PHARMACOGNOSTIC EVALUATION AND CHRYSAZIN QUANTITATION OF *XYRIS INDICA* FLOWERING HEADS

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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

College of Public Health Sciences

Chulalongkorn University

Academic Year 2013

การประเมินข้อกำหนดทางเภสัชเวทและปริมาณสารคริสะซินในช่อดอกกระถินทุ่ง

นางสาวชวนชม ขุนเอียด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์สาธารณสุข
วิทยาลัยวิทยาศาสตร์สาธารณสุข จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2556

Thesis Title	PHARMACOGNOSTIC EVALUATION AND CHRYSAZIN QUANTITATION OF *XYRIS INDICA* FLOWERING HEADS
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Accepted by the College of Public Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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ชวนชม ขุนเอียด : การประเมินข้อกำหนดทางเภสัชเวทและปริมาณสารคริสะซินในช่อดอกกระถินทุ่ง. (Pharmacognostic Evaluation and Chrysazin Quantitation of *Xyris indica* Flowering heads) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ดร. ชนิดา พลานุเวช, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. นิจศิริ เรืองรังษี, 135 หน้า.

กระถินทุ่ง มีชื่อทางวิทยาศาสตร์ว่า *Xyris indica* L. กระถินทุ่งเป็นเครื่องยาสมุนไพรที่ใช้ในการแพทย์แผนไทย มีสรรพคุณรักษาโรคกลาก อาการท้องผูก และท้องอืด การศึกษานี้มีวัตถุประสงค์เพื่อจัดทำข้อกำหนดทางเภสัชเวทและวิเคราะห์หาปริมาณสารคริสะซินของช่อดอกกระถินทุ่ง จาก 15 แหล่งในประเทศไทย วาดภาพลายเส้นแสดงลักษณะทั้งต้นของกระถินทุ่ง เตรียมเครื่องยาโดยการอบแห้งและบดผง ลักษณะทางมหภาคของเครื่องยามีรูปร่างเป็นช่อดอก มีใบประดับสีน้ำตาลและดอกสีเหลือง รสขมฝาดและกลิ่นเฉพาะ ลักษณะเด่นทางจุลภาคของเครื่องยาคือ กลีบดอก เมล็ด ละอองเรณู และเกสรตัวผู้ที่เป็นหมัน การศึกษาเอกลักษณ์ทางเคมี-ฟิสิกส์พบว่า มีปริมาณน้ำหนักที่หายไปเมื่อทำให้แห้ง ปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด ปริมาณสารสกัดด้วยน้ำ ปริมาณสารสกัดด้วยเอทานอล และปริมาณน้ำ เท่ากับ 6.899 ± 0.165, 2.497 ± 0.033, 0.409 ± 0.027 , 6.592 ± 0.474, 4.030 ± 0.486 และ 11.121 ± 1.132 โดยน้ำหนัก ตามลำดับ การศึกษาด้วยเทคนิคทางทินเลเยอร์โครมาโตกราฟีโดยมีแผ่นซิลิกาเจลเป็นวัฏภาคคงที่ และใช้ตัวทำละลายปิโตรเลียมอีเทอร์และเอทิลแอซิเทต (8:1) เป็นเฟสเคลื่อนที่ ตรวจสอบภายใต้แสงอัลตราไวโอเลต (254 นาโนเมตร และ 365 นาโนเมตร) และภายใต้แสงธรรมชาติหลังจากย้อมด้วยสารละลาย 5 % โพแทสเซียมไฮดรอกไซด์ พบแถบคริสะซินเรืองแสงสีเหลือง ภายใต้แสงอัลตราไวโอเลต 365 นาโนเมตร ที่ค่า hRf เท่ากับ 59 วิเคราะห์ปริมาณสารคริสะซินในสารสกัดหยาบด้วยเบนซีนจากช่อดอกโดยวิธีทินเลเยอร์โครมาโตกราฟีเดนซิโตเมตรีและการวิเคราะห์เชิงภาพ โดยใช้เฟสเคลื่อนที่ข้างต้น การวิเคราะห์สารคริสะซินโดยวิธีทินเลเยอร์โครมาโตกราฟีเดนซิโตเมตรี มีช่วงความเป็นเส้นตรงระหว่าง 15.0-75.0 ไมโครกรัม/มิลลิกรัม และมีค่าสัมประสิทธิ์สหสัมพันธ์เท่ากับ 0.9997 ค่าเฉลี่ยการคืนกลับระหว่างร้อยละ 90.67-99.16 ระดับความเที่ยงของการวิเคราะห์ ประเมินจากค่าสัมประสิทธิ์ของการกระจาย มีค่าระหว่างร้อยละ 0.76 -14.28 ขีดจำกัดของการตรวจพบและขีดจำกัดของการหาปริมาณ มีค่า 5.48 และ16.62 ไมโครกรัม ระดับความคงทนของวิธีการ ประเมินจากค่าสัมประสิทธิ์ของการกระจายมีค่าร้อยละ 4.44 วิเคราะห์สารคริสะซินโดยวิธีการวิเคราะห์เชิงภาพทางทินเลเยอร์โครมาโตกราฟี มีช่วงความเป็นเส้นตรงระหว่าง 15.0-75.0 ไมโครกรัม/มิลลิกรัม และมีค่าสัมประสิทธิ์สหสัมพันธ์เท่ากับ 0.9985 ค่าเฉลี่ยการคืนกลับระหว่างร้อยละ 91.87-96.00 ระดับความเที่ยงของการวิเคราะห์ ประเมินจากค่าสัมประสิทธิ์ของการกระจาย มีค่าระหว่างร้อยละ 0.78-3.95 ขีดจำกัดของการตรวจพบและขีดจำกัดของการหาปริมาณ มีค่า 5.90 และ 17.89 ไมโครกรัม ระดับความคงทนของวิธีการ ประเมินจากค่าสัมประสิทธิ์ของการกระจายมีค่าร้อยละ 3.60 ปริมาณสารคริสะซินในช่อดอกกระถินทุ่งโดยวิธีทินเลเยอร์โครมาโตกราฟีเดนซิโตเมตรีและการวิเคราะห์เชิงภาพทางทินเลเยอร์โครมาโตกราฟีมีค่าระหว่าง 0.0223 ± 0.0011 และ 0.0219-0.0007 กรัม/ 100 กรัมของช่อดอกแห้ง ตามลำดับ การเปรียบเทียบปริมาณสารคริสะซินระหว่าง 2 วิธี ถูกทดสอบโดยใช้สถิติ paired t-test พบว่าปริมาณสารคริสะซินโดยสองวิธีไม่แตกต่างกัน ศึกษาฤทธิ์ต้านเชื้อจุลชีพของสารสกัดเบนซีนจากช่อดอกและสารมาตรฐานคริสะซินโดยวิธี agar-overlay bioautography พบว่า สารสกัดเบนซีนบนแผ่นทีแอลซีมีฤทธิ์ต้านการเจริญเติบโตของเชื้อแบคทีเรียแกรมบวก 4 ชนิด คือ *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* และ *Micrococcus luteus*. สารคริสะซินในสารสกัดและสารมาตรฐานคริสะซินมีฤทธิ์ต้านการเจริญเติบโตของ *Bacillus subtilis* ผลการศึกษาในครั้งนี้สามารถนำไปใช้จัดทำเป็นข้อกำหนดมาตรฐานของสมุนไพรกระถินทุ่งในประเทศไทยซึ่งเป็นประโยชน์ในการควบคุมคุณภาพและเป็นข้อมูลพื้นฐานของฤทธิ์ต้านจุลชีพของพืชชนิดนี้ต่อไป

