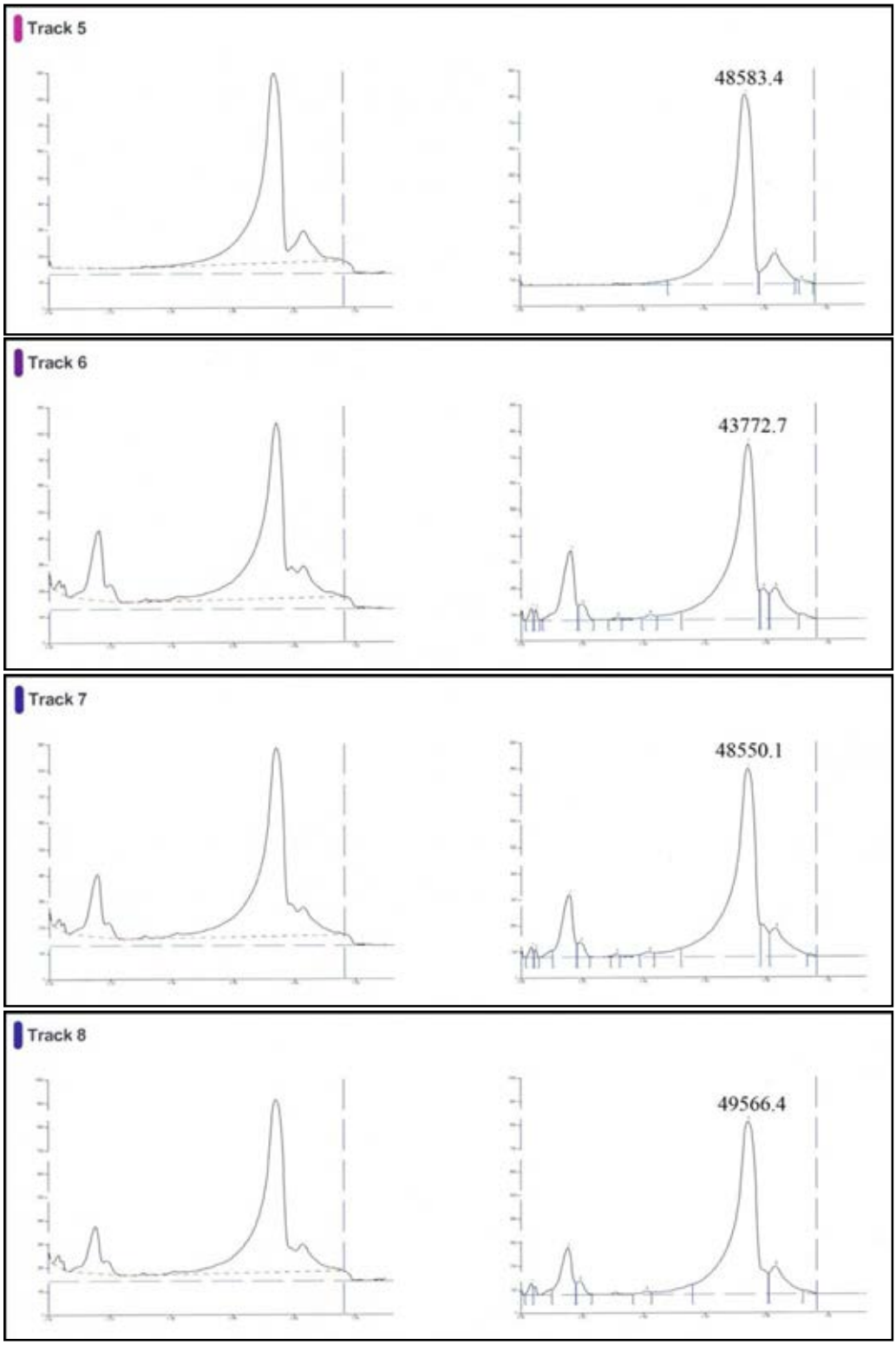



**Figure 27. (Continue)** TLC-densitometric chromatogram (Precision No.1); Track 5 was usnic acid 1.0 mg/ml, Track 6 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml, Track 7 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml, Track 8 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml

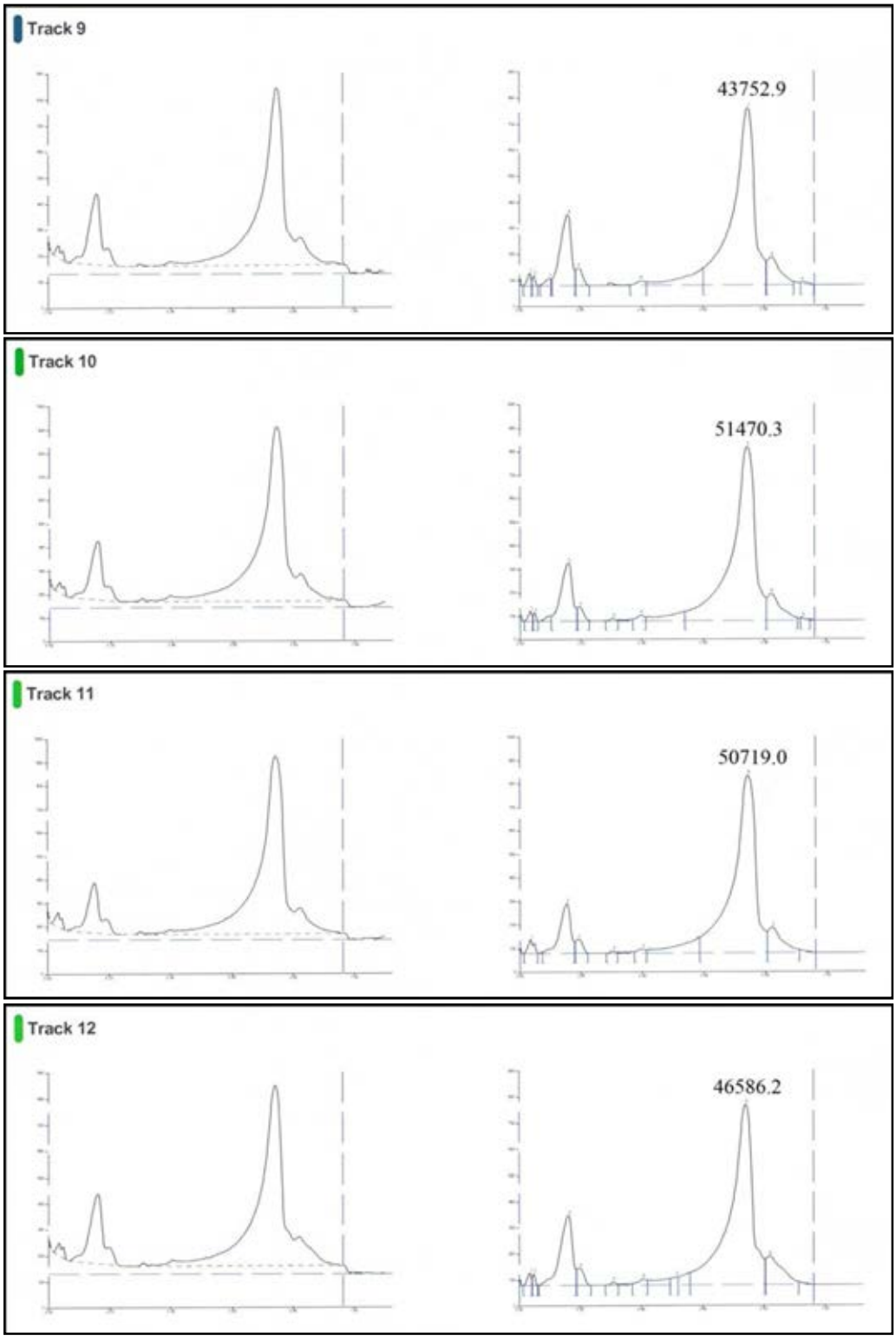



**Figure 27. (Continue)** TLC-densitometric chromatogram (Precision No.1); Track 9 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml, Track 10 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml, Track 11 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml, Track 12 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml

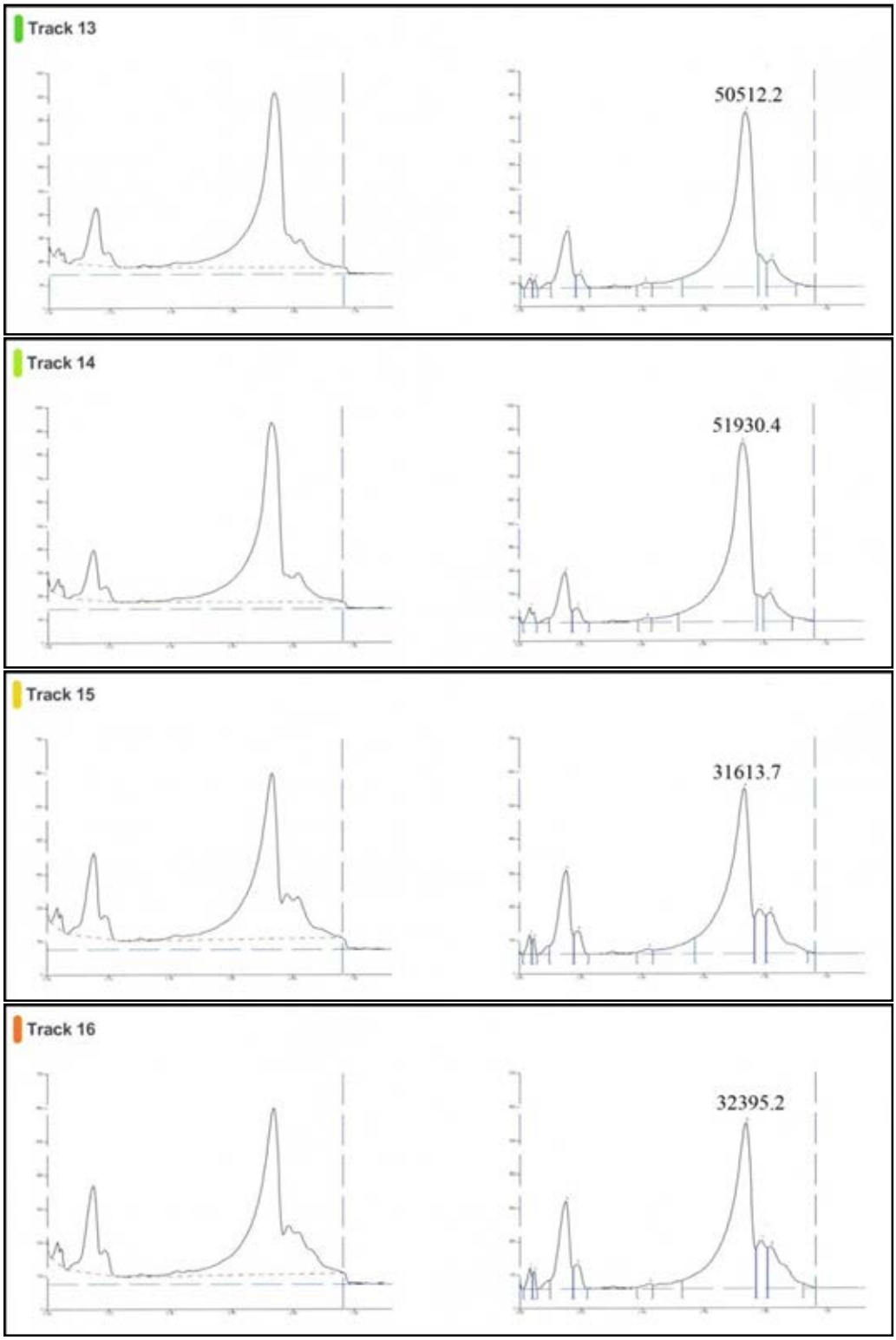



**Figure 27. (Continue)** TLC-densitometric chromatogram (Precision No.1); Track 13 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml, Track 14 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml, Track 15 was sample 1.0 mg/ml, Track 16 was sample 1.0 mg/ml

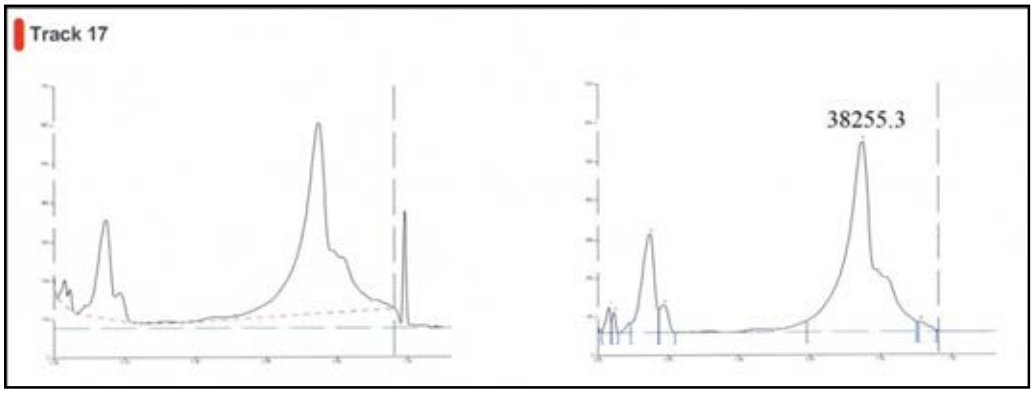



**Figure 27.** (Cont.) TLC-densitometric chromatogram (Precision No.1); Track 17 was sample 1.0 mg/ml

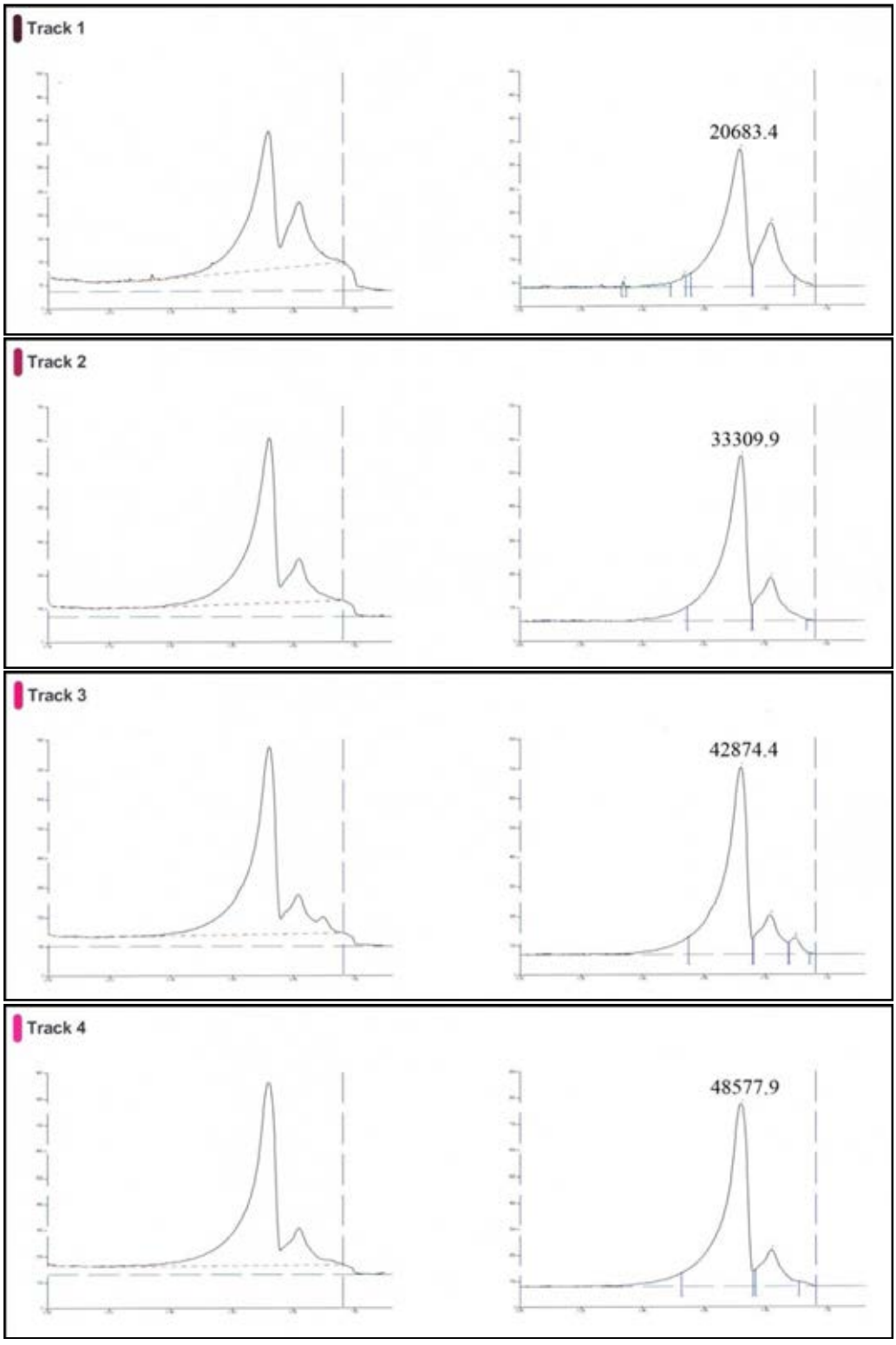



**Figure 28.** TLC-densitometric chromatogram (Precision No.2); Track 1 was usnic acid 0.2 mg/ml, Track 2 was usnic acid 0.4 mg/ml, Track 3 was usnic acid 0.6 mg/ml, Track 4 was usnic acid 0.8 mg/ml

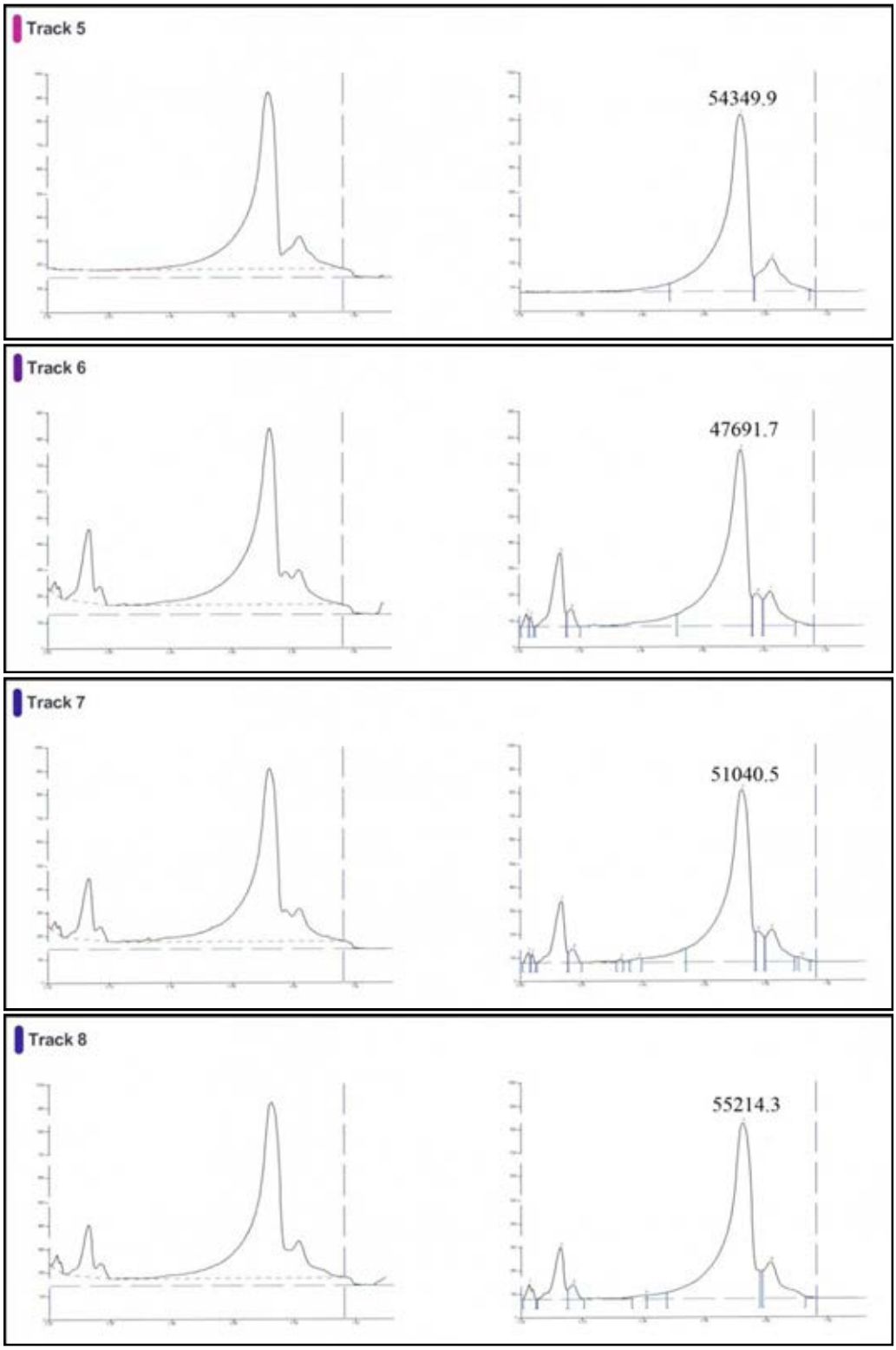



**Figure 28. (Continue)** TLC-densitometric chromatogram (Precision No.2); Track 5 was usnic acid 1.0 mg/ml, Track 6 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml (first time), Track 7 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml (first time), Track 8 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml (first time)

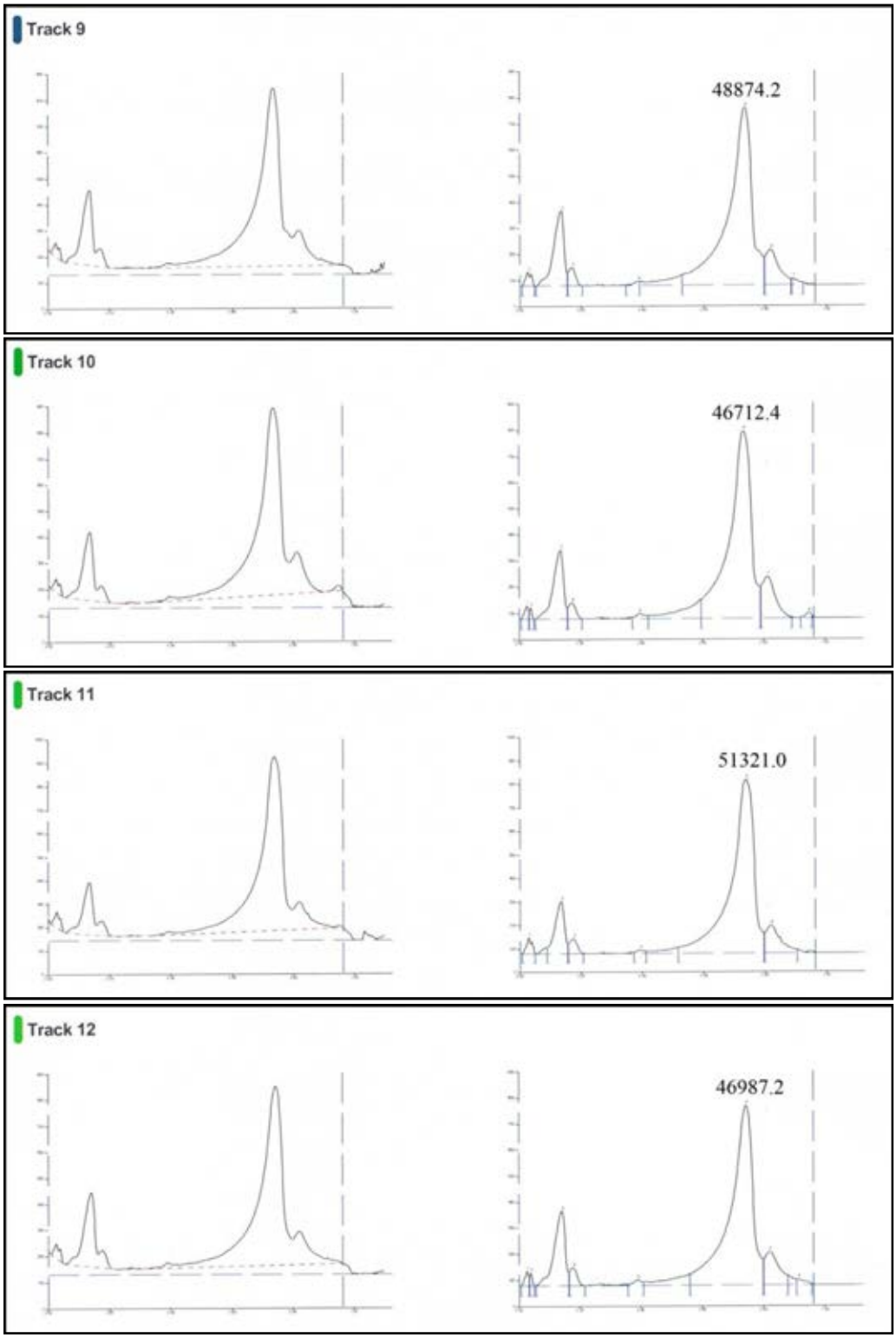



**Figure 28. (Continue)** TLC-densitometric chromatogram (Precision No.2); Track 9 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml, Track 10 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml, Track 11 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml, Track 12 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml

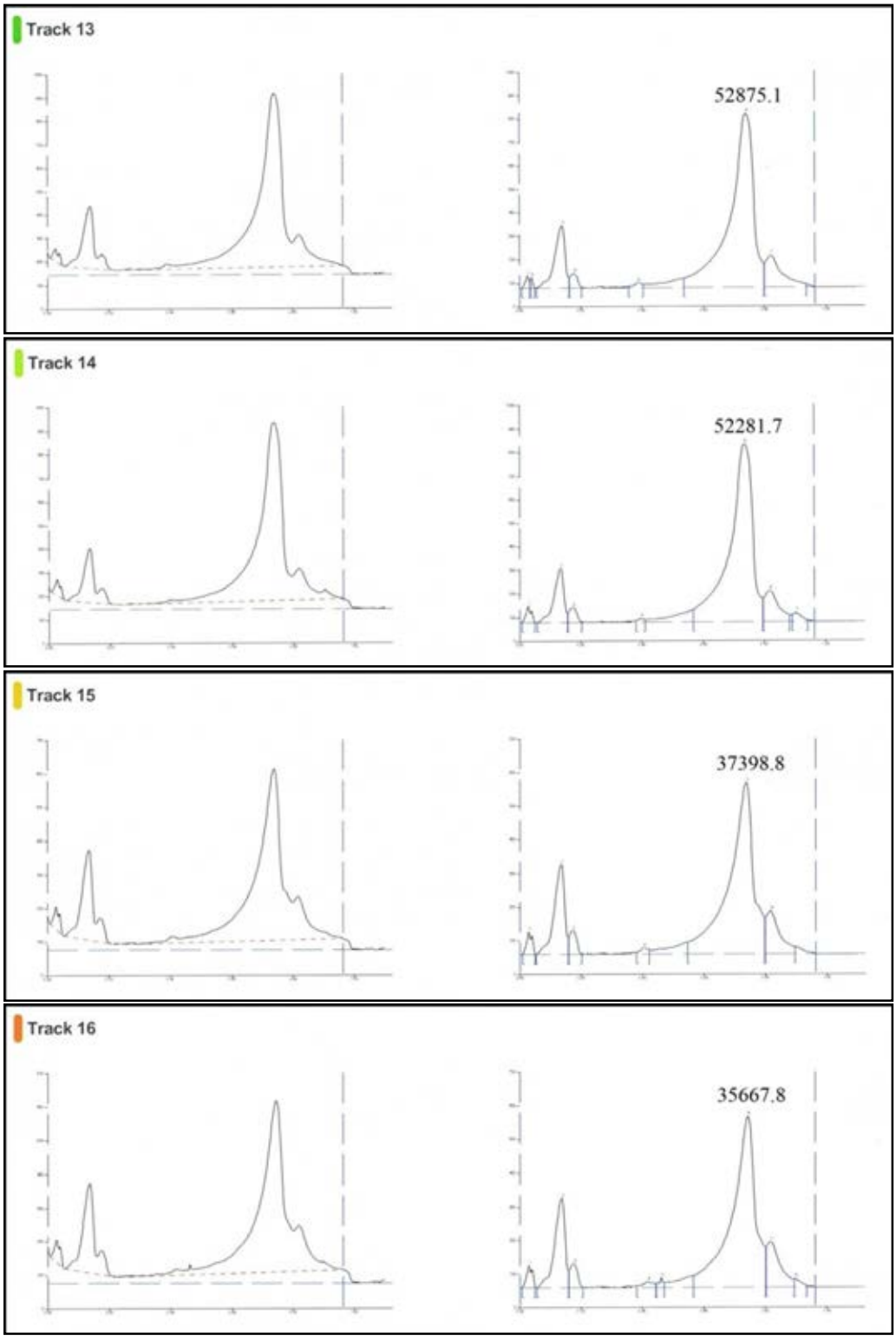



**Figure 28. (Continue)** TLC-densitometric chromatogram (Precision No.2); Track 13 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml, Track 14 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml, Track 15 was sample 1.0 mg/ml, Track 16 was sample 1.0 mg/ml

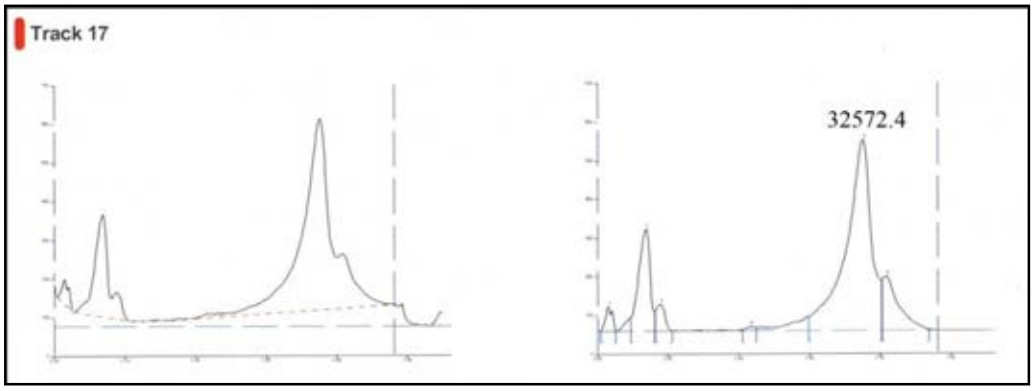



**Figure 28. (Continue)** TLC-densitometric chromatogram (Precision No.2); Track 17 was sample 1.0 mg/ml

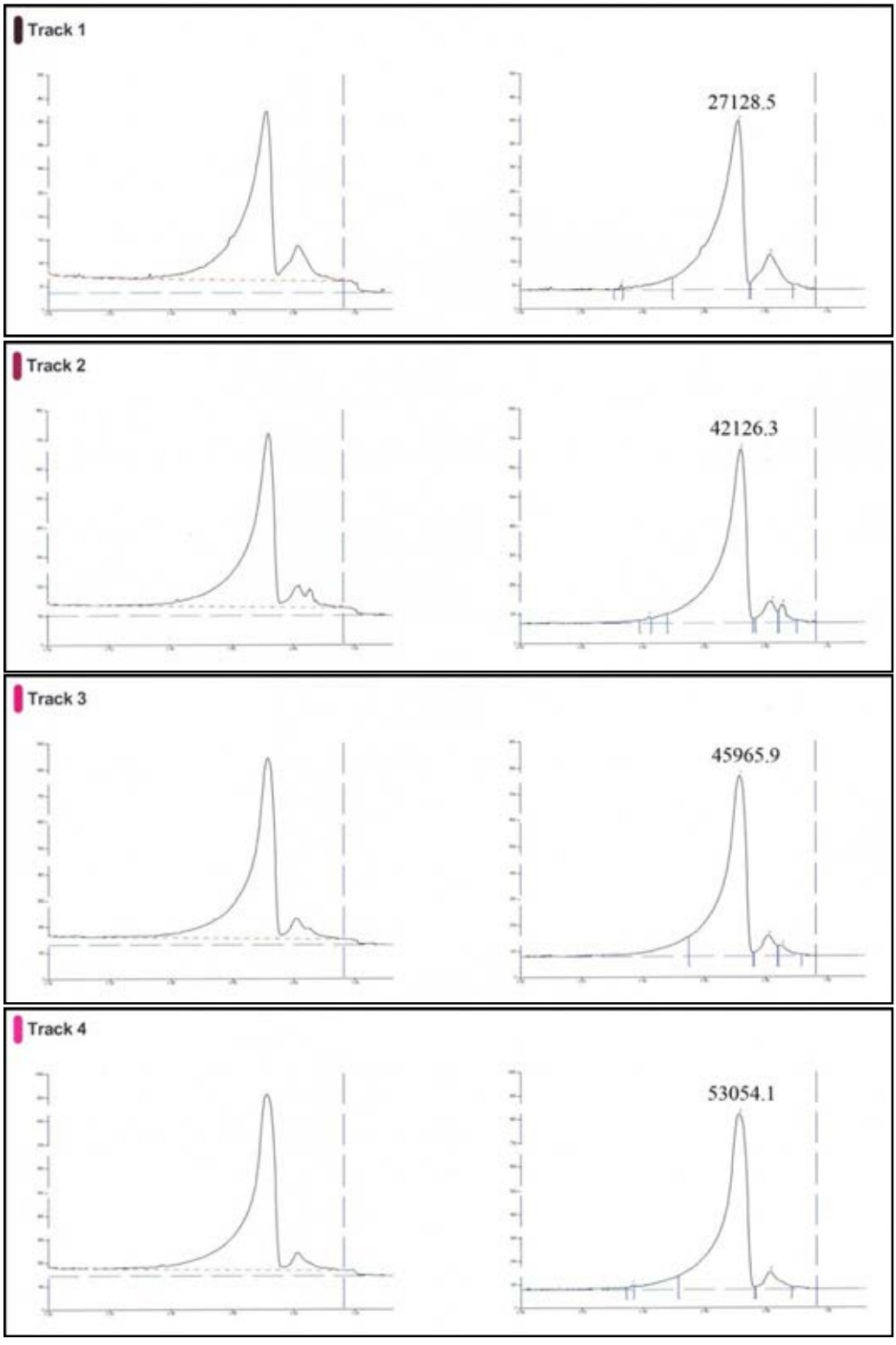



**Figure 29.** TLC-densitometric chromatogram (Precision No.3); Track 1 was usnic acid 0.2 mg/ml, Track 2 was usnic acid 0.4 mg/ml, Track 3 was usnic acid 0.6 mg/ml, Track 4 was usnic acid 0.8 mg/ml

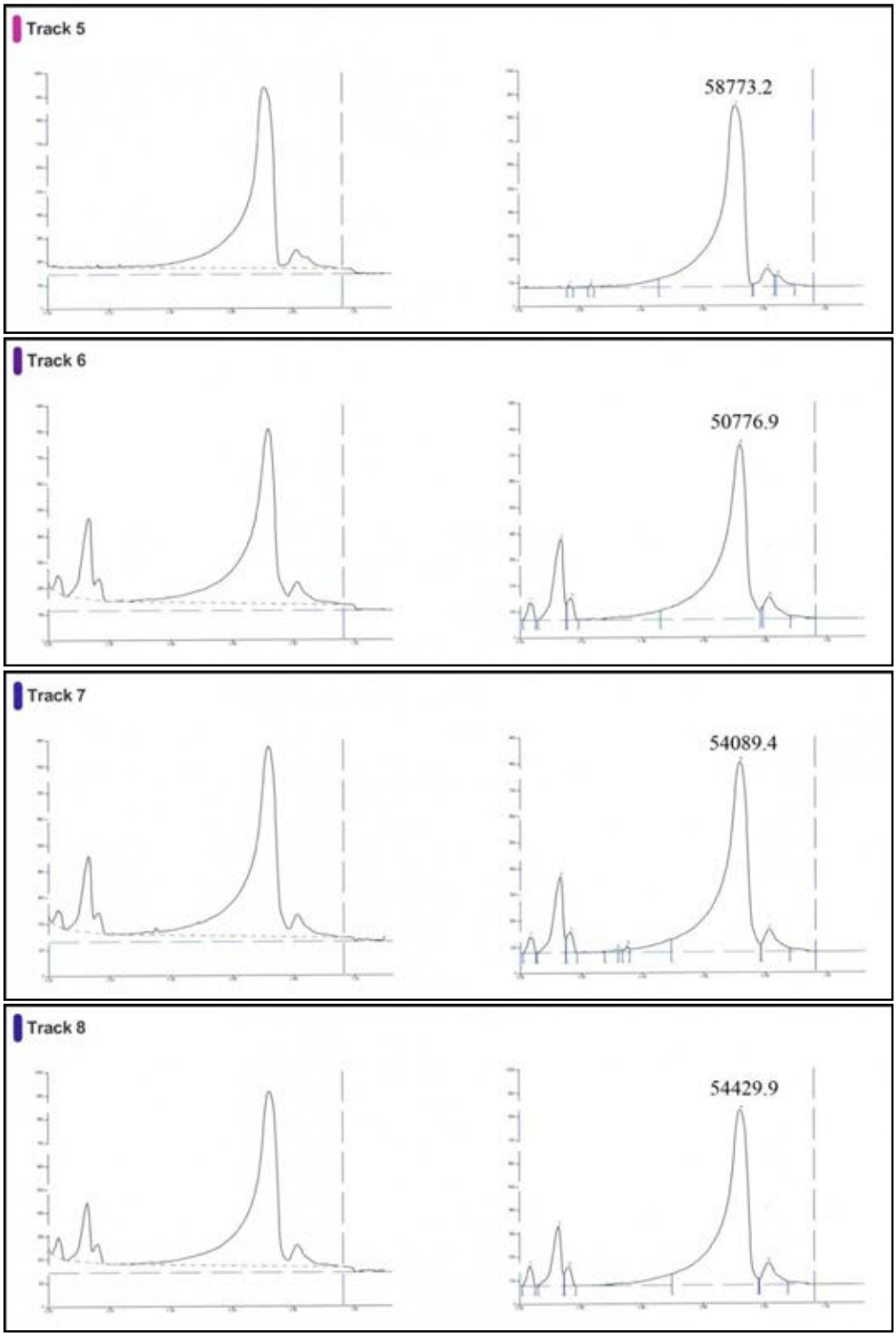



**Figure 29. (Continue)** TLC-densitometric chromatogram (Precision No.3); Track 5 was usnic acid 1.0 mg/ml, Track 6 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml (first time), Track 7 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml (first time), Track 8 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml (first time)

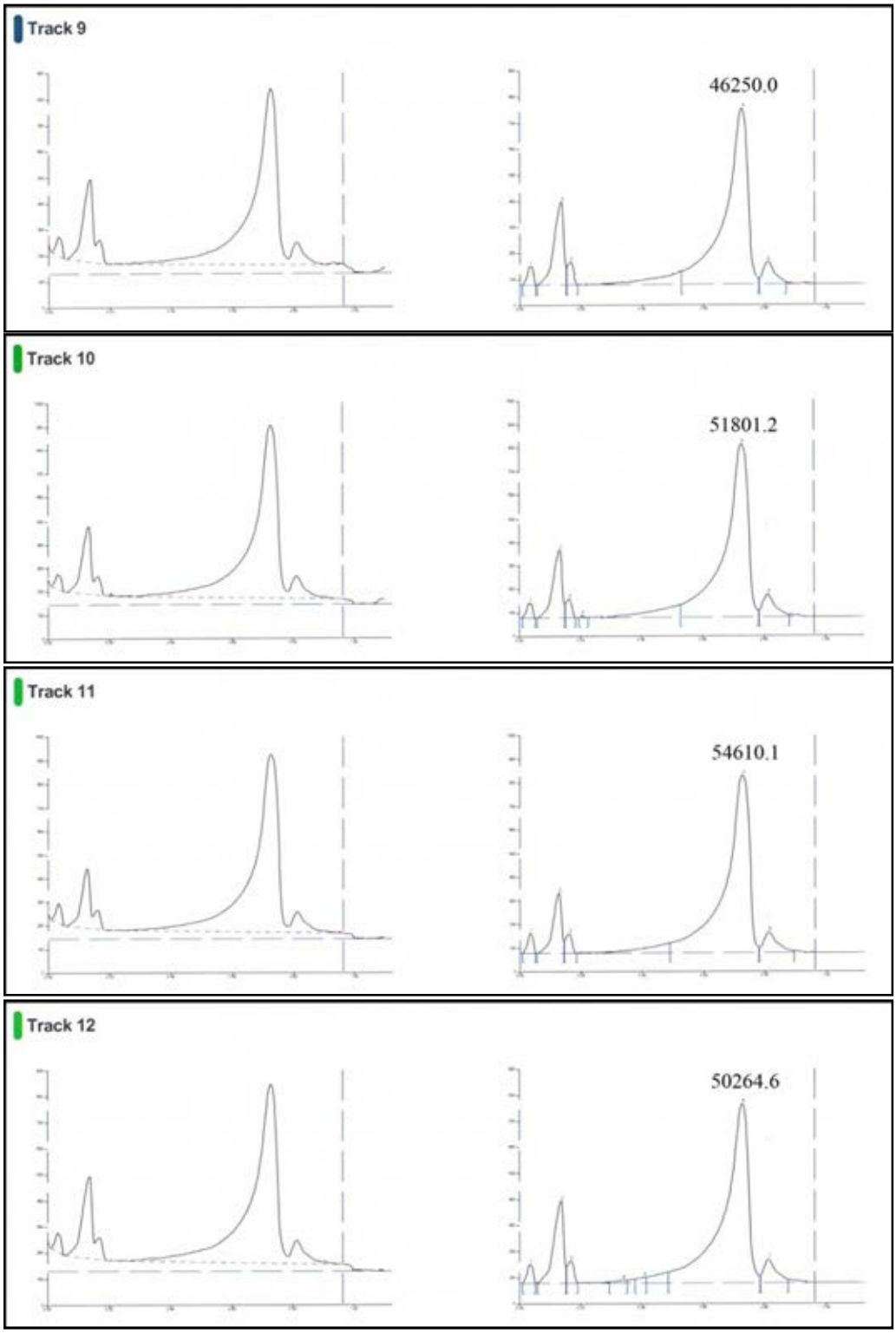



**Figure 29. (Continue)** TLC-densitometric chromatogram (Precision No.3); Track 9 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml, Track 10 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml, Track 11 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml, Track 12 was sample 1.0 mg/ml and spiked usnic acid 0.3 mg/ml