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต.....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5578954553 : MAJOR PUBLIC HEALTH SCIENCES

KEYWORDS : *XYRIS INDICA* / PHARMACOGNOSTIC SPECIFICATION / CHRYSAZIN / QUANTITATIVE ANALYSIS / ANTIMICROBIAL ACTIVITIES

CHUANCHOM KHUNIAD : PHARMACOGNOSTIC EVALUATION AND CHRYSAZIN QUANTITATION OF *XYRIS INDICA* FLOWERING HEADS. ADVISOR : CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR : ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 135 pp.

Flowering heads of *Xyris indica* L. have been used as crude drug in Traditional Thai medicine for a long time. It has traditionally been used to treat ringworm, constipation and flatulence. The aim of this study was to investigate the pharmacognostic specification and analysed the content of chrysazin in *X. indica* flowering heads. The flowering heads were collected from 15 different sources in Thailand. The drawing of whole plant of *X. indica* was illustrated in detail. The crude drug was dried in hot air oven and ground to powders. The macroscopic characteristics were dried flowering heads with brown bract, yellow flowers, variable in sizes, slightly characteristic odour and slightly bitter, astringent taste. The histological characteristics were fragment of corolla, seeds, pollen grain and staminode. The loss on drying, total ash, acid-insoluble ash, water soluble extractive matter and ethanol soluble extractive matter and water content were 6.899 ± 0.165, 2.497 ± 0.033, 0.409 ± 0.027 , 6.592 ± 0.474, 4.030 ± 0.486 and 11.121 ± 1.132 % dry weight, respectively. TLC fingerprint of ethanolic extracts of *X. indica* flowering heads were performed using silica gel as stationary phase and petroleum ether and ethyl acetate (8:1) as mobile phase. Detection under ultraviolet light (254 nm and 365 nm) as well as under visual light after with 5% potassium hydroxide solution showed chrysazin yellow fluorescent band under UV 365 nm at hRf = 59. The chrysazin content in of *X. indica* flowering heads was determined by TLC-densitometry and TLC image analysis using aforementioned mobile phase. The linearity of chrysazin by TLC-densitometry was 15.0-75.0 µg/ml with R^2=0.9997, % recovery was in range of 90.67-99.16 %, the precision was between 0.76 -14.28 % RSD, LOD and LOQ were 5.48 and 16.62 µg, respectively and % RSD of robustness was 4.44 %. The linearity of chrysazin by TLC image analysis was 15.0-75.0 µg/ml with R^2=0.9986, % recovery was in range of 91.87-96.00 %, the precision was between 0.78-3.95 % RSD, LOD and LOQ were 5.90 and 17.89 µg, respectively and % RSD of robustness was 3.60 %. The quantitative analysis showed that chrysazin content of *X. indica* flowering heads by TLC-densitometry was 0.0223 ± 0.0011 g/100 g of dried crude drug whereas chrysazin content by TLC image analysis was 0.0219 ± 0.0007 g/100 g of dried crude drug. Both methods were compared statistically using paired t-test statistical analysis. It was indicated that the chrysazin content from both methods were not significantly different. Antimicrobial activities of benzene extract of *X. indica* flowering heads and standard chrysazin were tested by agar-overlay bioautography. The benzene extract on TLC plate inhibited the growth of 4 tested gram positive bacteria, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Micrococcus luteus*. The chrysazin in benzene extract and standard chrysazin inhibited the growth of *Bacillus subtilis*. This study is useful for identification, authentication and standardization of *X. indica* crude drug. Besides, it provides scientific evidences in antimicrobial potential of *X. indica* flowering heads.

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

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

Co-advisor's Signature .....

ACKNOWLEDGEMENTS

The author wishes to express her heartily gratitude and appreciation to her thesis advisor, Dr. Chanida Palanuvej, for her valuable suggestion, continue guidance and support throughout the course of this study.

The author is sincerely grateful to her thesis co-advisor, Associate Professor Dr. Nijsiri Ruangrungsri, for his kindness, encouragement and valuable suggestion to complete this study.

Gratitude is sincerely grateful to the thesis committee members, Assistance Professor Naowarat Kanchanakhan and Associate Professor Dr. Uthai Sotanaphun for their important and constructive suggestion in finalizing this thesis.

The author is thankful to the Herbal Remedies and Alternative Medicine Task Force of STAR: Special Task Force for Activating Research under 100 years Chulalongkorn University Fund for financial support as well as gratitude to Thaksin University for the scholarships of M.Sc. program.

The author would like to express her gratitude to all staff members, her colleagues in College of Public Health Sciences, Chulalongkorn University for necessary assistance, instrumental support and friendships.

The author also wishes to express her special thanks to Mr. Worathat Thitikornpong from Faculty of Pharmaceutical Sciences, Chulalongkorn University for providing researches facilities and his kind assistance in techniques of TLC-densitometry throughout this study.

The author’s final thank goes to her family, especially her dear parents for their love, understanding, encourage and support her to concentrate on her study.

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LIST OF ABBREVIATIONS

bw	=	Body weight
CCD	=	Charged-coupled devices
cm	=	Centimeter
g	=	Gram
HPLC	=	High performance liquid chromatography
HPTLC	=	High performance thin layer chromatography
ICH	=	The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
IR	=	Infrared
kg	=	Kilogram
l	=	Liter
LD_{50}	=	Lethal dose 50%
LOD	=	Limit of detection
LOQ	=	Limit of quantitation
MBC	=	Minimum bactericidal concentration
MFC	=	Minimum fungicidal concentration
mg	=	Milligram
MIC	=	Minimum inhibitory concentration
ml	=	Milliliter
mm	=	Millimeter
MS	=	Mass spectrometry
nm	=	Nanometer
NMR	=	Nuclear magnetic resonance
PEC	=	Planar electrochromatography

R^2	=	Correlation coefficient
RSD	=	Relative standard deviation
SD	=	Standard deviation
TLC	=	Thin layer chromatography
WHO	=	World Health Organization
UV	=	Ultraviolet
v/v	=	Volume by volume
w/w	=	Weight by weight
%	=	Percent
µg	=	Microgram
µl	=	Microliter
ºC	=	Degree Celsius

CHAPTER 1
INTRODUCTION

Background and significance of the study

Herbal medicines have been used in medicine from time immemorial, for the treatment or prevention of diseases and in all cultures throughout history. The humanity has depended on the diversity of plant resources for medicine to cure myriads of ailments. Primitive people treated illness by using plants, animal parts and minerals. They learned by instinct, taste, experience, trial and error to distinguish useful plants with beneficial effects from those which were toxic and inactive [1-3].

In meaning, Herbal medicines are plant-, animal- and mineral- derived medicines that usually contain one or more active chemical compounds, or a complex mixture of chemical compounds that act individual or in synergistic combination in the body to treat, prevent or manage various diseases [3].

Herbal medicines are a major part in all the traditional systems of medicine. Over the last few decades, people have been turning back to herbal medicines for remedy that are more harmonious with the human metabolism, easily accessible, inexpensive, safety than synthetic drugs and the most important reason is that modern medicine has serious side effects in addition to the high cost of medications. Therefore, using herbal medicines becomes more popular [2, 4-5].

In many parts of the world, plant-based remedies in the form of herbal tea, capsule, tincture, decoctions and extract are still an important part of pharmaceutical care, whether administered by a pharmacist, a physician, a tribal healer or relative. It is estimated that 80% of the world's population depends on botanical resources for their primary care needs [6].

To be accepted as safe herbal medicines, herbal medicines have to be tested before taken by people. Most of the known side effects reported for herbal drugs are extrinsic to the preparation and are related to several manufacturing problems such as misidentification of plants, lack of standardization, failure of good manufacturing practice, contamination, substitution and adulteration of plants, incorrect preparations and/or dosage, etc [7].

Thus, quality control and development of standardization herbal medicines with consistent safety and efficacy should be key issued and need to be prioritized from the earliest stage of the development process. Quality control involves processes that maintain the quality of a manufactured product. A high quality herbal medicine will contain the correct plant material, or mixture of plant materials, or their extracts, exactly as stated on the product label, free from contamination with other undeclared plant materials and free from pesticides, radioactive particles, heavy metals, microbes and mycotoxins [3]. From this issue, WHO published the guideline called Quality control method for medicinal plant materials to ensure the quality of medicinal plant products by using modern control techniques and apply suitable standard [8].

In addition, there is another important point that should be considered. Because of overuse of antimicrobial drugs, some bacteria start to develop resistance to them. It is important to develop new antimicrobial agents and look for substances from other sources to replace the current regimens. The medicinal plants remain the most common source of antimicrobial agents [9-10].

The Kingdom of Thailand has its own system of traditional medicine called "Traditional Thai medicine" (TTM). Herbal medicines play a significant role in TTM. The historical evidence shows that Thai people began to use herbal medicines for health promotion and the treatment of various symptoms and diseases before the Sukhothai period or before 1238 A.D. [11].

Traditional Thai medicine has many herbal medicines use to treat the ailments. One of them is *Xyris indica* (Kra thin thung) that is taken to treat ringworm, constipation and flatulence [12].

Although in Thailand, there are plenty of various herbal medicines which are used to treat and prevent diseases from time immemorial. However, the information of scientific study is deficient and still need to explain mechanism or prove effect of herbal medicines.

The phytochemical studies of *X. indica* flowering heads have shown two isocoumarins; xyridin A and xyridin B, two sterols; stigmasterol and spinasterol and three anthraquinones; chrysazin as a main compound, 3-methoxychrysazin and 3-hydroxy-chrysazin [13-16]. Nevertheless, there is no report on standardization of this herbal medicine and antimicrobial activities test have not complete.

Thereby, the aim of this study is to assess the pharmacognostic specification of *X. indica* flowering heads and analyze chrysazin content in all samples by thin layer chromatography (TLC) to standardize and control quality of raw materials and examine antimicrobial activities by bioautography for evidence based efficacy of this crude drug.

Objectives of the study

1. To establish the pharmacognostic specification of *X. indica* flowering heads.

2. To investigate the content of chrysazin in *X. indica* flowering heads by TLC image analysis using ImageJ software compared to TLC-densitometry.

3. To examine the *in vitro* antimicrobial activities of standard chrysazin as well as benzene extracts of *X. indica* flowering heads against selected bacterial and fungal pathogens by bioautography.