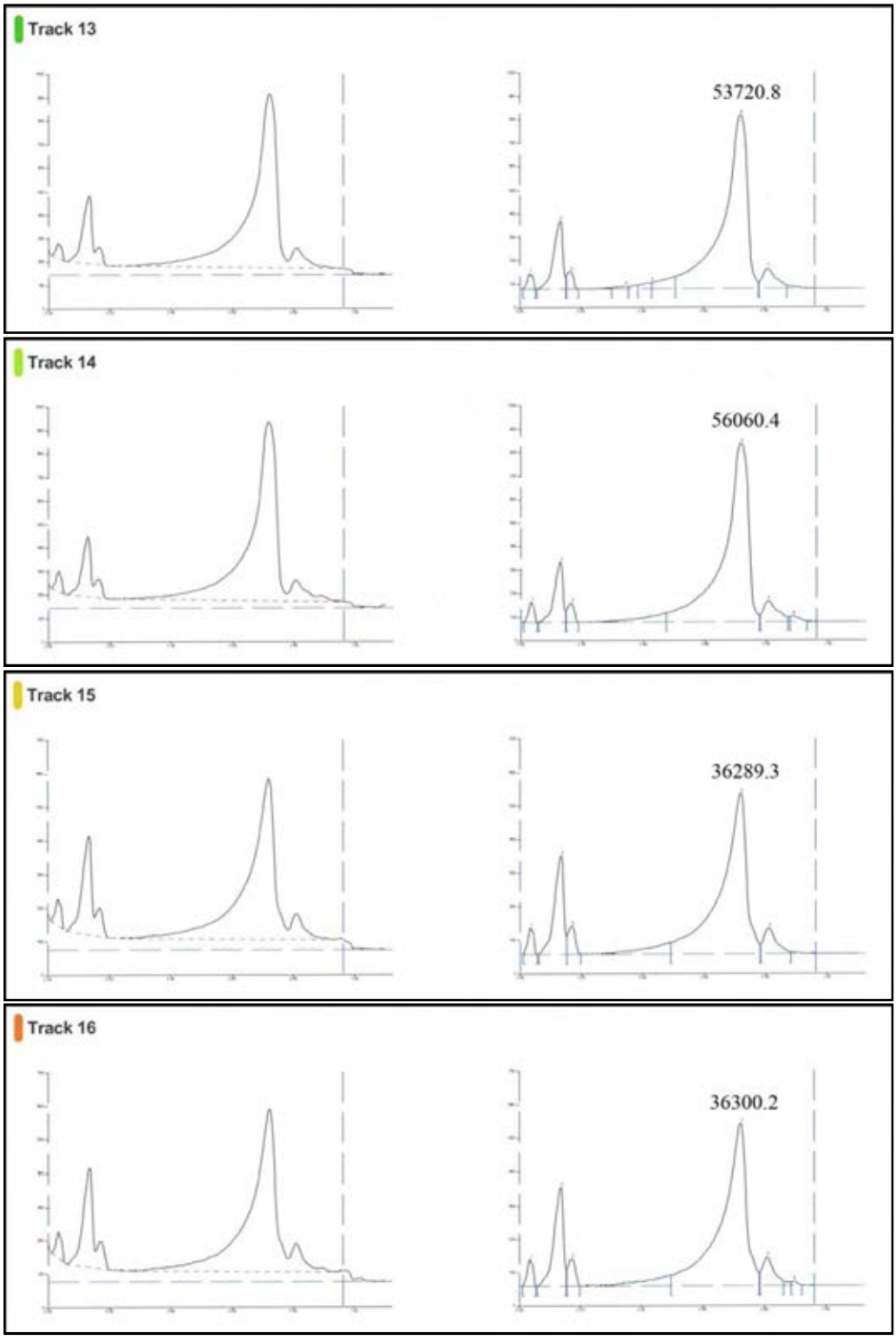



**Figure 29. (Continue)** TLC-densitometric chromatogram (Precision No.3); Track 13 was sample 1.0 mg/ml and spiked usnic acid 0.5 mg/ml, Track 14 was sample 1.0 mg/ml and spiked usnic acid 0.7 mg/ml, Track 15 was sample 1.0 mg/ml, Track 16 was sample 1.0 mg/ml

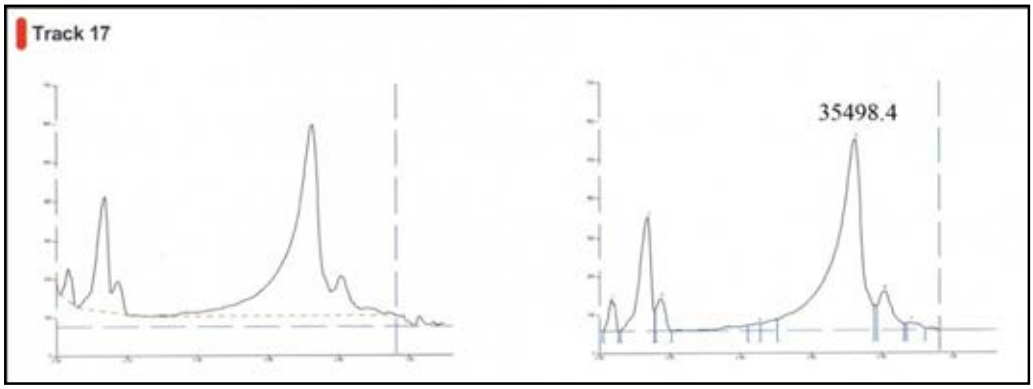



**Figure 29. (Continue)** TLC-densitometric chromatogram (Precision No.3); Track 17 was sample 1.0 mg/ml

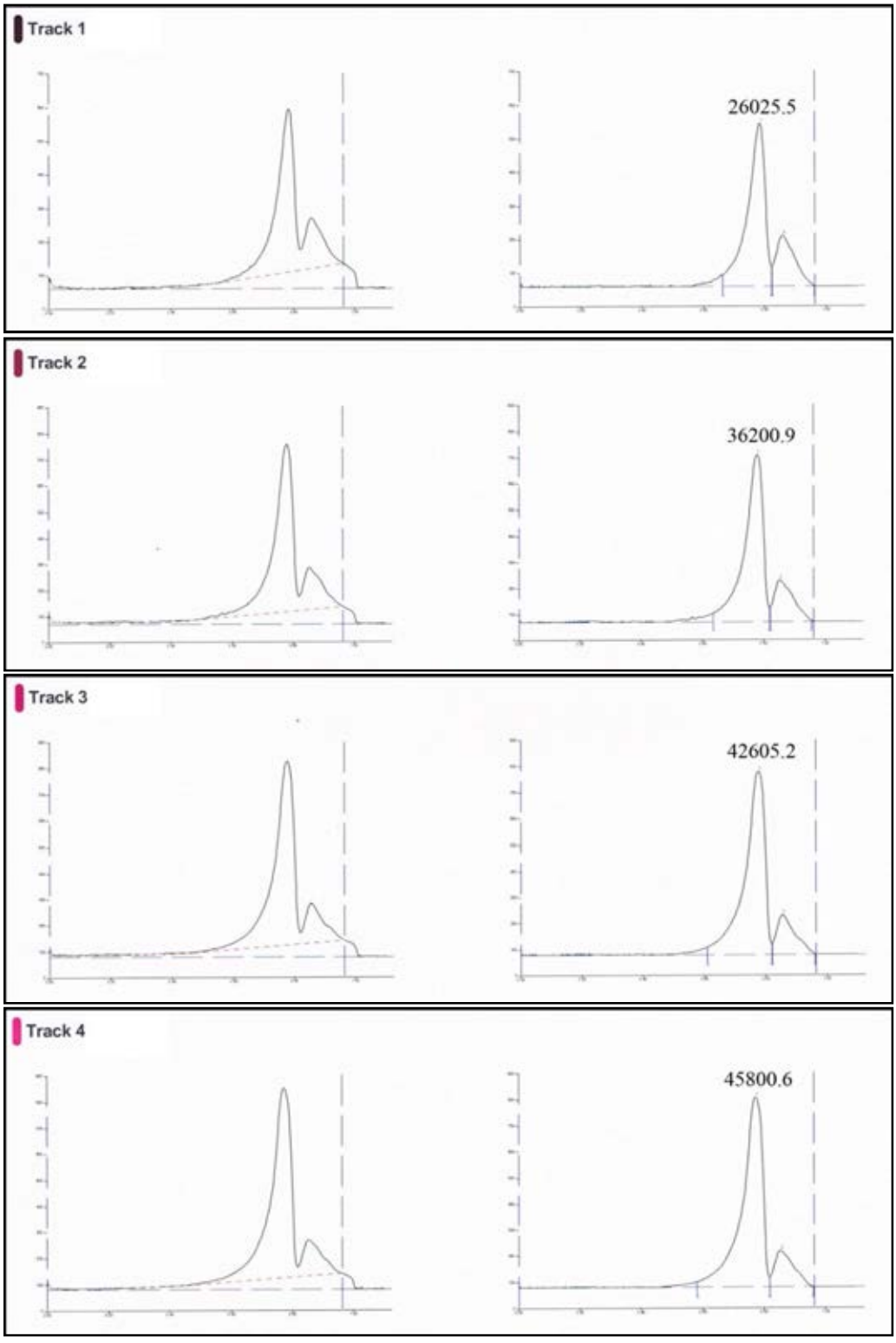



**Figure 30.** TLC-densitometric chromatogram (Sample Plate 1); Track 1 was usnic acid 0.2 mg/ml, Track 2 was usnic acid 0.4 mg/ml, Track 3 was usnic acid 0.6 mg/ml, Track 4 was usnic acid 0.8 mg/ml

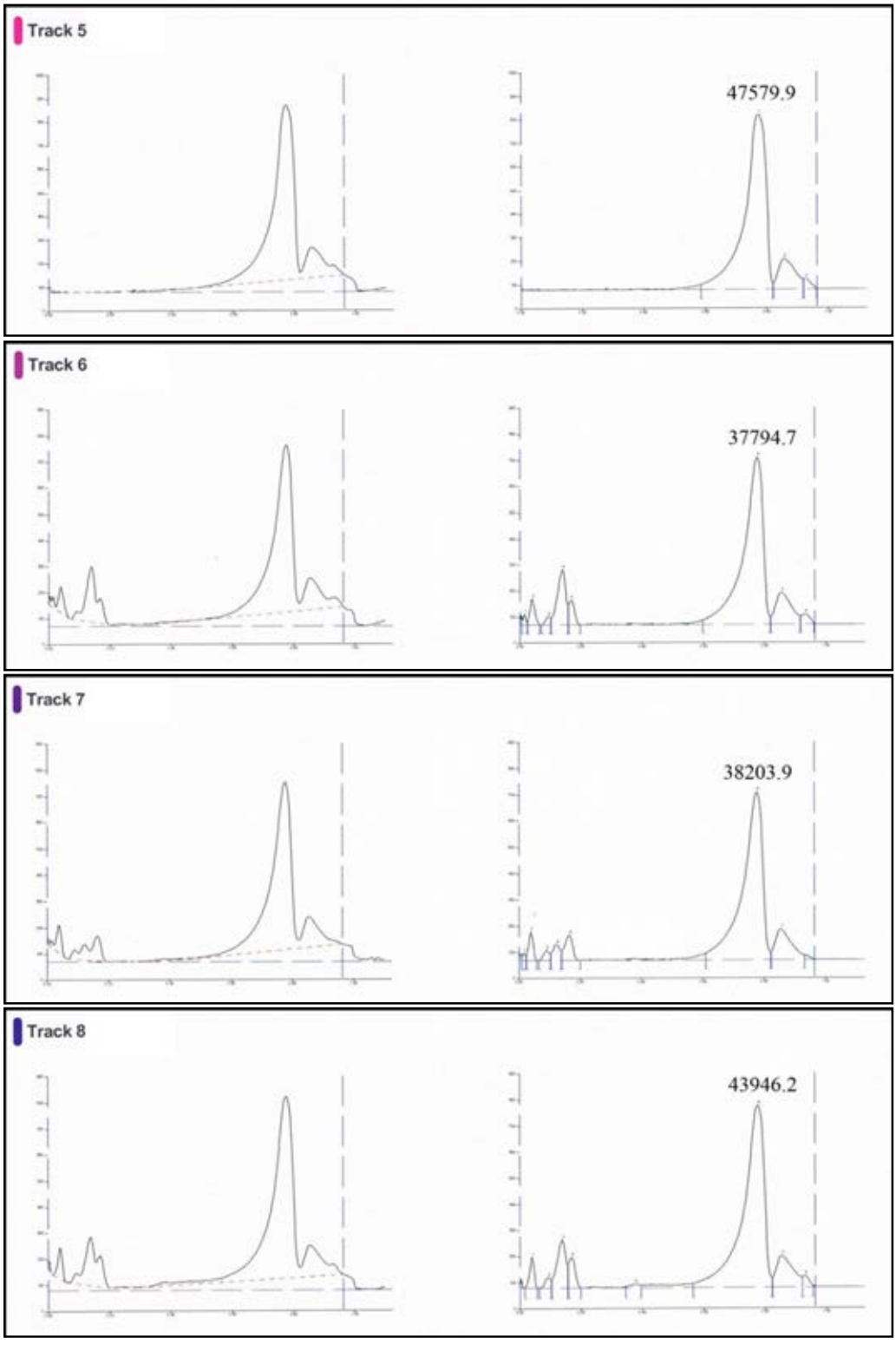



**Figure 30. (Continue)** TLC-densitometric chromatogram (Sample Plate 1); Track 5 was usnic acid 1.0 mg/ml, Track 6 was sample from Phichit 1.0 mg/ml, Track 7 was sample from Bangkok 1 1.0 mg/ml, Track 8 was sample from Bangkok 2 1.0 mg/ml

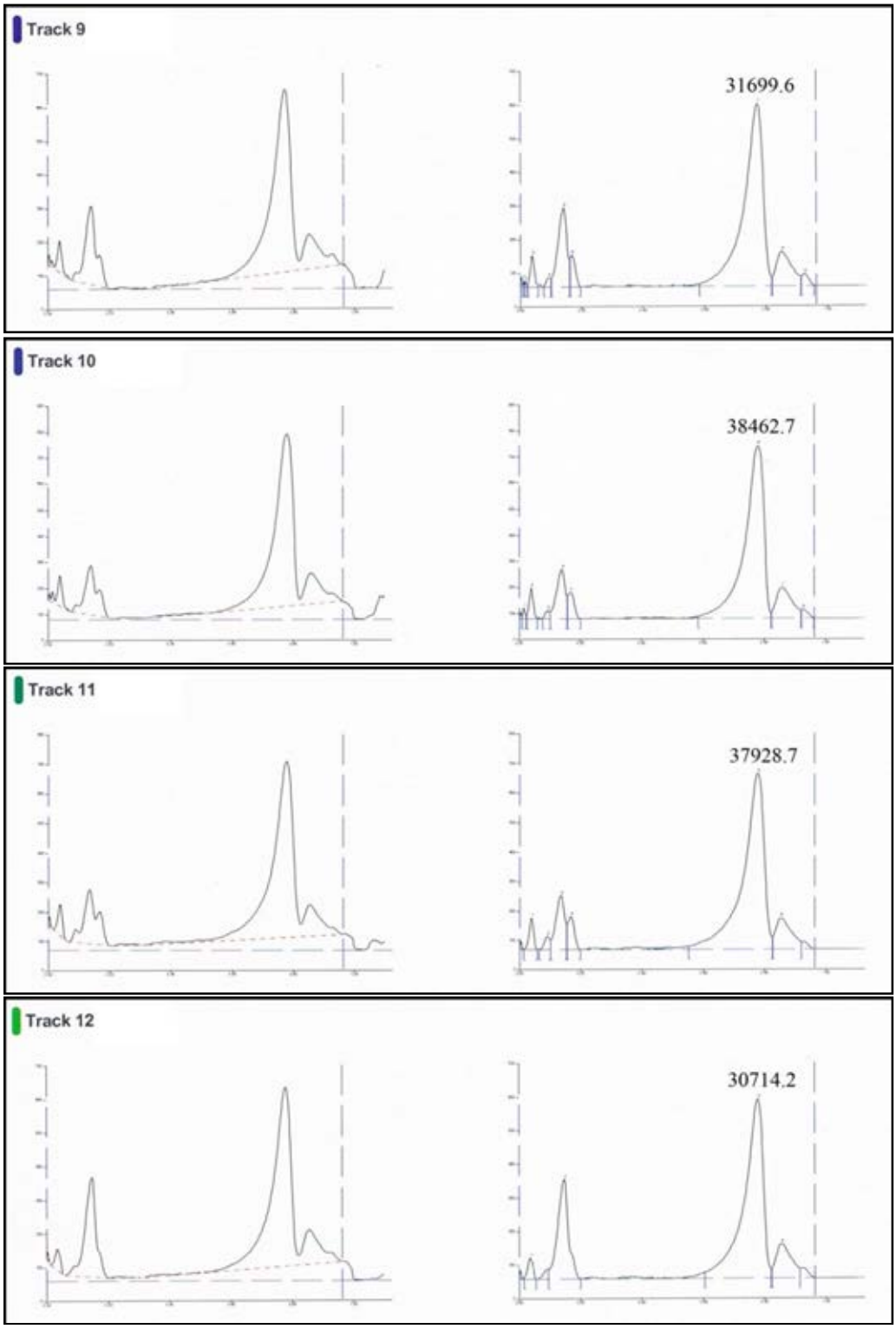



**Figure 30. (Continue)** TLC-densitometric chromatogram (Sample Plate 1); Track 9 was sample from Buri Ram 1 1.0 mg/ml, Track 10 was sample from Buri Ram 2 1.0 mg/ml, Track 11 was sample from Ranong 1.0 mg/ml, Track 12 was sample from Suphan Buri 1.0 mg/ml