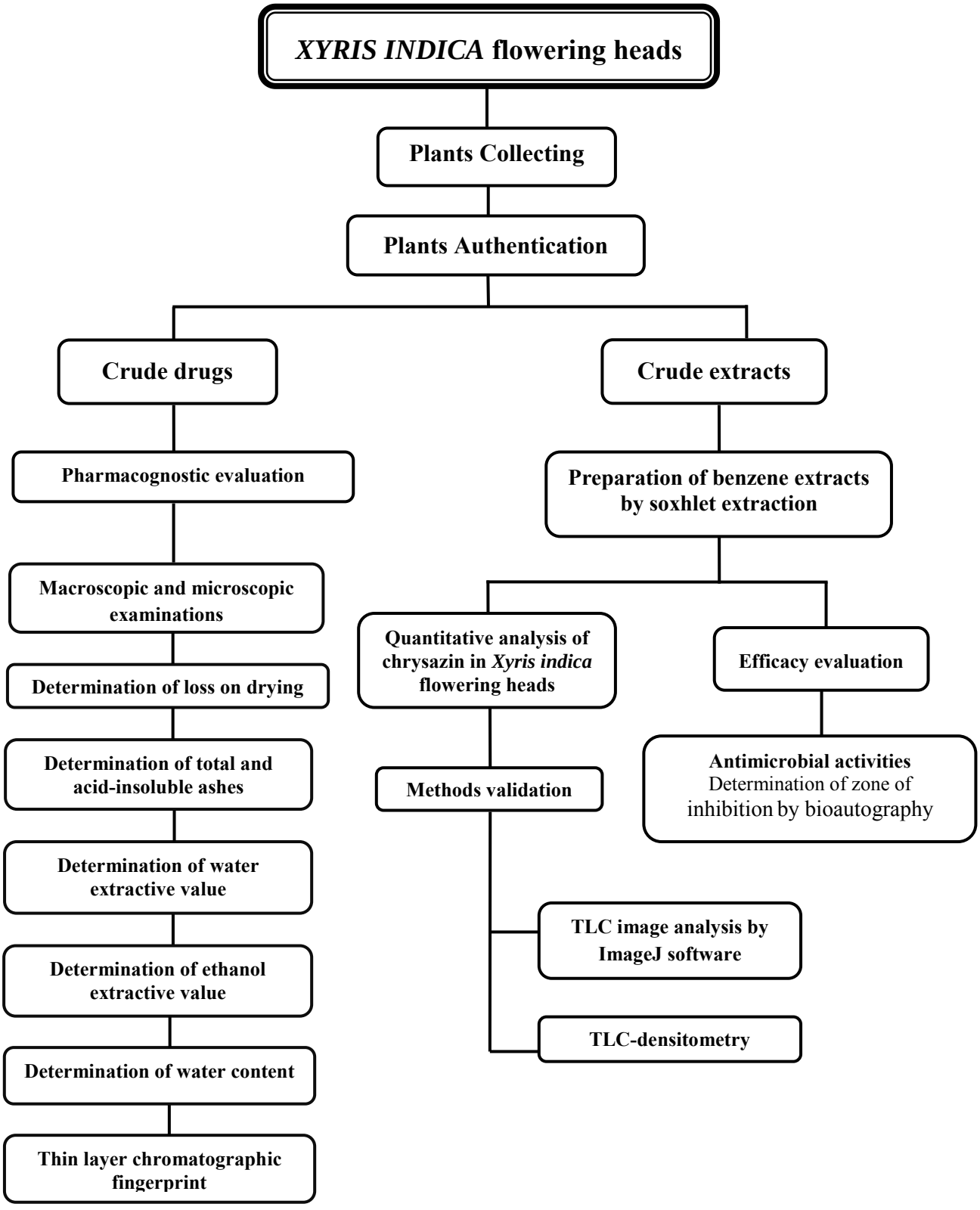

Figure 1. The conceptual framework

CHAPTER II
REVIEW OF RELATED LITERARURES

Genus *Xyris*

Xyridaceae is a small tropical and subtropical family, comprising 385 species in 5 genera: *Abolboda* Humb. & Bonpl. (1813), *Achlyphila* Maguire & Wurdack (1960), *Aratitiyopea* Steyerm. & P.E.Berry (1984), *Orectanthe* Maguire (1958), and *Xyris* L. (1753). *Xyris* is the largest genus of the family [17]. In Thailand, there is only 1 genus which is *Xyris*.

The genus *Xyris* contains about 280 species that are mainly in tropical and subtropical America, some species in Africa, Asia, and Australia [18]. Information of this family is published in the flora of Thailand volume 5 Part 1, including 11 species [19-20], there are:

1. *Xyris bancana* Miq.: Krathin thung (Central, Trat)

2. *Xyris capensis* Thunb.: Ya hua khot (Loei)

3. *Xyris complanata* R.Br: Tan (Nakhon Si Thammarat), Ya krathin (Trat), Ya kratiam (Prachun Buri), Ta tan (Narathiwat)

4. *Xyris grandis* Ridl.: Ya bua yai (North-eastern)

5. *Xyris indica* L.: Kra jub dang (Narathiwat), Krathin thung (Central, Trat), Krathin na (Chanthaburi), Ya krathiam (Prachun Buri), Yak hi klak (Saraburi), Ya bua (Prachun Buri, Ubon Ratchathani)

6. *Xyris intersita* Malme: Krathin thung yai (Central)

7. *Xyris kradungensis* B.Hansen: Ya bua bae (North-eastern)

8. *Xyris lobbii* Rendle: Dao nuea (North)

9. *Xyris pauciflora* Willd.: Kung (Prachun Buri, Ubon Ratchathani), Ya khon kai (North-eastern, Loei), Ya bua (Prachun Buri, Ubon Ratchathani)

10. *Xyris tuberosa* Ridl.: Ya dok lueang (Loei)

11. *Xyris wallichii* Kunth: Ya bua chuen (Central)

Recent studies showed that they were 6 new species of *Xyris* in Thailand that was exhibited below [17,21]:

1. *Xyris borneensis* Rendle: Kathin sai

2. *Xyris linifolia* P.Royen: Kathin nam khong

3. *Xyris bituberosa* Phonsena & Chantar.: Kathin phu wua

4. *Xyris buengkanensis* Phonsena & Chantar.: Kathin bueng kan

5. *Xyris emarginata* Phonsena & Chantar.: Kathin khok

6. *Xyris thailandica* Phonsena & Chantar.: Kathin phu

So the number of *Xyris* species is 17 species for nowadays.