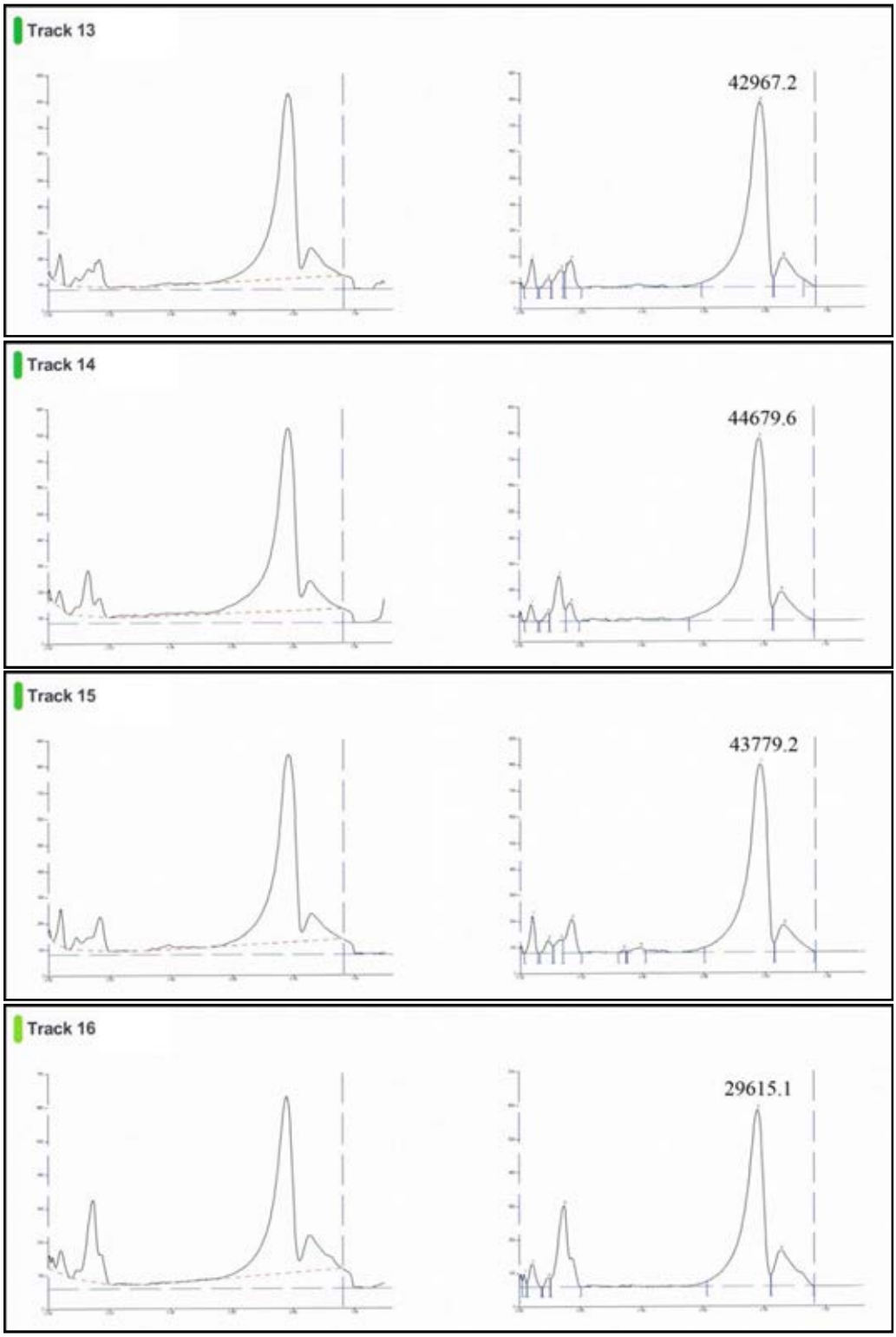



**Figure 30. (Continue)** TLC-densitometric chromatogram (Sample Plate 1); Track 13 was sample from Phrae 1.0 mg/ml, Track 14 was sample from Nakhon Sawan 1.0 mg/ml, Track 15 was sample from Udon Thani 1.0 mg/ml, Track 16 was sample from Khon Kaen 1.0 mg/ml

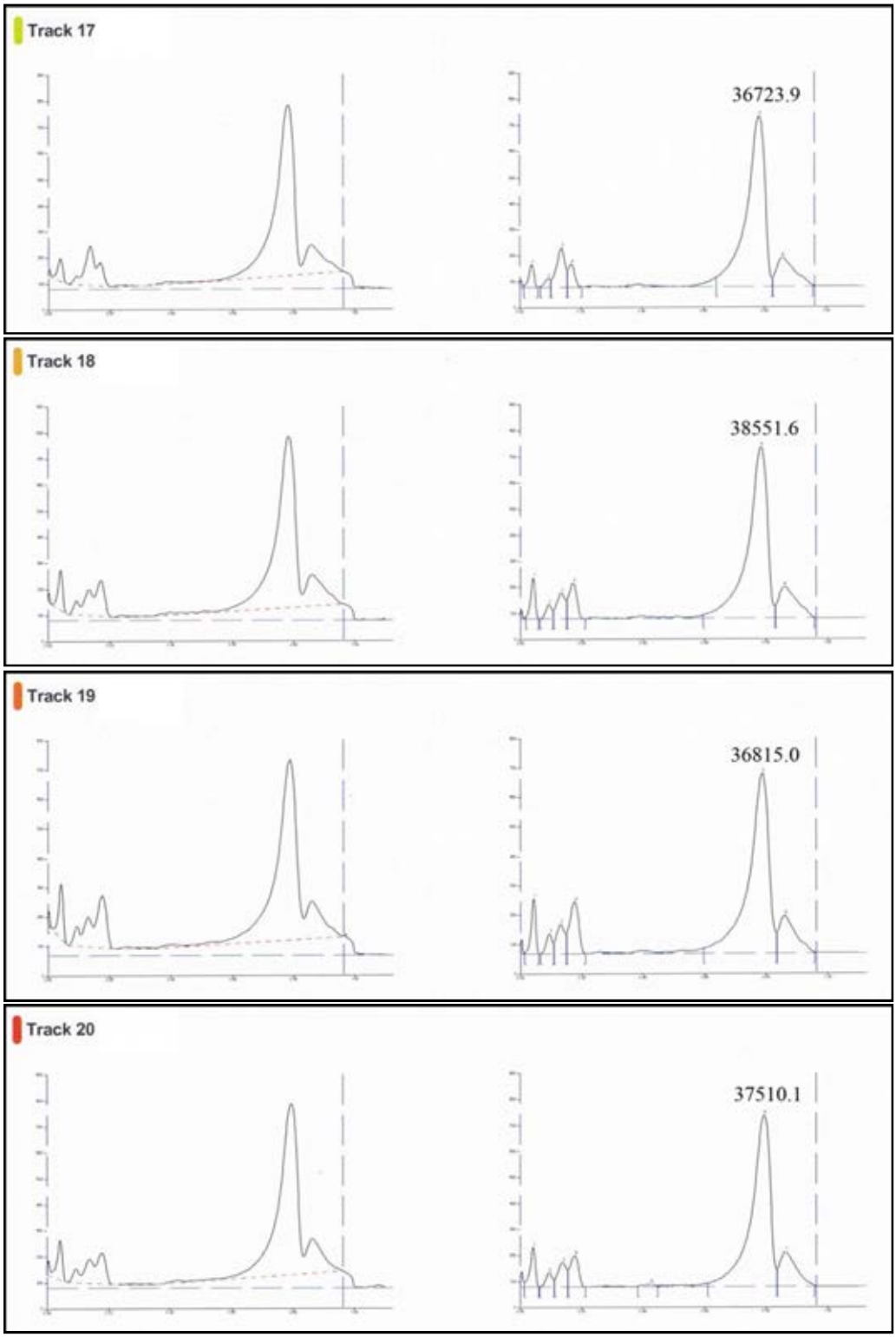



**Figure 30. (Continue)** TLC-densitometric chromatogram (Sample Plate 1); Track 17 was sample from Krabi 1.0 mg/ml, Track 18 was sample from Lop Buri 1.0 mg/ml, Track 19 was sample from Chiang Rai 1.0 mg/ml, Track 20 was sample from Uttaradit 1.0 mg/ml

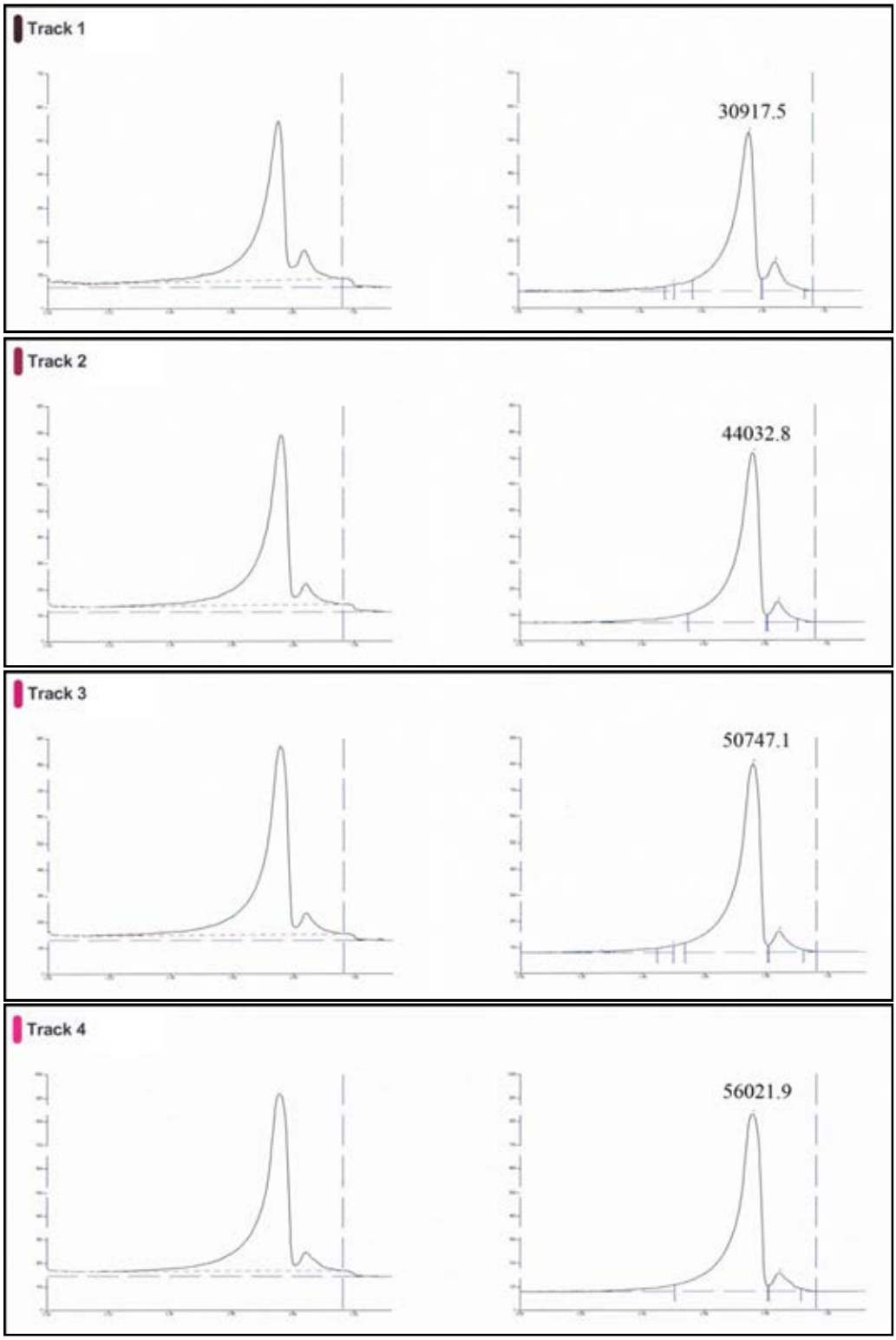



**Figure 31.** TLC-densitometric chromatogram (Sample Plate 2); Track 1 was usnic acid 0.2 mg/ml, Track 2 was usnic acid 0.4 mg/ml, Track 3 was usnic acid 0.6 mg/ml, Track 4 was usnic acid 0.8 mg/ml

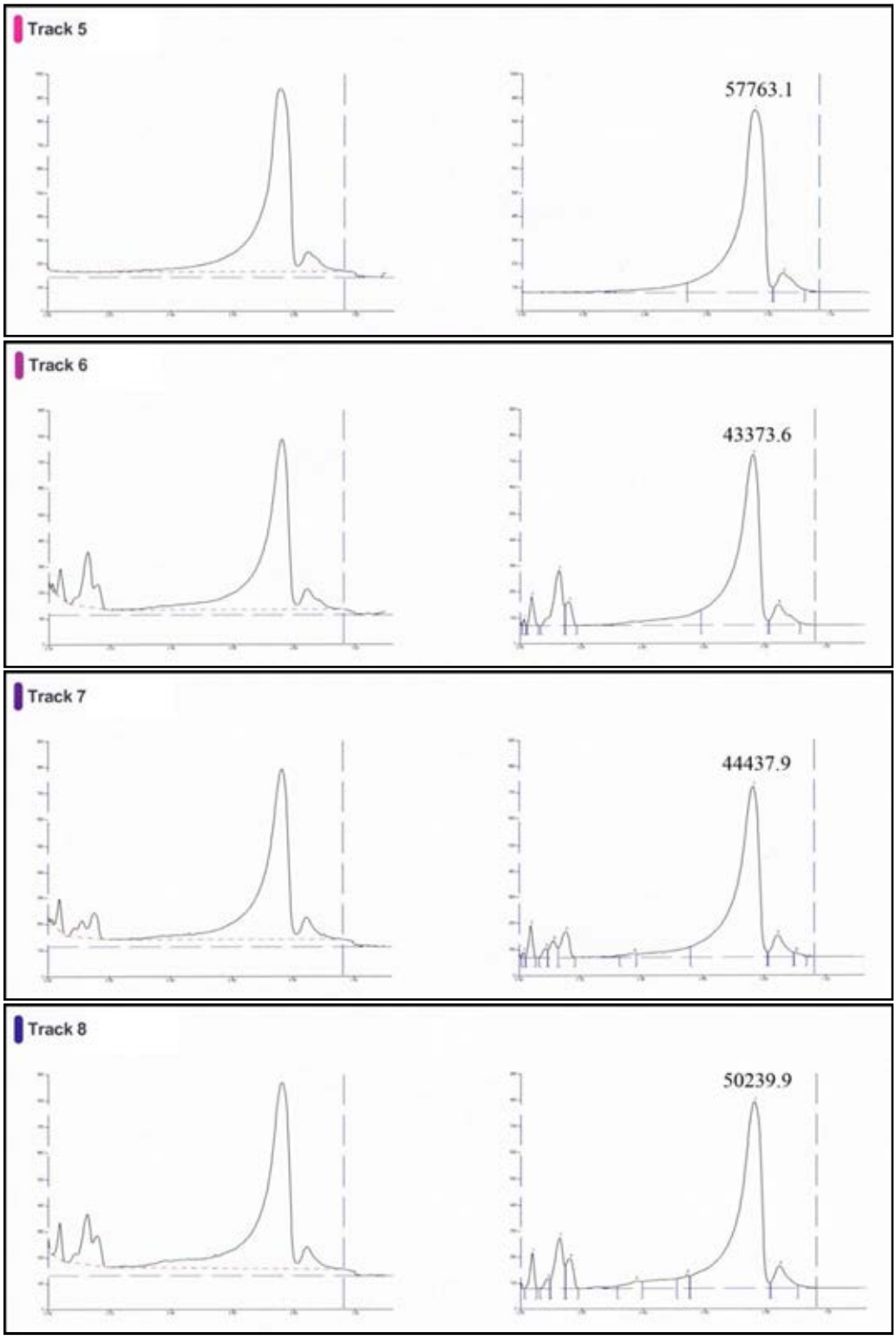



**Figure 31. (Continue)** TLC-densitometric chromatogram (Sample Plate 2); Track 5 was usnic acid 1.0 mg/ml, Track 6 was sample from Phichit 1.0 mg/ml, Track 7 was sample from Bangkok 1 1.0 mg/ml, Track 8 was sample from Bangkok 2 1.0 mg/ml

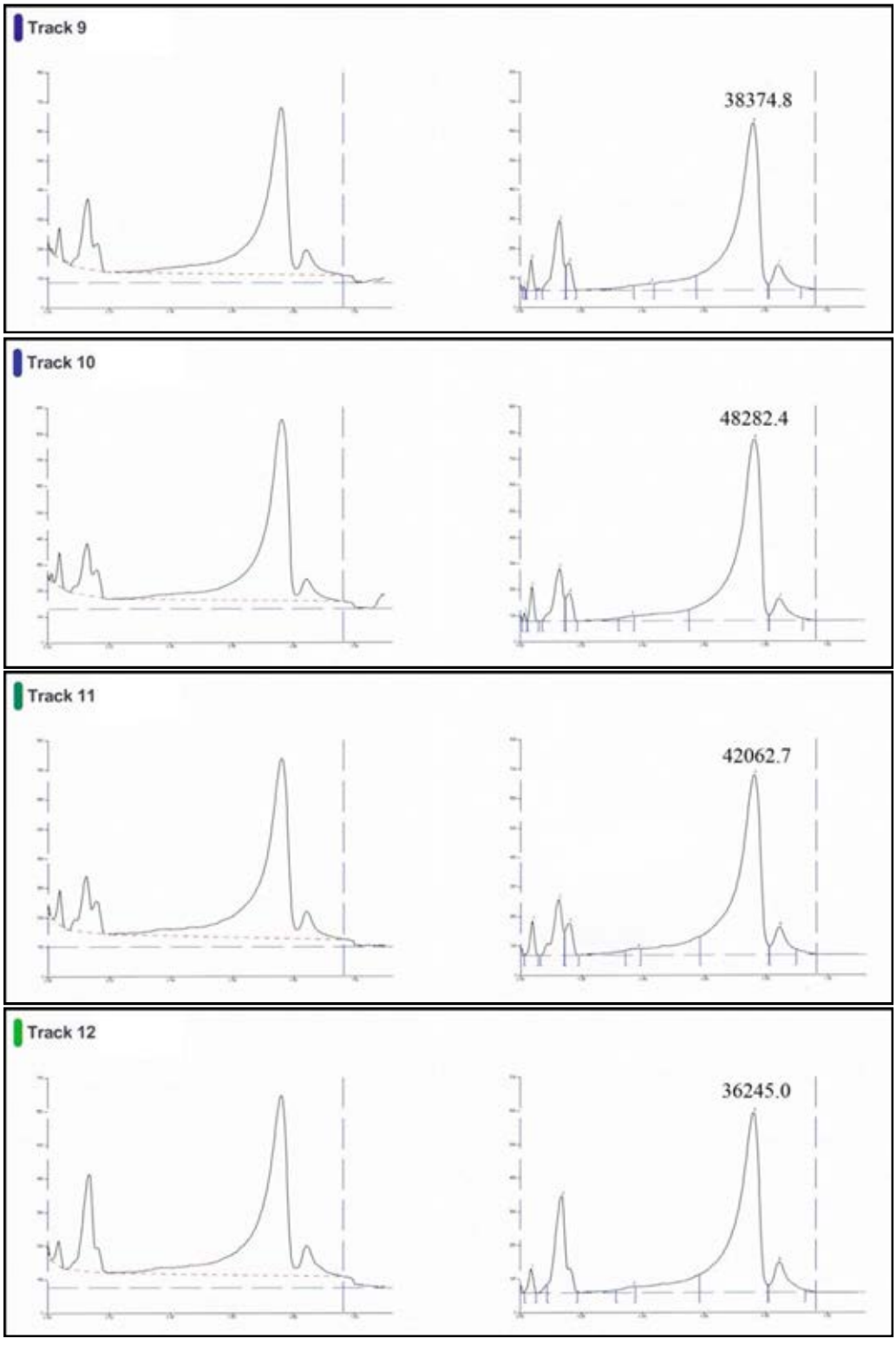



**Figure 31. (Continue)** TLC-densitometric chromatogram (Sample Plate 2); Track 9 was sample from Buri Ram 1 1.0 mg/ml, Track 10 was sample from Buri Ram 2 1.0 mg/ml, Track 11 was sample from Ranong 1.0 mg/ml, Track 12 was sample from Suphan Buri 1.0 mg/ml