Botanical characteristics of *Xyris indica* L.

Figure 2. The whole plant of *Xyris indica* L.

X. indica L. (Synonyms: *X. calocephala* Miquel, *X. capito* Hance, *X. paludosa* R. Brown, *X. robusta* Martius.) is widespread species in Thailand and a native plant of east India. Tall yellow-eyed grass is called to common name. Its Thai name is called Kra thin thung. *X. indica* can be mostly found in Cambodia, India, Indonesia, Laos, Malaysia, Philippines, Sri Lanka, Vietnam, Australia and Thailand. In Thailand, this species is found extensively in North-eastern: Udon Thani, Khon Kean, Surin; South-eastern: Chanthaburi, Prachin buri; Central: Nakhon Nayok, Suphan buri; Peninsular: Phangnga, Krabi, Songkhla, Yala and Surat Thani [18-19,22].

The ecology of this plant is almost exclusively restricted to and common in inundated rice fields, grows in swamps, moist localities, streams, pools and ponds in the lowland up to 100 m altitude. Flowering is on (August-) October to January (-February) [19,23-24].

Description as follows: *Leaves* without a ligule, margins not thickened; apex bluntly oblique to acute, (10-)30-50(-70) cm by (2.5)3-6(-8) mm; blade flat with conspicuous, short transverse ribs connecting the longitudinal nerves. *Scape* terete with 6-15 longitudinal ridges (18-)30-70(-110) cm by (1.2-)2-3(-4.5) mm. *Inflorescence* spherical to cylindrical, (0.7-)1-1.5(-3.5) by 0.8-1.4 cm. *Median bracts*

with margins entire, lighter or darker brown, numerous light spots scattered in triangularrhombic field below apex, field otherwise not different from surface of bract neither in color nor structure. *Lateral sepals* light brownish to almost hyaline, with coarsely serrate crest [19].

Medicinal uses

The natives of Bengal use this plant by mixing with vinegar to cure ringworm. Furthermore it is used as a remedy by boiling in oil for treatment of itch and leprosy [25-26]. Surveying traditional use of medicinal plants reveals that the local people of North East of India also use this plant to cure ringworm [27].

In traditional Thai medicine, this plant is taken to treat ringworm, constipation and flatulence. It is not only used in human but also used leaves to feed ruminant [12].

Chemical constituents

Previous phytochemical studies of *X. indica* began with the isolation of two new isocoumarins named xyridin A and xyridin B, two sterols named stigmasterol and spinasterol and three anthraquinones named chrysazin, 3-methoxychrysazin and 3-hydroxychrysazin from chloroform extract of *X. indica* flowering heads. The structures of these compounds were determined by spectroscopic methods (UV, IR, NMR, MS) after purification by silica gel column chromatography [13-16].

There is another phytochemical report on *Xyris* species, the result showed same group of compounds. The anthraquinones chrysazin and 3-methoxychrysazin were obtained from *X. semifuscata* [28]. 3-methoxychrysazin and 1, 5-dihydroxy-3-methoxy-anthraquinone has been isolated from *X. pilosa* [29]. Furthermore, flavonoids were obtained from *X. itatiayensis*, *X. longiscapa* and *X. obt*usiuscula [30].

Pharmacological activities

Selected isolated compounds of *X. indica* were tested for *in vitro* antimicrobial activities.

Three anthraquinones, chrysazin, 3-methoxychrysazin and 3-hydroxychrysazin showed only marginal or no antibacterial activity at the tested concentrations compared to miconazole as standard drug. However, 3-hydroxychrysazin showed good antifungal activity with MIC of 0.78 μg/ml against *Trichophyton mentagrophytes* and *Trichophyton rubrum* which were dermatophytes

causing ringworm and athelete's foot. Therefore, the researchers concluded that this compound was an antifungal principal in this medicinal plant [16].

Xyridin A, xyridin B, keto acid and (±)-dihydroxyridin A were screened *in vitro* for antibacterial activity against various gram positive and gram negative bacteria. The activity was determined *via* the growth inhibition of microorganism. The results indicated that these compounds showed moderate antibacterial activity. Xyridin B was slightly more potent than xyridin A and (±)-dihydroxyridin A was the most active compound as antibacterial. All of these compounds were also subjected to fungicidal screening against various human, plant and animal pathogens. It might be seen that (±)-dihydroxyridin A was more active as antifungal agent [31].

The petroleum ether extract and the ethanol extract from flowering heads were tested to determine inhibition zone, MIC, MBC and MFC. The petroleum ether extract from flowers showed the potential against three gram positive bacteria and one fungus. The ethanol extract demonstrated the activity against five gram positive bacteria, four gram negative bacteria and two fungi. The ethanol extract displayed large inhibition zone of 16.00±1.00 mm on *Staphylococcus epidermidis* while the petroleum ether extract showed the lowest MIC on *S. epidermidis* and the lowest MBC on *Bacillus subtilis* [32].

Moreover, some *Xyris* species were selected to examine antifungal activity. The result corroborated the ethnomedical use of *Xyris* species to treat dermatitis. The results showed that isolated compound from *X. pteygoblephara* aerial parts named dihydroisocoumarin were evaluated against clinical isolates of the dermatophytes. The inhibition zone of *Epidermophyton floccosum*, *T. mentagrophytes* and *T. rubrum* revealed similar strength to the positive control, amphotericin B [33].

Seven species of *Xyris* (*X. cipoensis, X. longiscapa, X. peregrina, X. pilosa, X. platystachia, X. pterygoblephara* and *X. tortula*) were assayed for *in vitro* antifungal activity by direct bioautography. Ethanol extracts from all seven species, except *X. cipoensis*, inhibited the growth of *Fusarium oxysporum*, whereas the extracts from *X. peregrina, X. platystachia, X. pterygoplephara* and *X. tortula* also inhibited *Aspergillus niger*. The bioactivity studies of *X. pilosa* presented two anthraquinones, 3-methoxychrysazin and 1,5-dihydroxy-3-methoxy-anthraquinone as active compounds against *F. oxysporum* [29].

Antibacterial study showed that the crude extracts of *X. platystachia* and *X. pterygoblephara* were active against *Micrococcus luteus*, while only *X. platystachia* inhibited the growth of *Staphylococcus aureus* [34]. The inhibitory activity of *X. pilosa* extract against *S. aureus* was shown with similar inhibition zone to chloramphenicol used as positive control [35].

Chrysazin

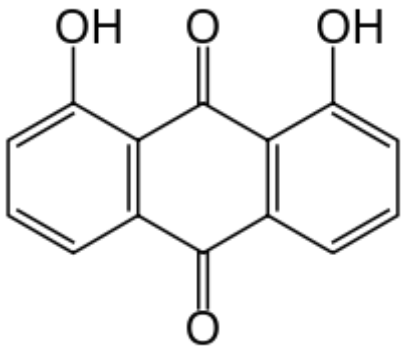

Figure 3. Structure of chrysazin

Synonyms: Chrysazine; Danthron; Dantron; 1,8-Dihydroxyanthraquinone

Formula: $C_{14}H_8O_4$

Scientific Name: 1,8-dihydroxy-9,10-anthracenedione

Molecular weight: 240.21 g/mol

Description: Red or reddish-yellow needles or leaves (From ethanol); orange crystalline powder

Melting point: 191 – 193°C

Solubility: Very soluble in aqueous alkali hydroxides; soluble in acetone, chloroform, diethyl ether and ethanol; almost insoluble in water [36].

Danthron or chrysazin has been used since the beginning of the 20th century as a laxative. In 1987, U.S. manufacturers voluntarily withdrew production of human drug products containing danthron, and in 1999, the U.S. Food and Drug Administration issued the final rule ordering the withdrawal of danthron-containing products from the U.S. market for use as laxatives. However, danthron has continued to be used as a pharmaceutical in the United Kingdom. To a lesser extent, danthron has been used as an intermediate in the manufacture of alizarine and indanthrene dyes [37].

Danthron is an anthraquinone and occurs naturally in several species of plants and insects e.g.,*Cassia* (senna), *Aloe*, *Rheum* and *Rhamnus* (cascara) species. It has been separated from *Xyris semifuscata* dried leaves and stems harvested in Madagascar and form the basic structure of the aglycones of naturally occurring laxative glycosides. Danthron has been identified in larvae of the elm-leaf beetle, *Pyrrhalta luteola*; it has been suggested that the insect biosynthesizes a mixture of anthraquinones and anthrones as protection from predators [36,37].

Carcinogenicity

Danthron is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in experimental animals [37].

Cancer studies in experimental animals

Oral exposure to danthron caused tumors in two rodent species and at several different tissue sites. Dietary administration of danthron caused liver cancer (hepatocellular carcinoma) in male mice and benign and malignant intestinal-tract tumors (adenoma and adenocarcinoma of the colon and adenoma of the cecum) in male rats [37].

Cancer studies in humans

The data available from studies in humans are inadequate to evaluate the relationship between human cancer and exposure specifically to danthron. One case report described the occurrence of cancer of the small intestine (leiomyosarcoma) in an 18-year-old girl with a history of prolonged exposure to danthron [37].

Studies on mechanisms of carcinogenesis

Danthron has been evaluated for its ability to promote tumors induced by other chemicals. When danthron was fed to male mice that also received 1,2-dimethylhydrazine as a tumor initiator, the incidence and multiplicity of colon tumors (adenoma or adenocarcinoma) and liver tumors (adenoma) were significantly increased. However, danthron did not promote the induction of tumors when either painted on the skin of mice pretreated with 7,12‑dimethylbenz[a]anthracene or fed to rats pretreated with 1,2‑dimethylhydrazine [37].

Toxic effects

The oral LD_{50} for dantron in male ARS/ICR mice was ~ 7 g/kg bw. Groups of four male and four female beagle dogs received either a vehicle capsule or a capsule containing dantron at 5 or 15 mg/kg bw daily for one year. No adverse effect was observed. The doses employed were reported to be several-fold higher than the usual clinical dose [36].

Male rats given dantron at 60 or 240 mg/kg diet for 26 weeks had enlarged lymph nodes in the mesocolon, which were brownish due to pigmentation of the accumulated mononuclear phagocytes. ln kidney, pigment deposition was seen in the cortical region [36].

Genetic and related effects

Danthron was found to cause genetic damage in a limited number of *in vitro* test systems, including *Salmonella typhimurium*, yeast, and mammalian cell cultures [37].

Quality control methods for herbal materials

All of pharmacognostic parameters are following World Health Organization (WHO) guideline quality control methods for medicinal plant materials [8].

Macroscopic and microscopic examinations

Macroscopic examinations of herbal materials refer to the evaluation of herbal materials by shape, size, color, odor, taste etc. Since the majority of information on the identity, purity and quality of the material can be drawn from these observations. This evaluation procedure provides the simplest and quickest means to establish the identity and purity and thereby ensure quality of a particular sample.

Microscopic examination is based on observation of cell and tissue structures of herbal material with application of the knowledge of plant histology and anatomy so as to authenticate plant species. The procedure of identification contains three main steps: selection of typical materials, preparation of sections or powder, and observation of features. The features will be drawn. This analysis is also used to identify some adulterants.

Determination of loss on drying

Loss on drying test is objected to evaluate the amount of water and volatile matters in herbal materials when the material is dried under specified conditions. Drying can be carried out by heating at 100-105 °C until reaches a constant weight. Loss on drying is the loss of mass expressed as percent w/w.

Determination of ash values

The residue remaining left after incineration of the herbal materials is designated as ash. The residue obtained usually represents the inorganic salts naturally occurring in the herbal material and adhering to it. It varies with in definite limits according to the soils. It may also include inorganic matter deliberately added for the purpose of adulteration. An ash value determination furnishes the basis for judging the identity and cleanliness of any herbal material and gives information relative to its adulteration/contamination with inorganic matter. Thus ash values are helpful in determining the quality and purity of herbal materials. Ash value can be determined by two different methods: Total ash and acid insoluble ash.