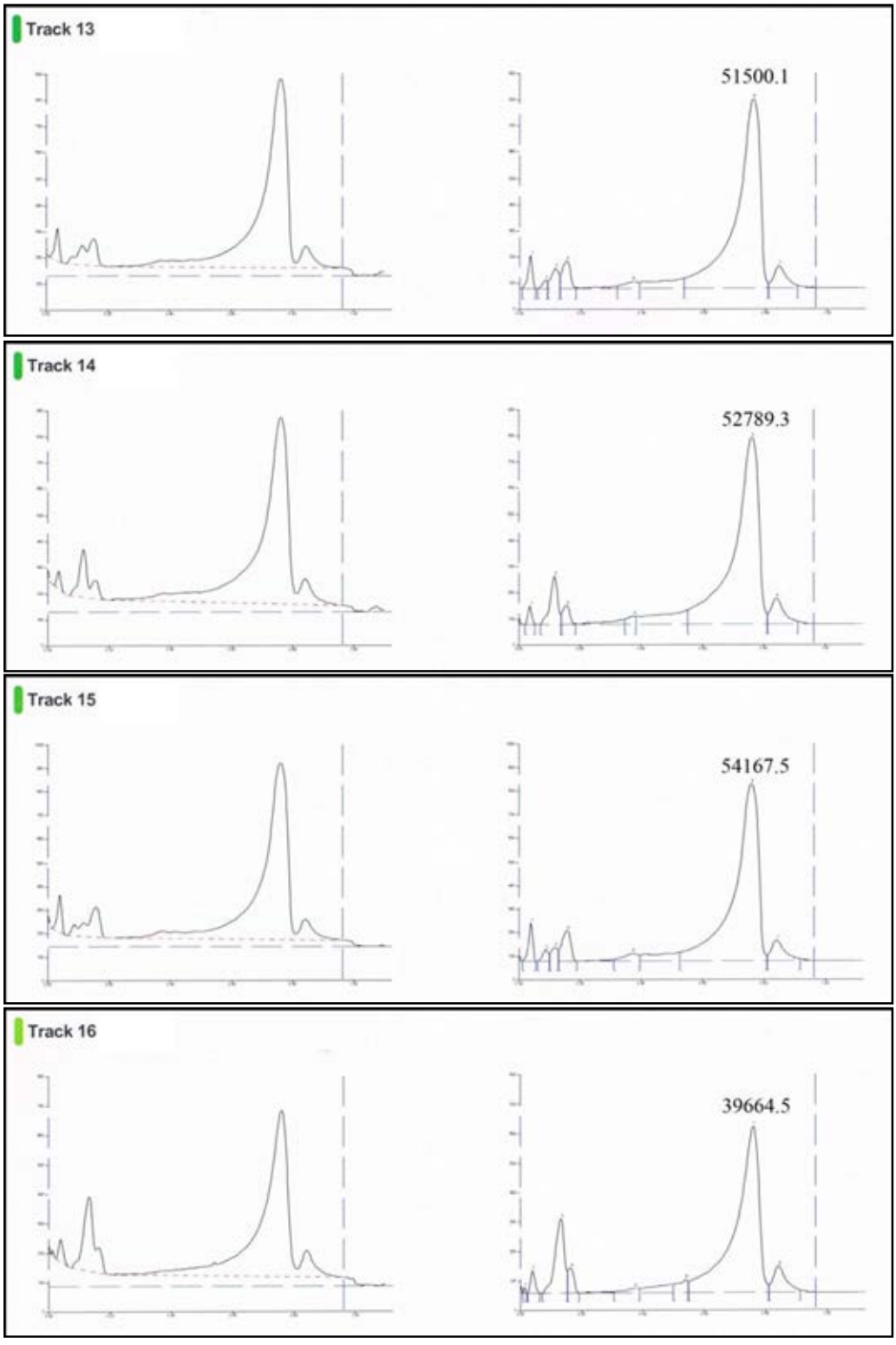



**Figure 31. (Continue)** TLC-densitometric chromatogram (Sample Plate 2); Track 13 was sample from Phrae 1.0 mg/ml, Track 14 was sample from Nakhon Sawan 1.0 mg/ml, Track 15 was sample from Udon Thani 1.0 mg/ml, Track 16 was sample from Khon Kaen 1.0 mg/ml

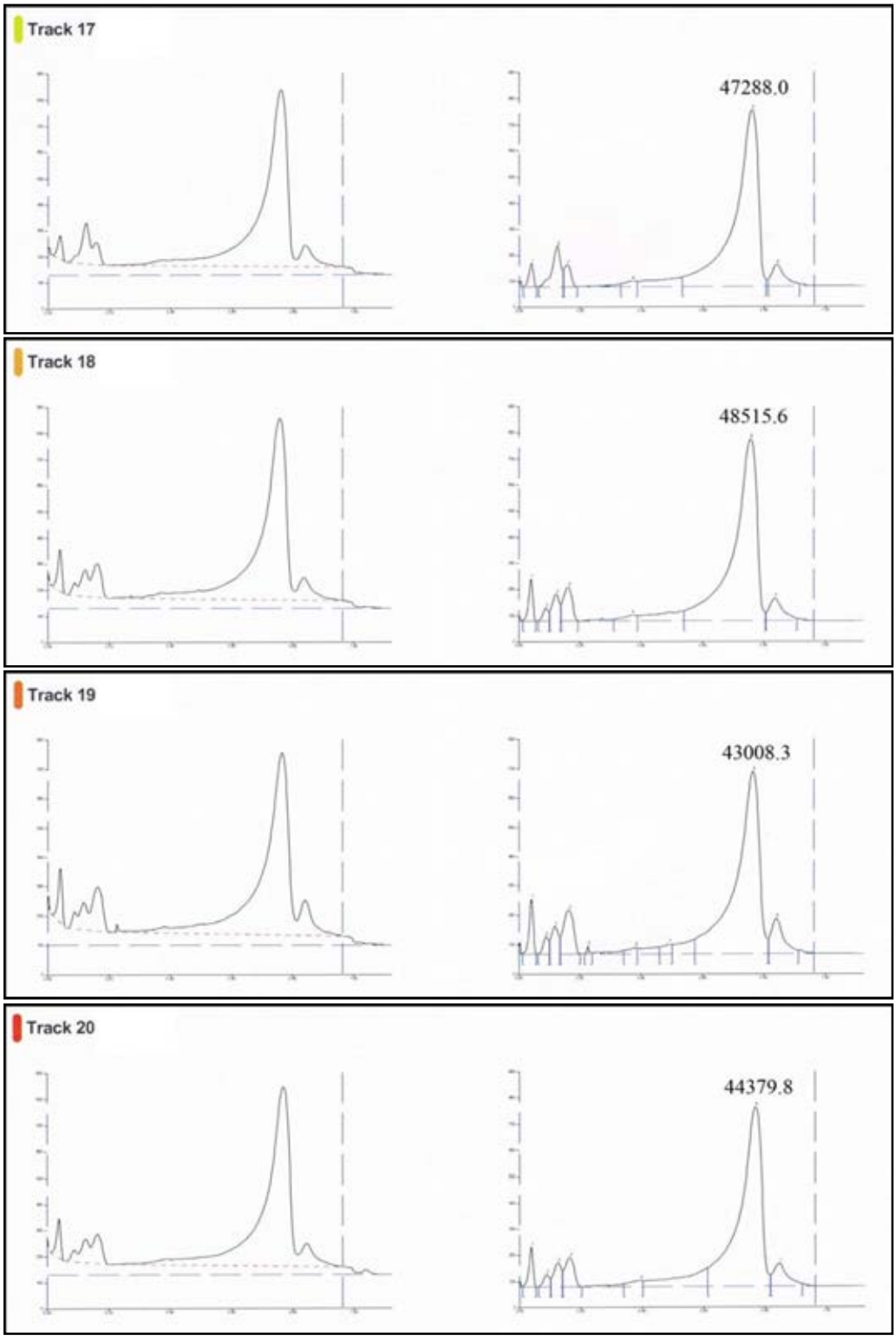



**Figure 31. (Continue)** TLC-densitometric chromatogram (Sample Plate 2); Track 17 was sample from Krabi 1.0 mg/ml, Track 18 was sample from Lop Buri 1.0 mg/ml, Track 19 was sample from Chiang Rai 1.0 mg/ml, Track 20 was sample from Uttaradit 1.0 mg/ml

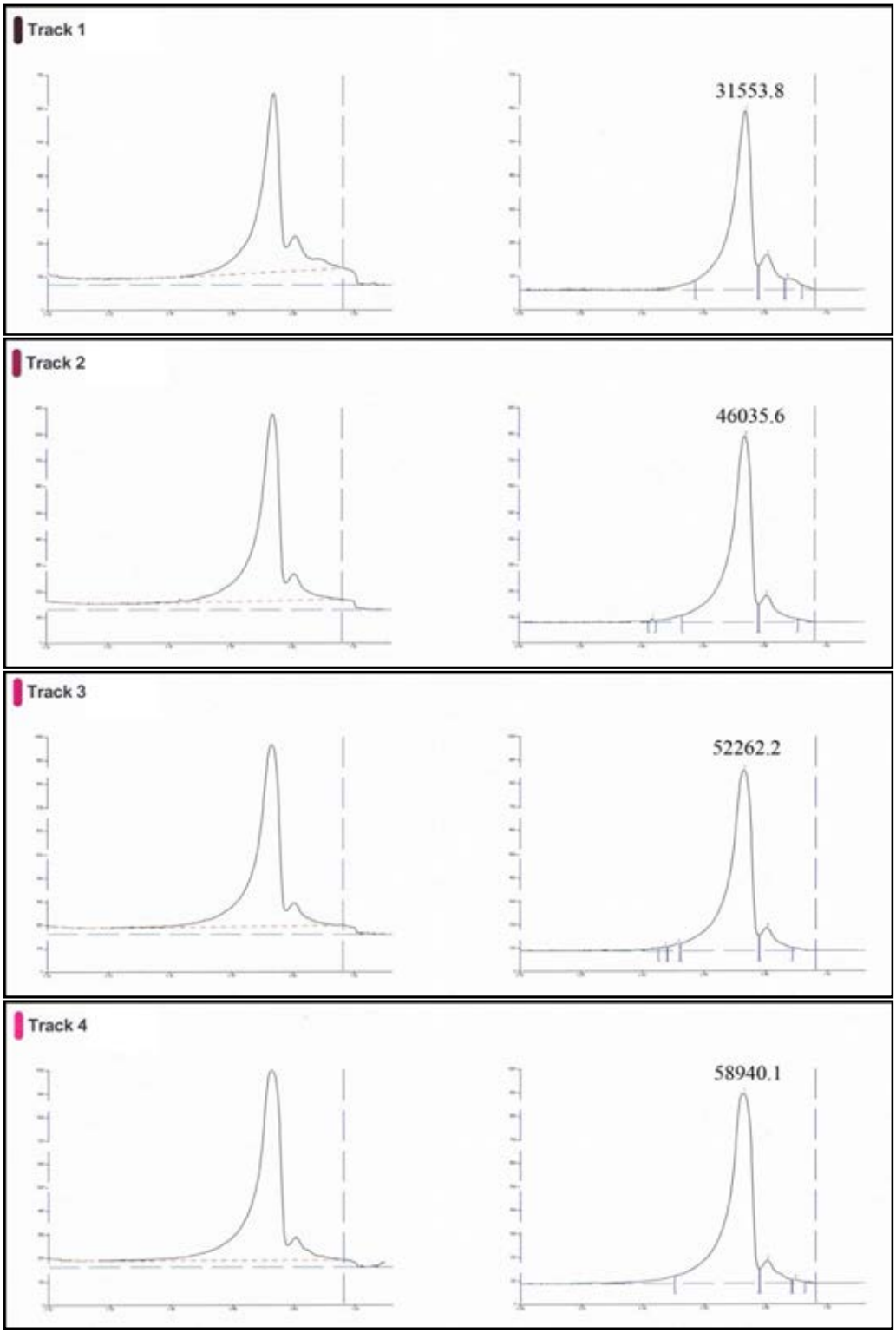



**Figure 32.** TLC-densitometric chromatogram (Sample Plate 3); Track 1 was usnic acid 0.2 mg/ml, Track 2 was usnic acid 0.4 mg/ml, Track 3 was usnic acid 0.6 mg/ml, Track 4 was usnic acid 0.8 mg/ml

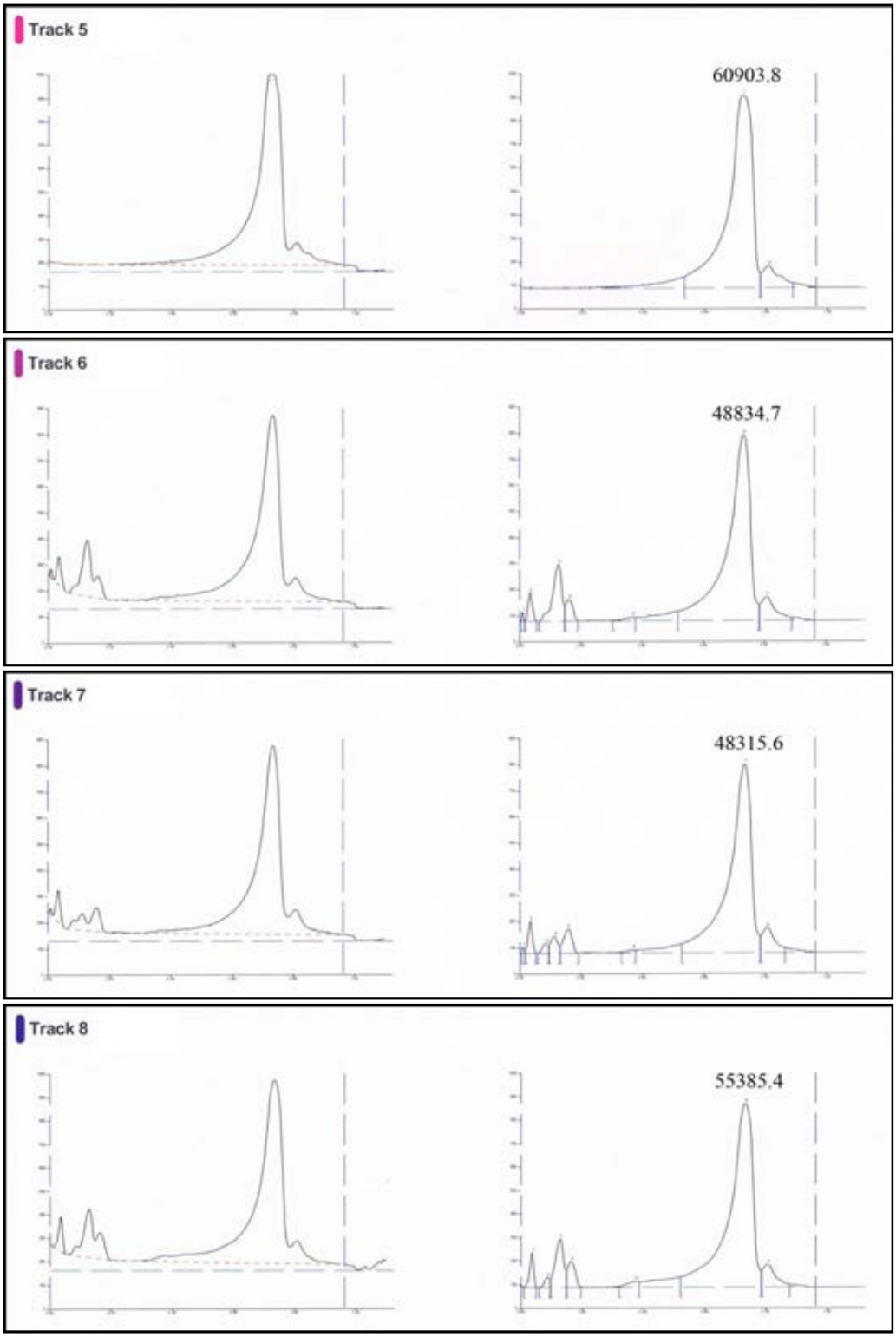



**Figure 32. (Continue)** TLC-densitometric chromatogram (Sample Plate 3); Track 5 was usnic acid 1.0 mg/ml, Track 6 was sample from Phichit 1.0 mg/ml, Track 7 was sample from Bangkok 1 1.0 mg/ml, Track 8 was sample from Bangkok 2 1.0 mg/ml

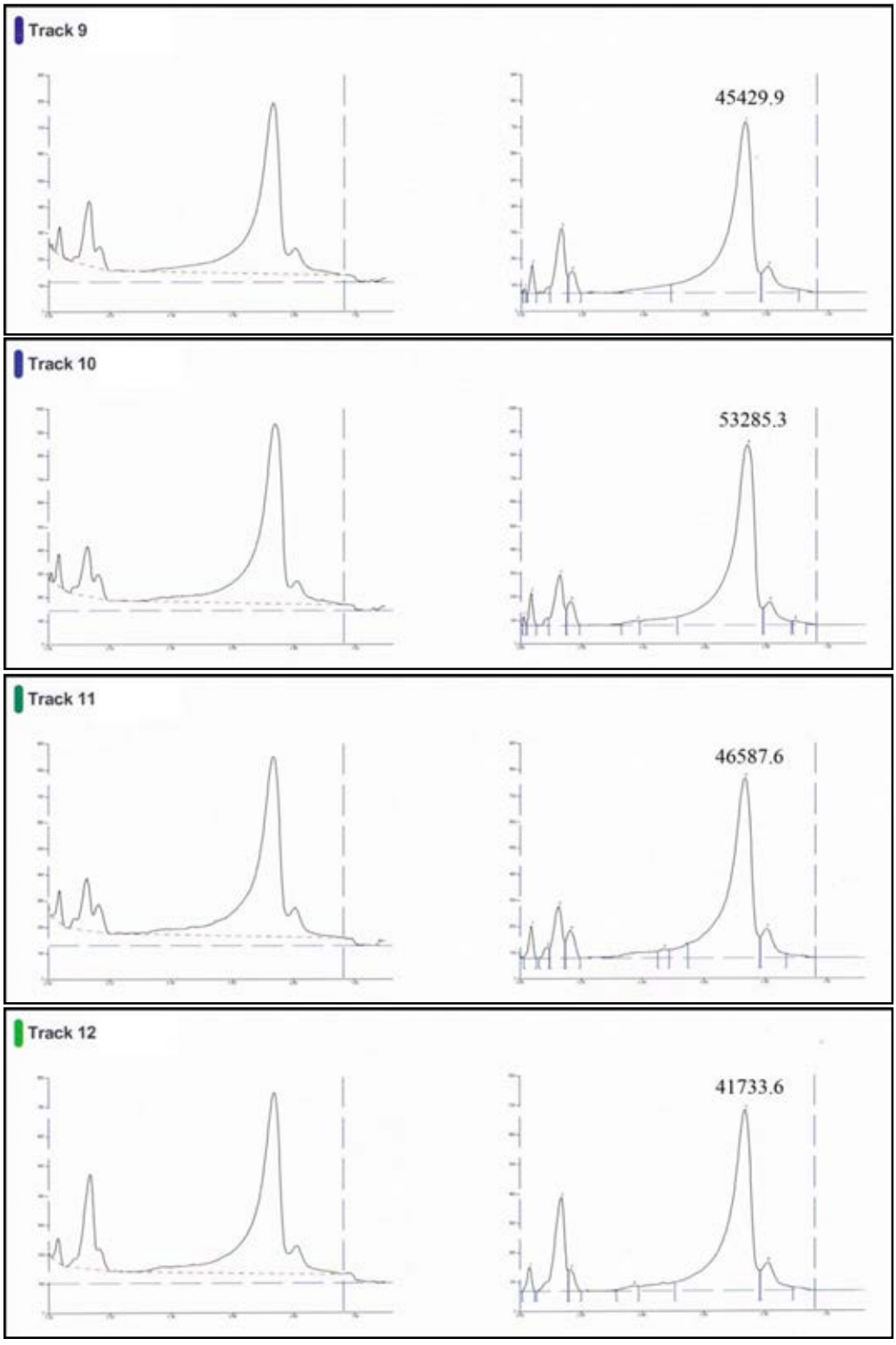



**Figure 32. (Continue)** TLC-densitometric chromatogram (Sample Plate 3); Track 9 was sample from Buri Ram 1 1.0 mg/ml, Track 10 was sample from Buri Ram 2 1.0 mg/ml, Track 11 was sample from Ranong 1.0 mg/ml, Track 12 was sample from Suphan Buri 1.0 mg/ml

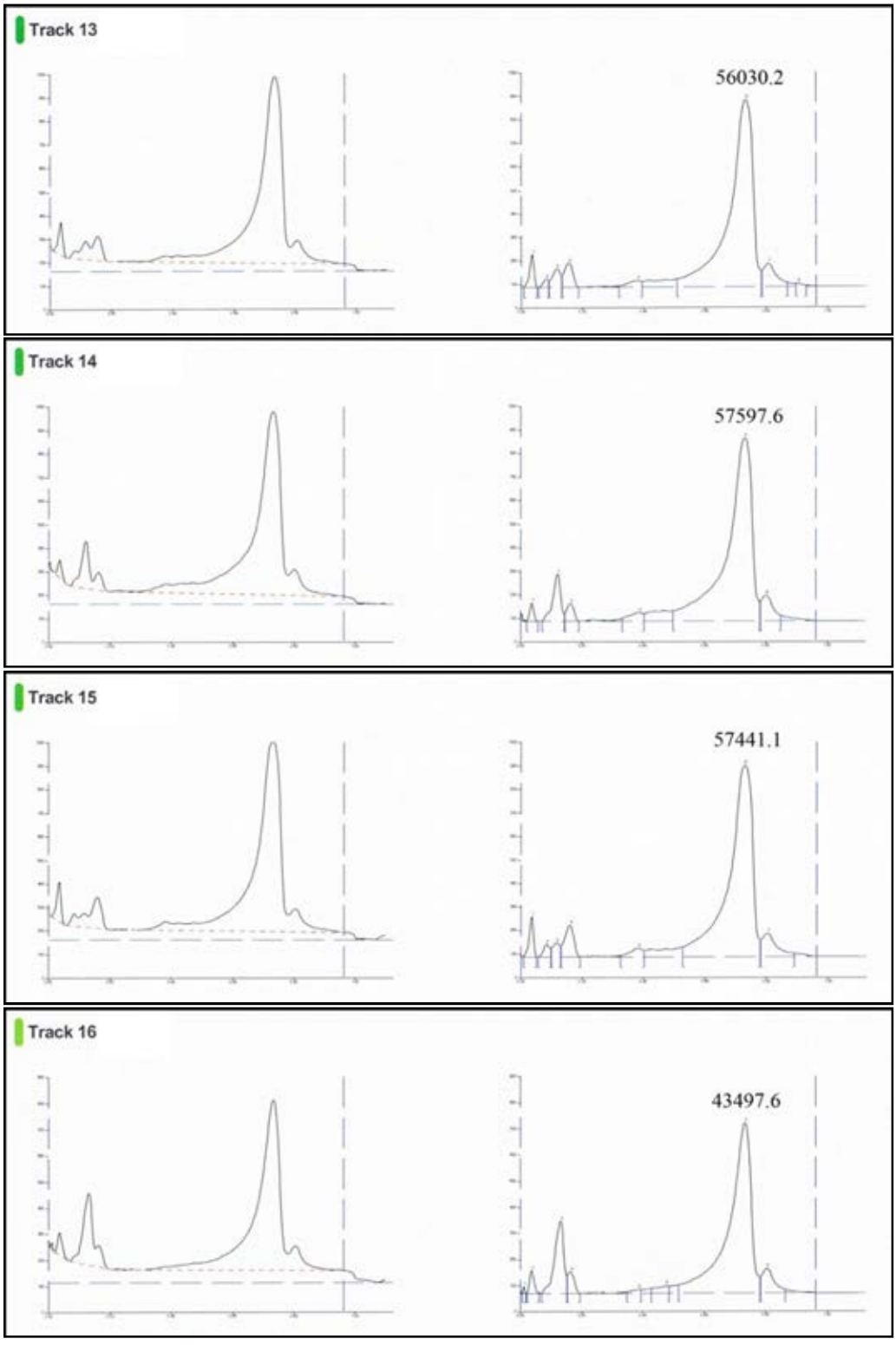



**Figure 32. (Continue)** TLC-densitometric chromatogram (Sample Plate 3); Track 13 was sample from Phrae 1.0 mg/ml, Track 14 was sample from Nakhon Sawan 1.0 mg/ml, Track 15 was sample from Udon Thani 1.0 mg/ml, Track 16 was sample from Khon Kaen 1.0 mg/ml

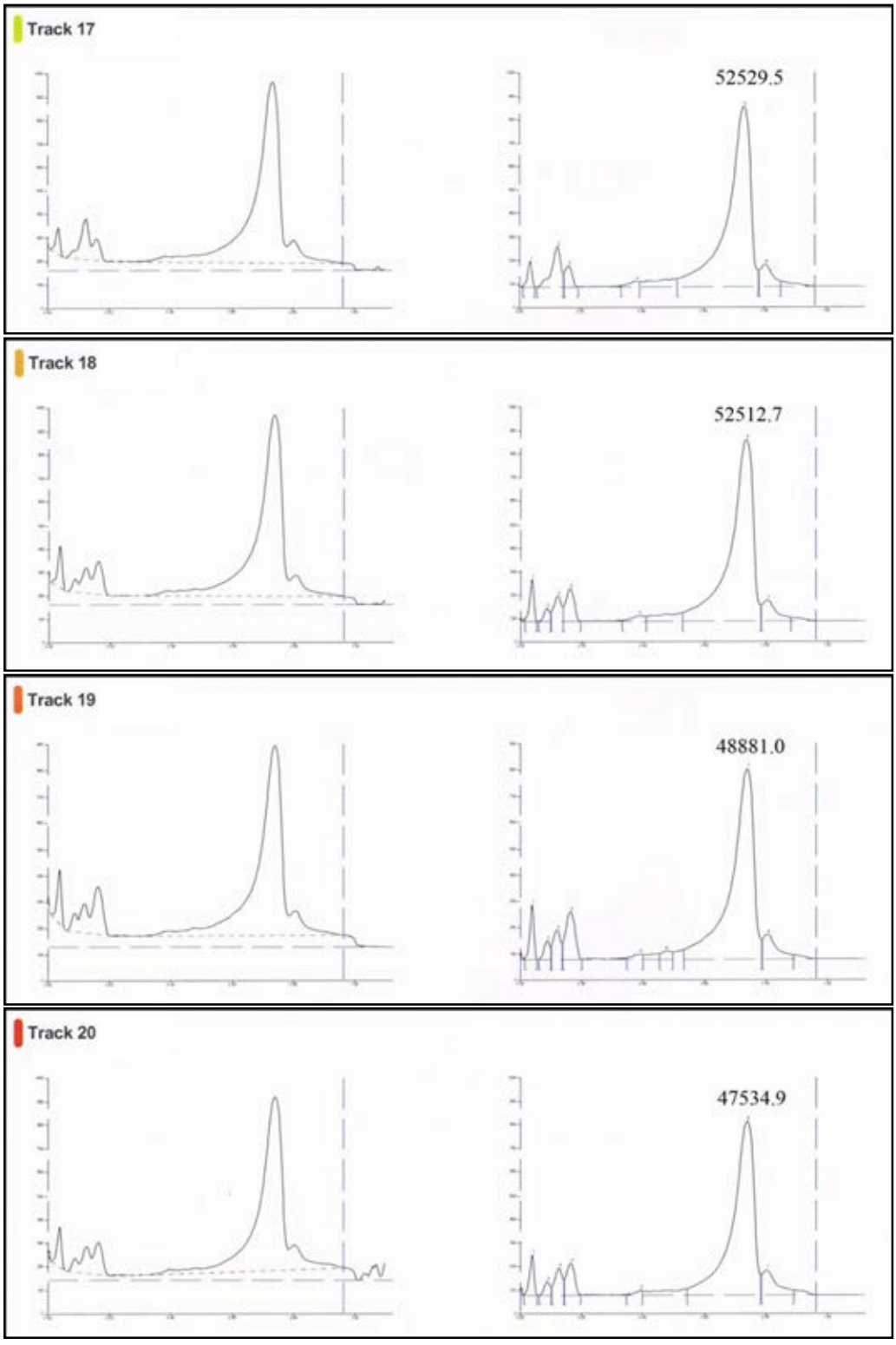
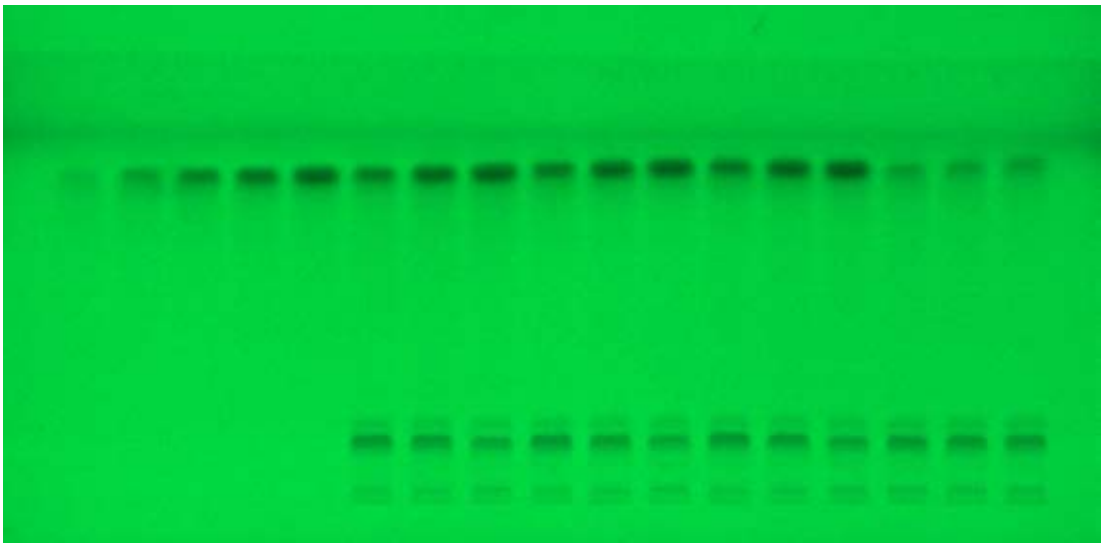
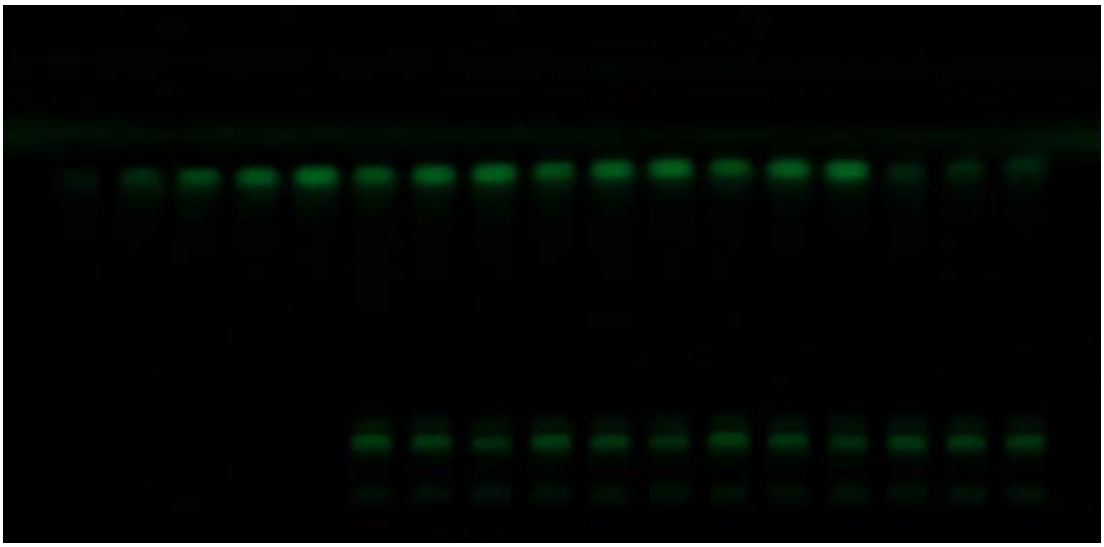



**Figure 32. (Continue)** TLC-densitometric chromatogram (Sample Plate 3); Track 17 was sample from Krabi 1.0 mg/ml, Track 18 was sample from Lop Buri 1.0 mg/ml, Track 19 was sample from Chiang Rai 1.0 mg/ml, Track 20 was sample from Uttaradit 1.0 mg/ml

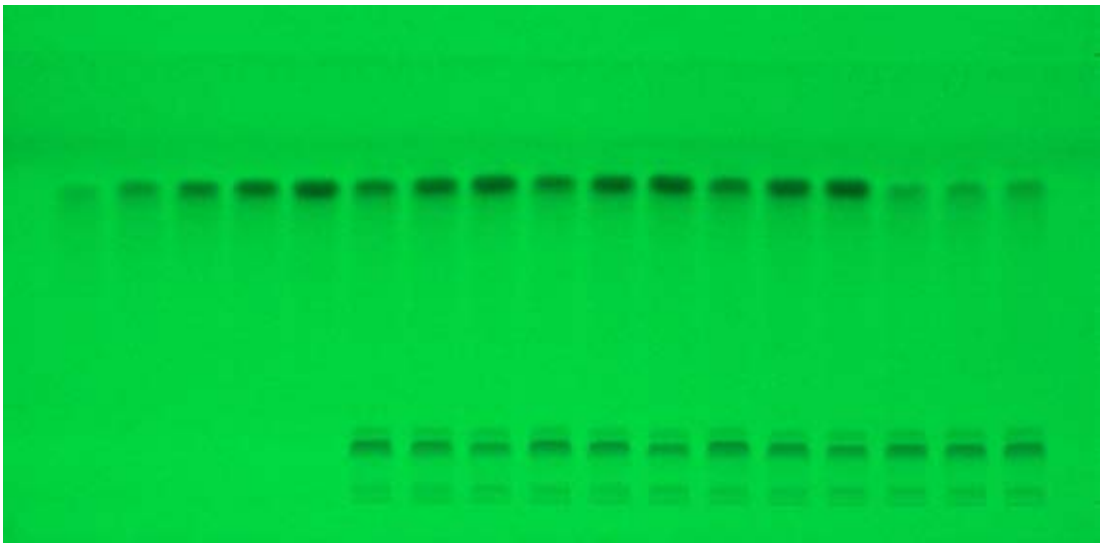


(A)

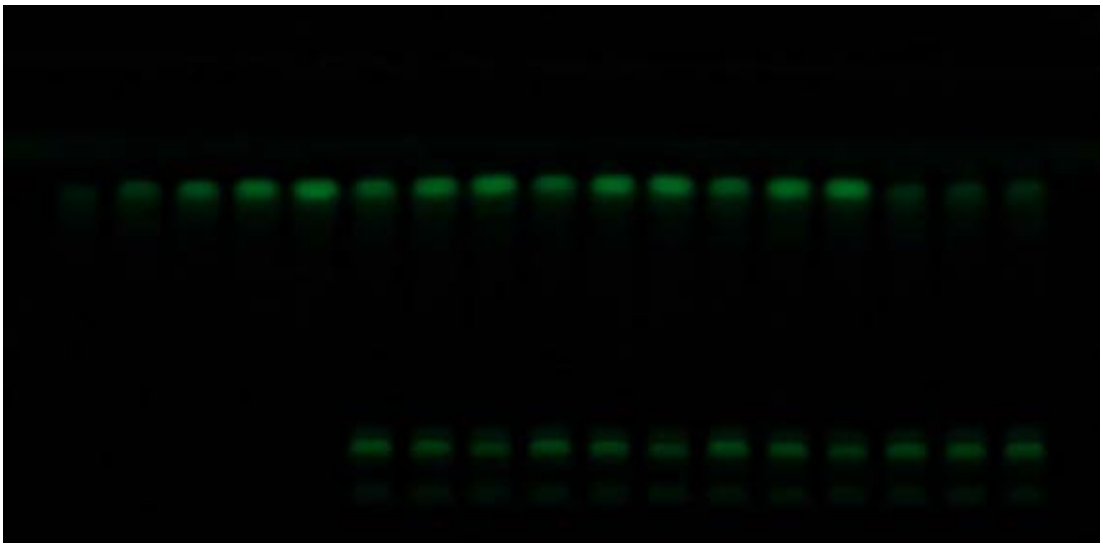


(B)

**Figure 33.** The TLC plates (Accuracy) developed with chloroform and methanol (9:1) visual under 254 nm wavelength original image (A), with subtract background by ImageJ software (B)

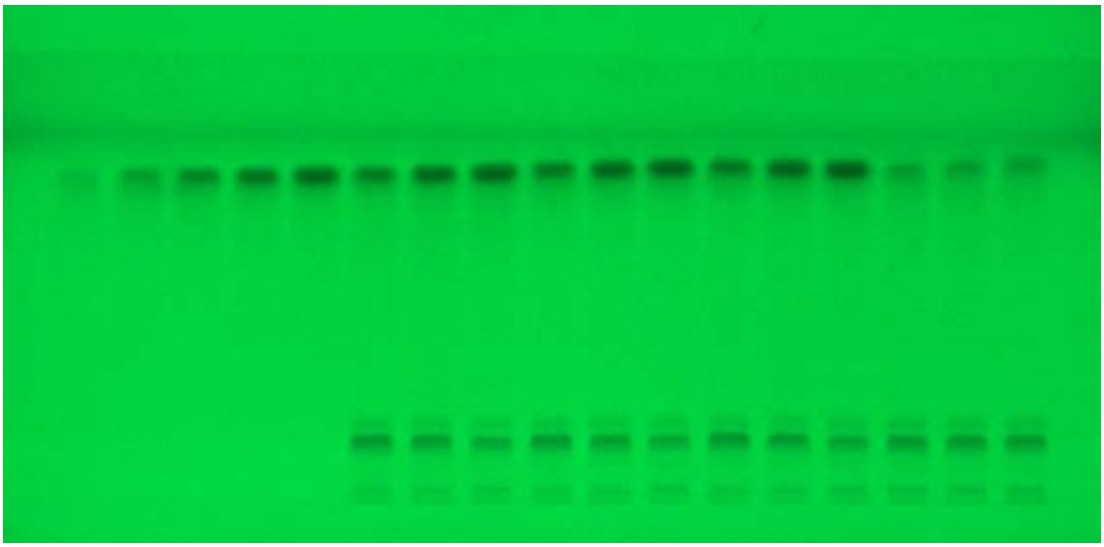


(A)

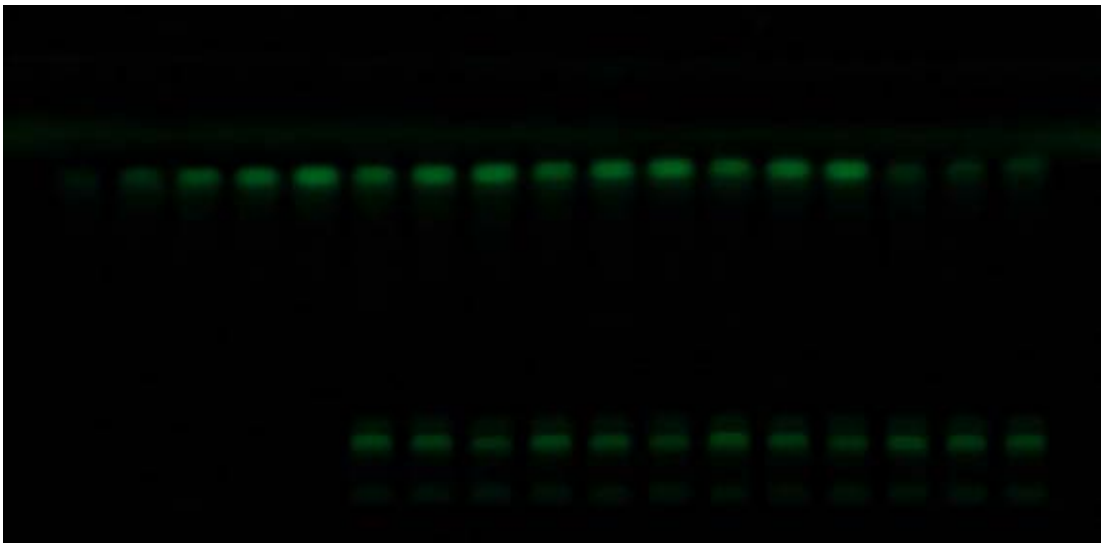


(B)

**Figure 34.** The TLC plates (Precision No.1) developed with chloroform and methanol (9:1) visual under 254 nm wavelength original image (A), with subtract background by ImageJ software (B)

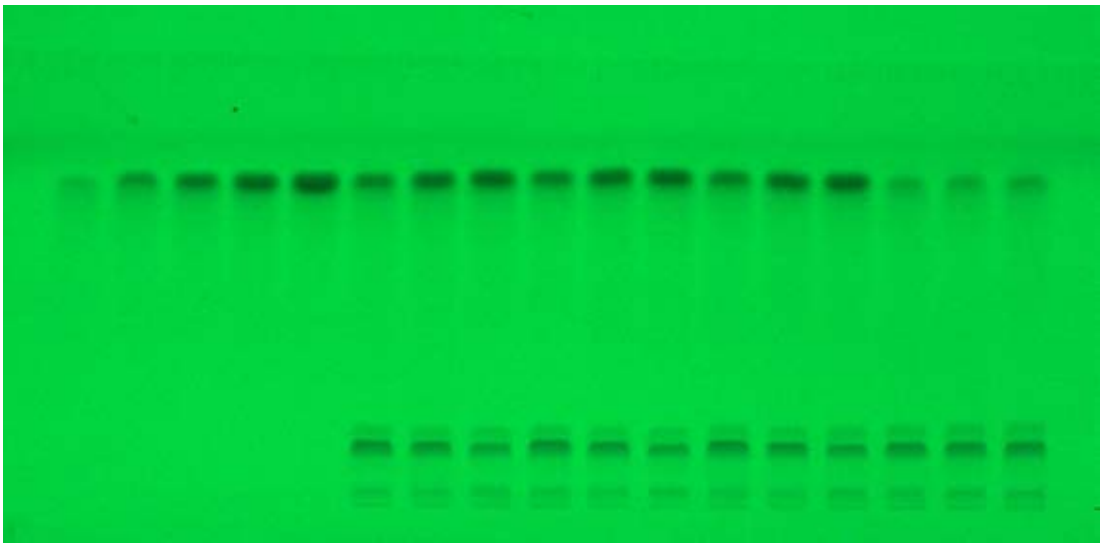


(A)