Determination of total ash is designed to measure the total amount of herbal materials produced after complete incineration of the ground sample at as high temperature as possible (about 500°C) to remove all the carbons. Total ash usually consists of carbonates, phosphates, silicates and silica. This ash includes both physiological ash which is obtained from the plant tissue itself and non-physiological ash which is the remains of the adhering material to the plant e.g. sand and soil.

Determination of acid-insoluble ash is frequently necessary to evaluate the herbal medicines, which indicates the residue obtained after treating the total ash with dilute HCl and weighing residue. This ash value particularly indicates contamination with silicious material e.g. earth and sand.

Determination of solvent extractive values

The herbal materials have their biological activity mainly due to active chemical constituents. These constituents may be soluble in different polar, semi polar or nonpolar solvent. The extraction of any herbal materials with a particular solvent yields a solution containing different phytoconstituents. The composition of these phytoconstituents in that particular solvent depends upon the nature of the herbal

materials and solvent used. The use of a single solvent can be the means of providing preliminary information on the quality of particular drug sample. This method determines the amount of active constituents in given amount of herbal material when extracted with solvents.

Determination of water content

Moisture is expected component of herbal materials, which must be eliminated as far as practicable. Moisture triggers the enzymatic activity or facilitates growth of microbes which leads to herbal materials destruction. The preparation of herbal materials from the harvested plants involves cleaning to remove soil or other extraneous material followed by drying which plays a very important role in the quality as well as purity of the material.

The azeotropic method gives a direct measurement of water present in herbal materials being examined. The water in sample is distilled together with an water-immiscible solvent, such as toluene. The water and solvent can be separated in the receiving tube on cooling.

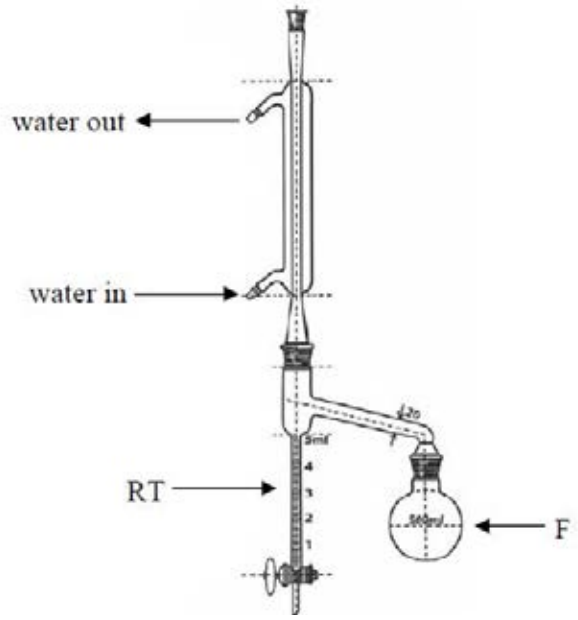

Figure 4. Apparatus used to determine water content by the azeotropic method

An appropriate quantity of ground material placed in flask (F), toluene is added, and the apparatus is connected as shown in Figure 4. The graduated receiving tube (RT) is filled with toluene, and then the flask is heated until no more water is distilled over. Both toluene and water distilled over, but the water being heavier than

toluene, sinks to bottom of the receiving tube (RT) displaying toluene which flows back into the flask (F). When the apparatus has cooled and the toluene and the water in tube (RT) have separated completely, the volume of water distilled over can be read and the % water initially present in the material can be calculated.

Determination of volatile oil content

Volatile oils are characterized by their odour, oil-like appearance and ability to volatilize at room temperature. They are usually composed of mixtures of chemicals for example, monoterpenes, sesquiterpenes and their oxygenated derivatives. Aromatic compounds predominate in certain volatile oils. In order to determine the volume of oil, the plant material is distilled with water and the distillate is collected in a graduated tube of Clevenger apparatus (Figure 5). 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.

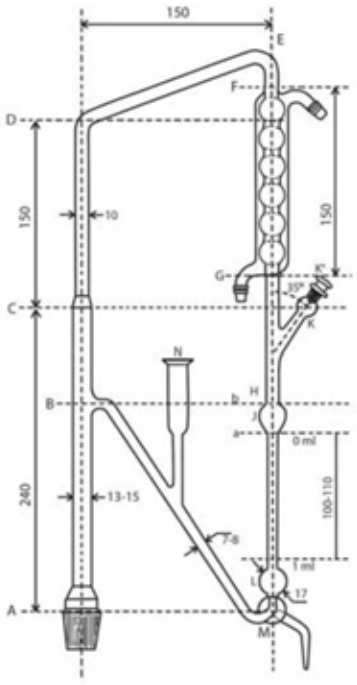

Figure 5. Apparatus used to determine volatile oils

Thin layer chromatography (TLC)

Thin layer chromatography is one of the most widespread analytical methods used for a long time in the organic chemistry laboratory [38].

At present, TLC is still most commonly used because of its advantages. TLC is the cheapest, quickest and easiest method that can be used for qualitative and quantitative analysis as well as identifying compound in herbal extracts [39].

TLC is an adsorption chromatography in which materials are separated based on the interaction between a thin layer of adsorbent and a selected solvent. A thin layer of an adsorbent attached on a plate is used as stationary phase, while the selected solvent is the mobile phase [3]. The commonly adsorbents for TLC plates include silica, alumina, octadecasilica, cellulose, dextran gels, polyamide, or other ion exchange polymeric resin. Siliga gel is most commonly used [39]. An adsorbent layered onto a glass, plastic or aluminum plate. The materials to be separated are dissolved in a solvent and spotted onto the plate near the bottom. The selected solvent is allowed to flow up the plate by capillary action called development step. After development, the solvent is removed from the plate by evaporation or heating and detection is performed in the plate [40].

The information provided by a finished TLC plate includes the migrating behavior of the separated substance. It is given in the form of the hRf value. hRf value is a parameter that often used for qualitative evaluation [2]. The hRf value is defined as follows:

$$\mathbf{hRf} = \frac{\textbf{Distance of compound from origin spot to the developed spot}}{\textbf{Distance of solvent from origin spot to the developed front}} \times \mathbf{100}$$

When TLC is used to identify the authenticity of herbal extracts, standard compounds of the known major of characteristic components in the herb are normally used as references for comparison. If the positions (hRf) and colors of major compounds in the test extract are the same as those of reference standards on three plates that are respectively developed with different solvent systems, the species of material for extract can be confirmed [39].