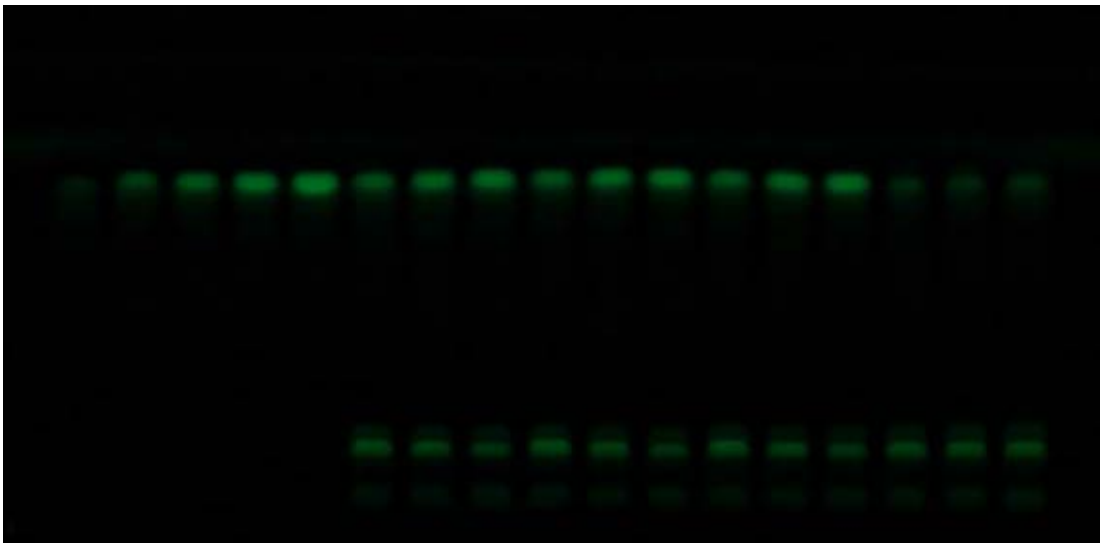


(B)

**Figure 35.** The TLC plates (Precision No.2) developed with chloroform and methanol (9:1) visual under 254 nm wavelength original image (A), with subtract background by ImageJ software (B)

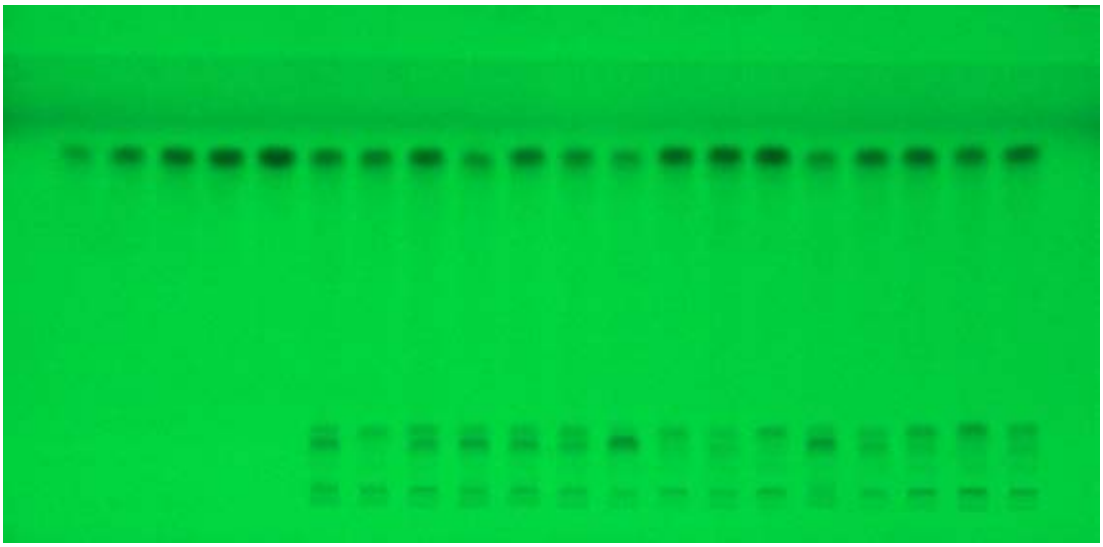


(A)

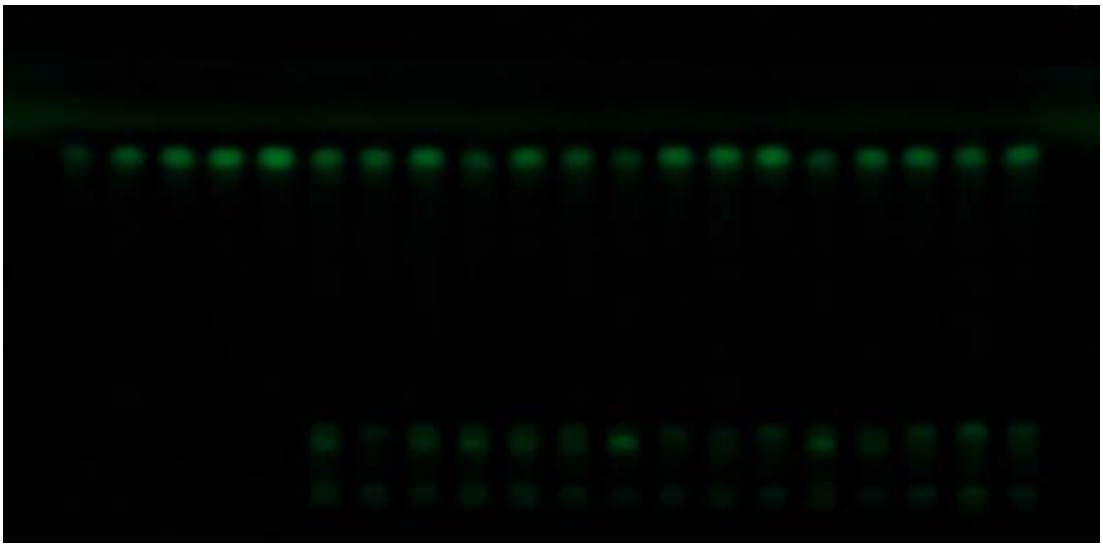


(B)

**Figure 36.** The TLC plates (Precision No.3) developed with chloroform and methanol (9:1) visual under 254 nm wavelength original image (A), with subtract background by ImageJ software (B)

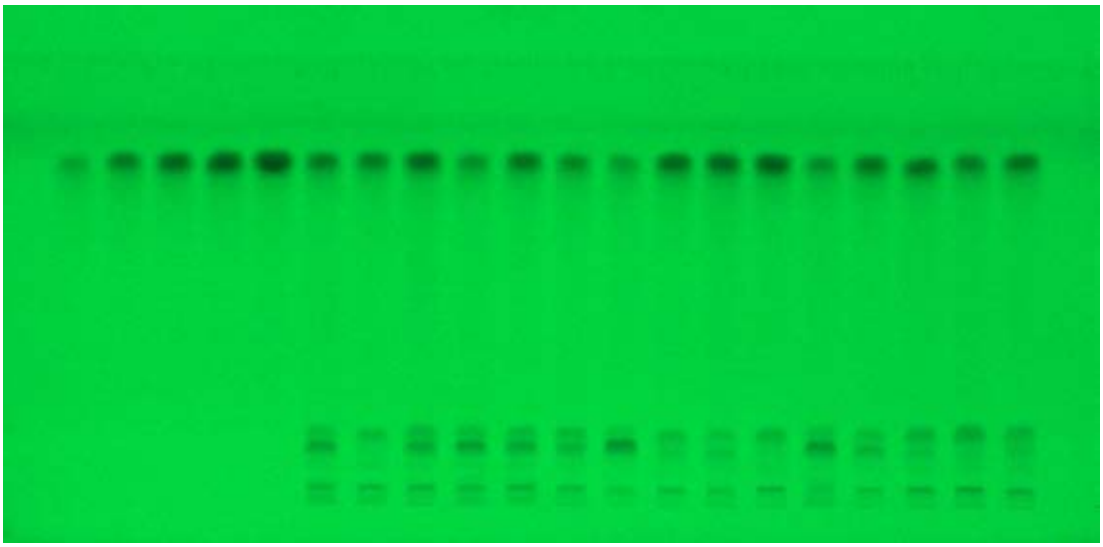


(A)

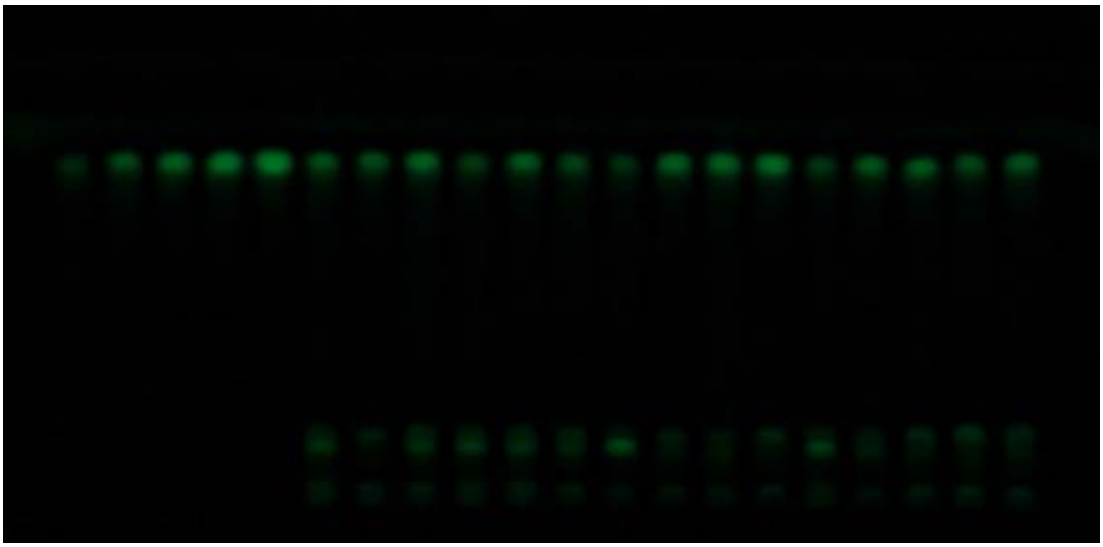


(B)

**Figure 37.** The TLC plates (Sample Plate 1) developed with chloroform and methanol (9:1) visual under 254 nm wavelength original image (A), with subtract background by ImageJ software (B)

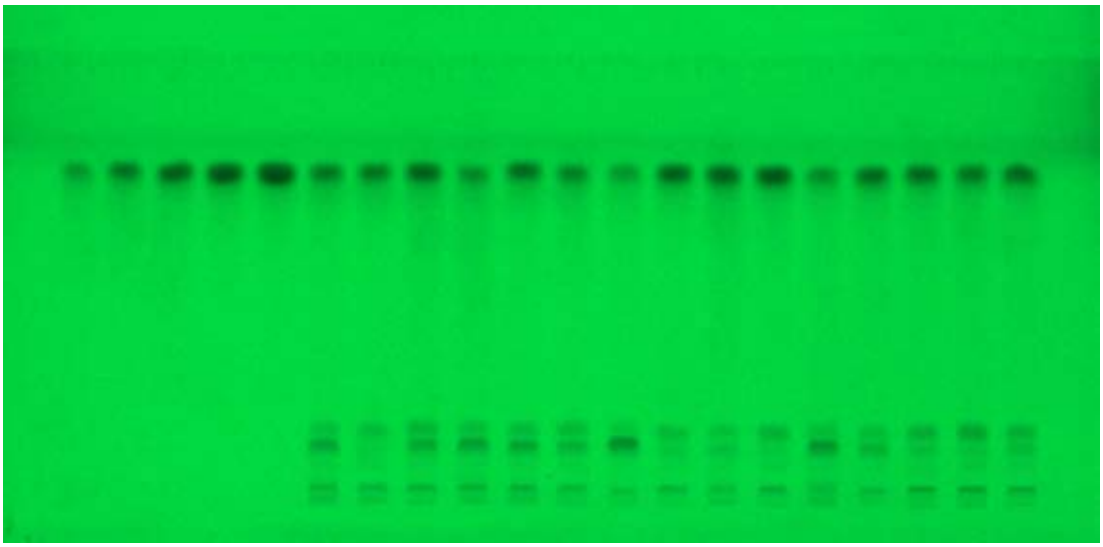


(A)

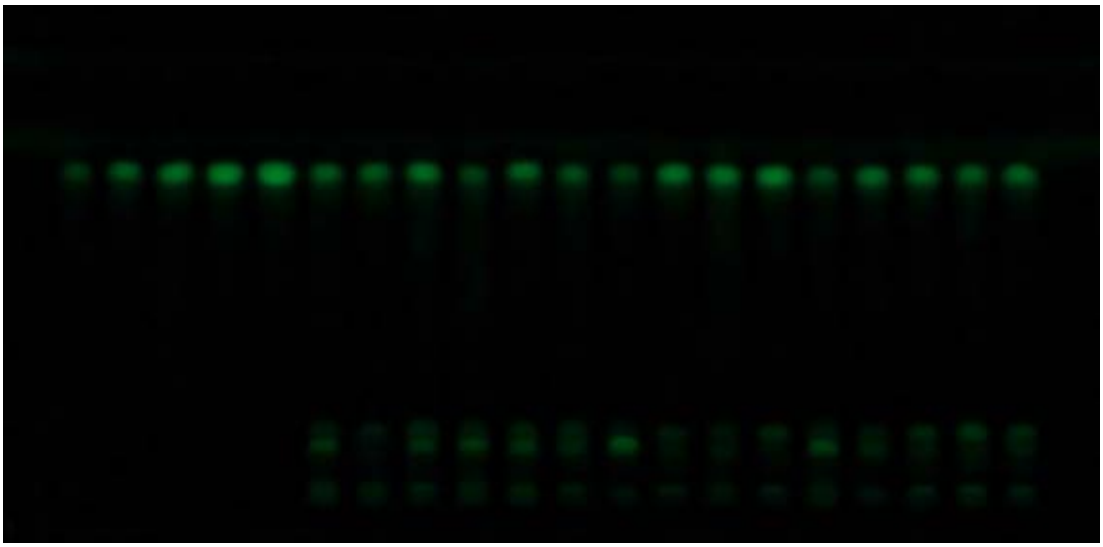


(B)

**Figure 38.** The TLC plates (Sample Plate 2) developed with chloroform and methanol (9:1) visual under 254 nm wavelength original image (A), with subtract background by ImageJ software (B)



(A)



(B)

**Figure 39.** The TLC plates (Sample Plate 3) developed with chloroform and methanol (9:1) visual under 254 nm wavelength original image (A), with subtract background by ImageJ software (B)

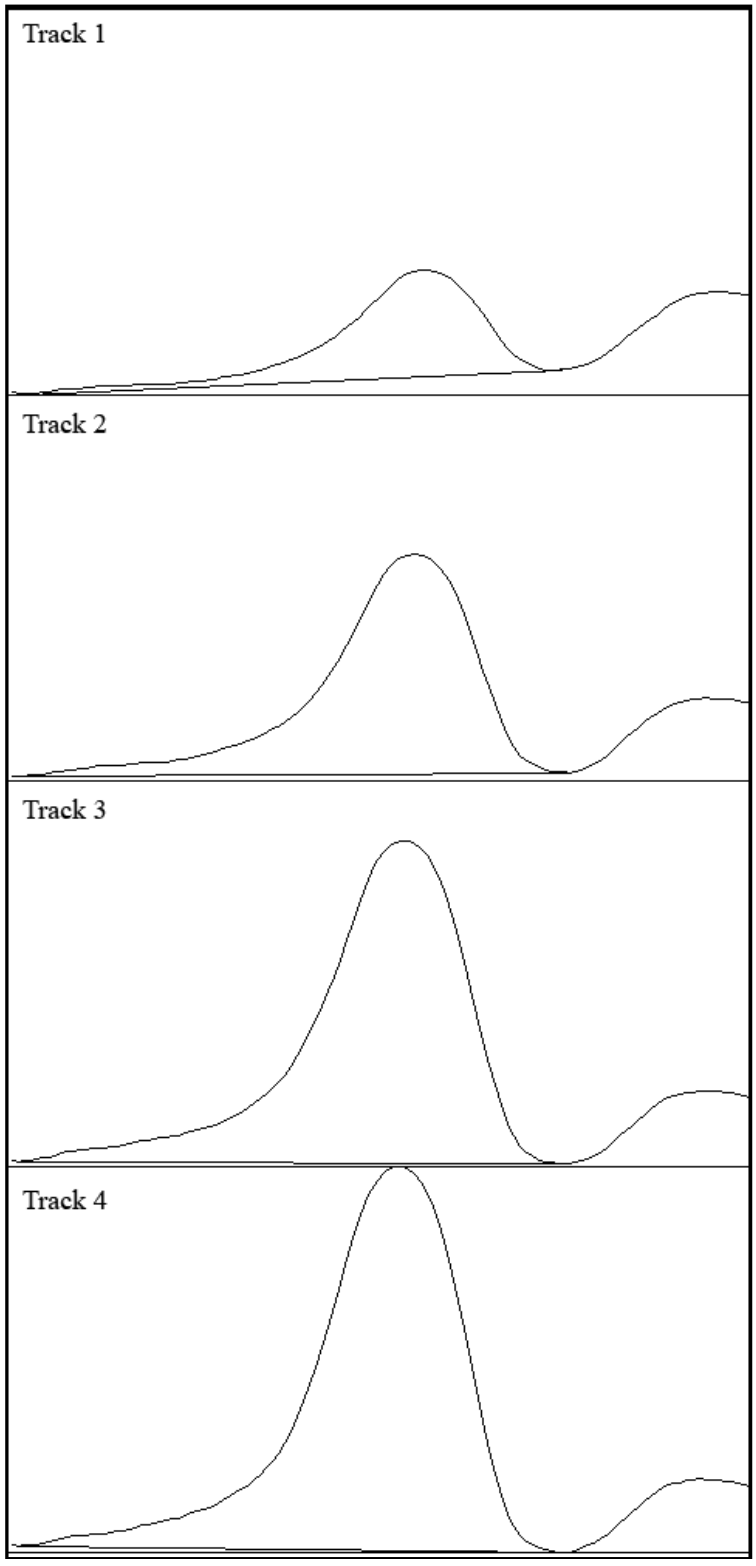



**Figure 40.** TLC image analysis chromatogram (ImageJ software); Track 1 was usnic acid 0.2 mg/ml, Track 2 was usnic acid 0.4 mg/ml, Track 3 was usnic acid 0.6 mg/ml, Track 4 was usnic acid 0.8 mg/ml

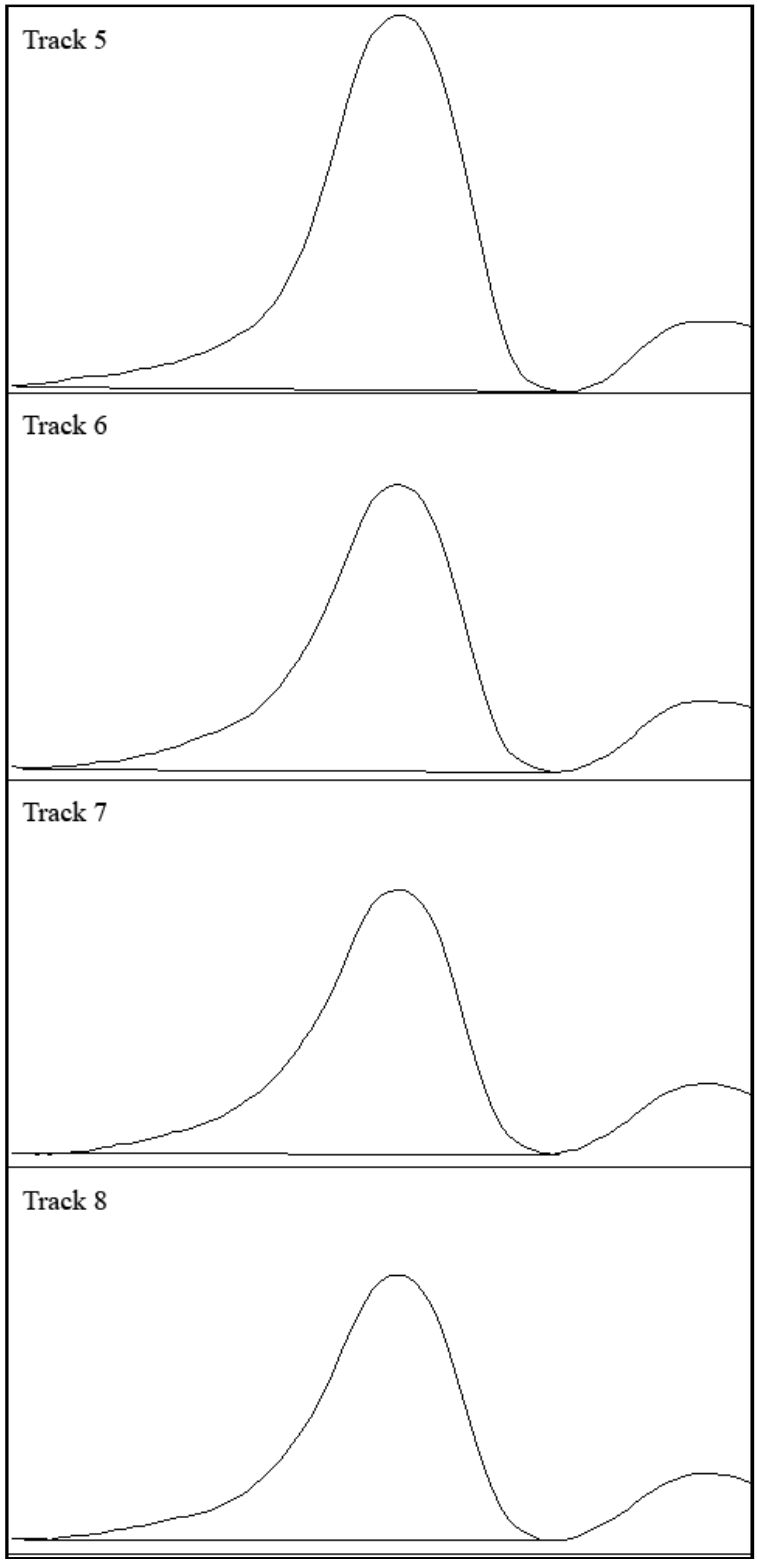



**Figure 40. (Continue)** TLC image analysis chromatogram (ImageJ software); Track 5 was usnic acid 1.0 mg/ml, Track 6 was sample from Phichit 1.0 mg/ml, Track 7 was sample from Bangkok 1 1.0 mg/ml, Track 8 was sample from Bangkok 2 1.0 mg/ml

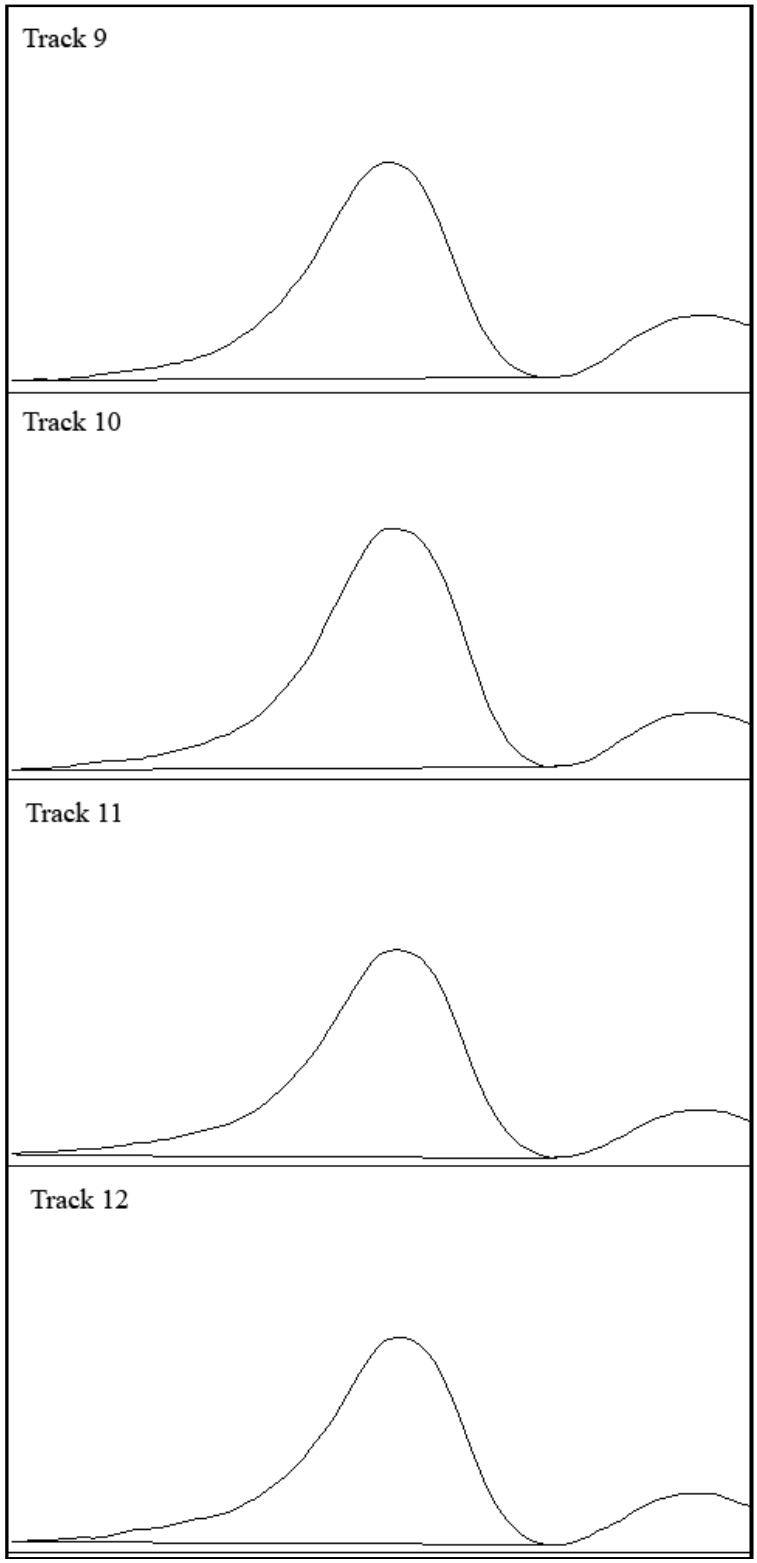



**Figure 40. (Continue)** TLC image analysis chromatogram (ImageJ software); Track 9 was sample from Buri Ram 1 1.0 mg/ml, Track 10 was sample from Buri Ram 2 1.0 mg/ml, Track 11 was sample from Ranong 1.0 mg/ml, Track 12 was sample from Suphan Buri 1.0 mg/ml

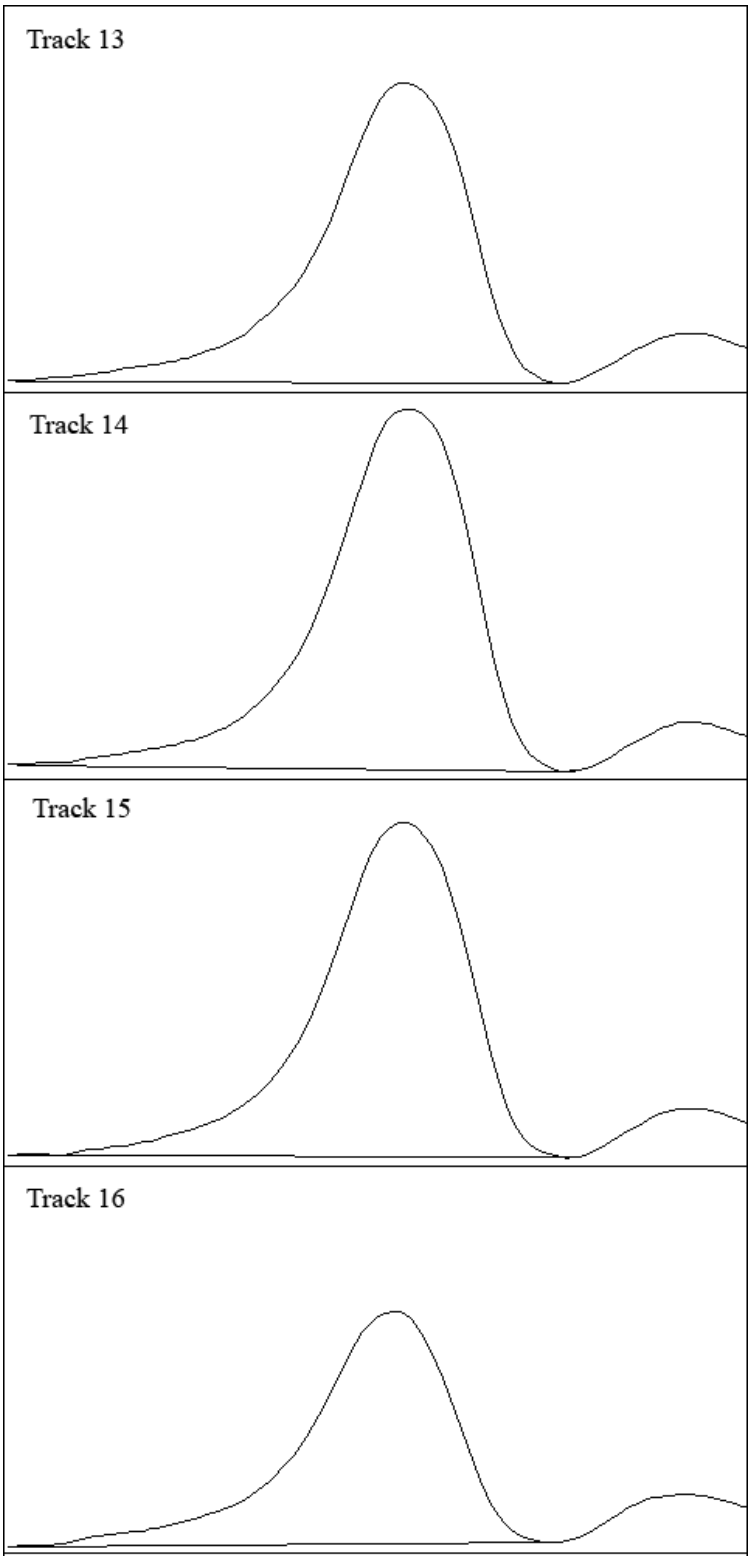



**Figure 40. (Continue)** TLC image analysis chromatogram (ImageJ software); Track 13 was sample from Phrae 1.0 mg/ml, Track 14 was sample from Nakhon Sawan 1.0 mg/ml, Track 15 was sample from Udon Thani 1.0 mg/ml, Track 16 was sample from Khon Kaen 1.0 mg/ml

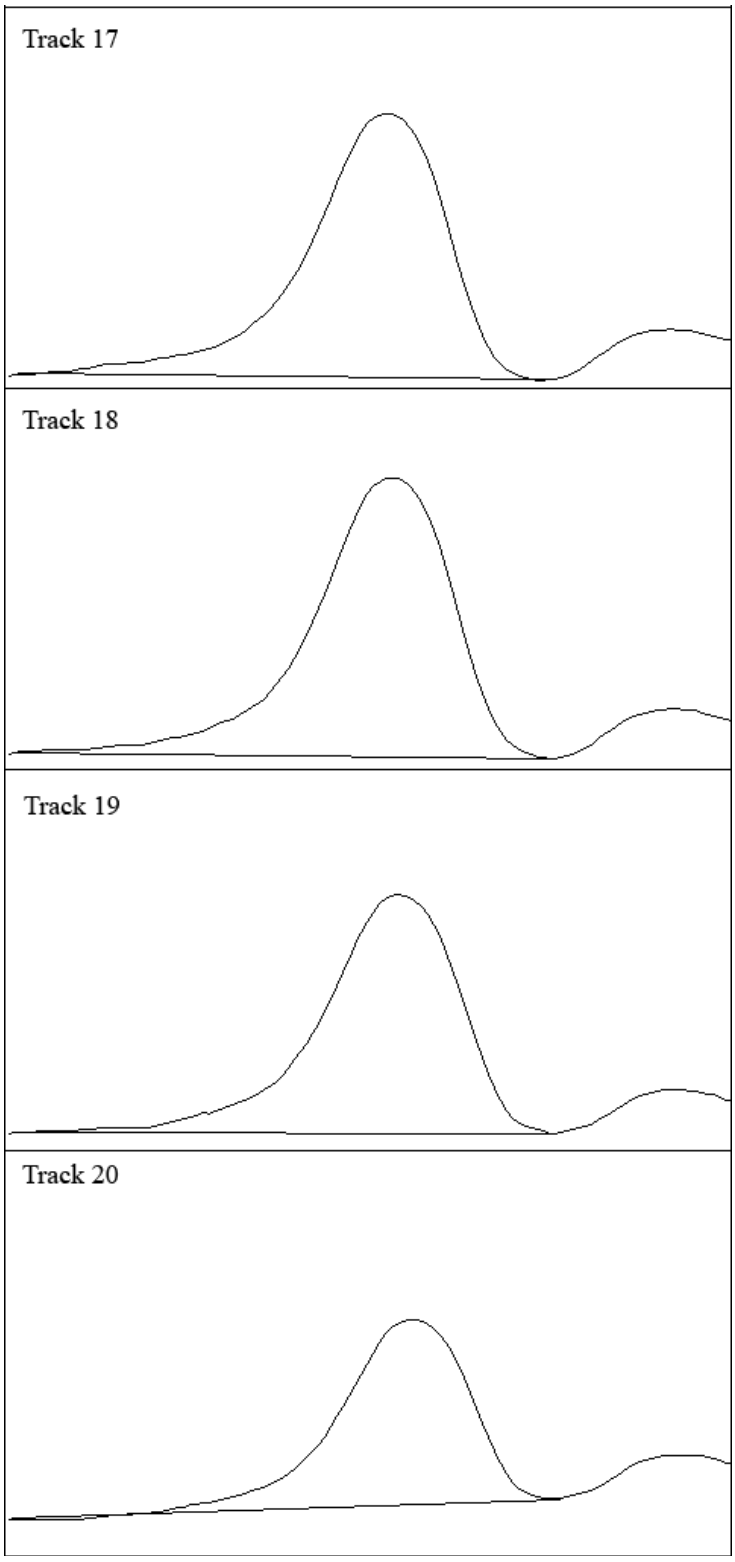
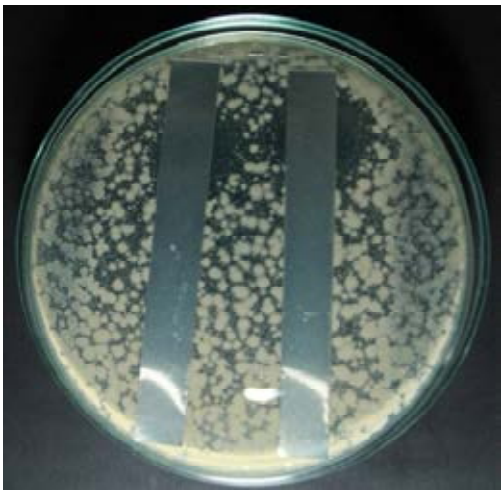
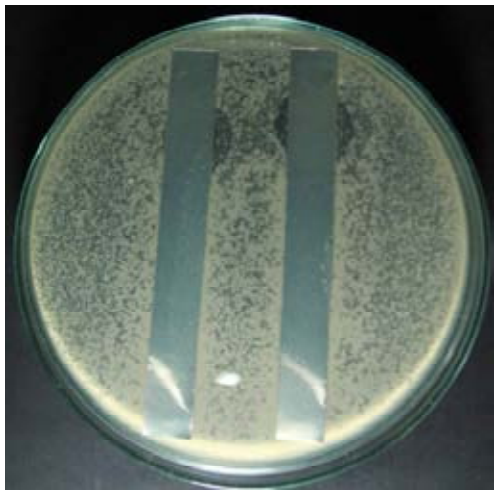


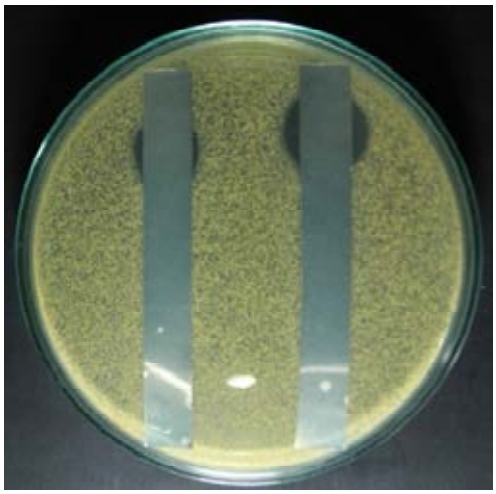

**Figure 40. (Continue)** TLC image analysis chromatogram (ImageJ software); Track 17 was sample from Krabi 1.0 mg/ml, Track 18 was sample from Lop Buri 1.0 mg/ml, Track 19 was sample from Chiang Rai 1.0 mg/ml, Track 20 was sample from Uttaradit 1.0 mg/ml



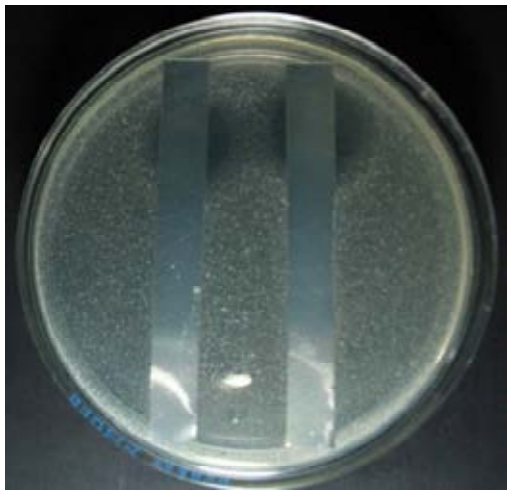
*Basillus cereus*



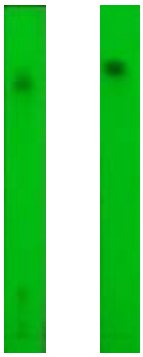
*Bacillus subtilis*



*Micrococcus luteus*

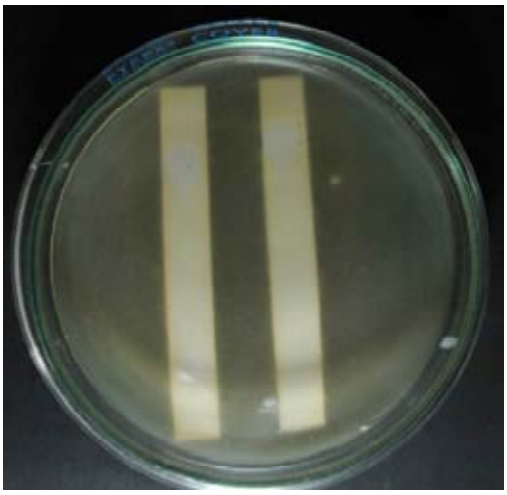


*Staphylococcus epidermidis*

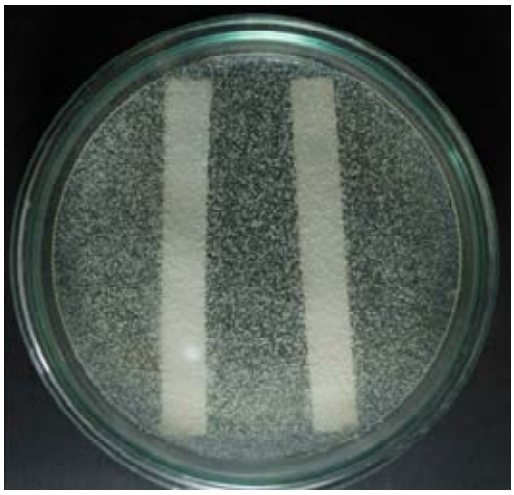


Left – benzene extract, Right – usnic acid

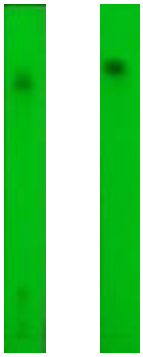
**Figure 41.** Zone of inhibition by bioautography, left - benzene extract, right - usnic acid



*Staphylococcus aureus*



*Candida albicans*



Left – benzene extract, Right – usnic acid

**Figure 41. (Continue)** Zone of inhibition by bioautography, left - benzene extract, right - usnic acid

# VITAE

Mr. Chayanon Chaowuttikul was born on July 16, 1988 in Trang, Thailand. He received his Bachelor's degree in Science (Thai Traditional Medicine) from Prince of Songkla University, Thailand in 2011. He attended to study Master of Science Program in Public Health Sciences in 2012 at College of Public Health Sciences, Chulalongkorn University, Thailand.

**Publication**

Chaowuttikul, C., Palanuvej, C., and Ruangrungsi, N. Pharmacognostic specification, usnic acid content and antibacterial activity of *Usnea siamensis*. Proceedings of the 39th Congress on Science and Technology of Thailand. Bangkok, 2013.