TLC provides the first characteristic fingerprints of herbal extracts. The TLC fingerprint with a visible pattern of bands provides fundamental data and is typically used to demonstrate the consistency and stability of herbal materials. The advantages

of using TLC to construct the fingerprints of herbal medicines are its simplicity, versatility, high velocity, specific sensitivity and simple sample preparation [3,41-42].

For detection of TLC plate after development, spot of compounds are detected on the plate. The first detection is direct observation of colored materials with eyes but most of compounds are colorless under daylight. Thus, they must be detected under other conditions [43].

Detection can be done by illuminating TLC plate under UV light or by color reaction with suitable reagent. The adsorbent containing fluorescent additive can glow under specified UV light, usually at the wavelength of 254 nm. Many aromatic compounds having double bond can absorb this short wave UV (quenching) resulting in dark spot on a bright background detection. Some compounds with native fluorescence can be excited to fluorescence appear as bright spots, often differently colored, on a dark background under UV 365 nm. For example, chlorophylls have red fluorescence, anthraquinones appear as yellow fluorescence, coumarins show blue fluorescence. The compounds that do not absorb UV light at 254 nm or 365 nm can be invisible and require detection with reagent. The detection reagent solution is usually applied by spraying or dipping the layer. The mechanism of reagent detection is color reaction between the compound of interest on TLC and the selected reagent. In some cases, heat is required to assist the color reaction on TLC plates and this can be supplied in the form of a hair dryer or a drying oven [2,39,41]. The staining reagents can be separated into two types, the universal staining reagents and the specific staining reagents. For known compounds, the specific staining reagents for the compounds of interest should be used. In case of crude extracts with unknown compound, universal spray reagents are recommended. The most common used staining reagents are shown in Table 1.

Table 1. Commonly used TLC staining reagent for detection of natural compounds [39,44]

staining reagent	Preparation	Treatment	Detection
Ferric (III) chloride	2.7 g of salt dissolved in 100 ml 2M hydrochloric acid.		Phenols and phenolic acids.
p-Anisaldehype/ sulfuric acid	0.5 ml p-anisaldehyde in 50 ml glacial acetic acid and 1 ml conc. sulfuric acid.	Heat to 105°C until maximum visualization of spots. Enhance background with water vapor spray. Components give violet, blue, red, grey or green spots.	Phenols, sugars, steroids, and terpenes
Vanillin/sulfuric acid	Dissolve 1 g of vanillin in 100 ml conc. sulfuric acid or 0.5 g vanillin in 80 ml sulfuric acid and 20 ml ethanol	Dry at 120°C until color spot appears. Components give red and blue spot.	A universal spray. Many terpenoids, steroids and saponins.
Dragendorff's reagent	Dissolve 0.11 g potassium iodide and 0.18 g bismuth subnitrate ($OBiNO_3$) in 20 ml acetic acid and make up to 100 ml.	Generally, color reaction occurs rapidly, but heat is required. Components give dark orange to red.	Alkaloids and quaternary nitrogen compounds
Ninhydrin	Dissolve 0.2 g ninhydrin in 100 ml ethanol, or in 94 ml water and 6 ml acetone.	Heat to 110°C until reddish spots appears.	Amino acids, amines, amino sugars

TLC quantification

The amount of substances separated by TLC can be determined directly on a developed TLC plate by measurement the intensity of UV/Vis absorption, fluorescence, or fluorescence quenching. Spectroscopic methods of detection have been used since an early stage of chromatography. Substances separated by TLC were investigated by reflectance and transmission spectrometry in some 50 years ago. From the pioneering work of Salganicoff, Polak, Goodall, Goldman, and Ebel, modern

instruments, called densitometers, were developed. Today *in situ* quantitative and qualitative evaluation of developed TLC plates is performed with modern computer controlled densitometers and image-analyzing systems with charged-coupled devices cameras (CCD cameras) [42].

TLC-densitometry

Scanning densitometry is recommended as classical densitometry. The separation tracks on the plate are scanned with a light beam in the form of a slit selectable in length and width. The photosensor of the densitometer measures diffusely reflected light. The difference between the optical signal from the sample-free background and that from a sample zone (fraction) is correlated with the amounts of the respective fractions of calibration standards chromatographed on the same plate [42].

A typical scanning densitometer has the following operating characteristics:

- Reflectance or transmission modes
- Absorbance or fluorescence measurements
- Accommodates plates up to 20 x 20 cm
- Full spectra available for qualitative analysis, wavelength range: 190-800 nm
- Multiwavelength scanning, up to 31 channels
- Computer controlled and data processed [45].

In situ densitometry is a simple way of quantifying the desired sample components or amount directly applied on the plate. The resolution of compounds to be separated on the plate is followed by measuring the optical density of the separated spots directly on the plate. The sample amounts are determined by comparing them to a standard curve from reference materials chromatographed simultaneously under the same condition [2].

Scanning densitometers manufactured by different companies such as the CAMAG TLC Scanner 3, Desaga CD 60 and the CS9000 series of Shimadzu Corp., Tokyo, are slit-scanning, single-beam, single-wavelength instruments with powerful software for evaluation after scanning and sharing many features in common. They consist of an electronic part, a compartment for plate positioning, and the optical system, which is most important. The feature of the scanner principal design represented by TLC Scanner 3 is illustrated (Figure 6). There are three light sources

(mercury vapor lamp, deuterium lamp, or tungsten halogen lamp) positioned in the light path by motor drive. The deuterium and tungsten halogen lamps are continuum lamps. i.e., they emit light over a wide wavelength range. Different sources must be used to cover the entire UV-vis range. Halogen or tungsten lamps are light sources for visible wavelengths of 400-800 nm, deuterium lamps for UV wavelengths of 190-400 nm, and high-intensity mercury or xenon sources for fluorescence excitation [42,46].

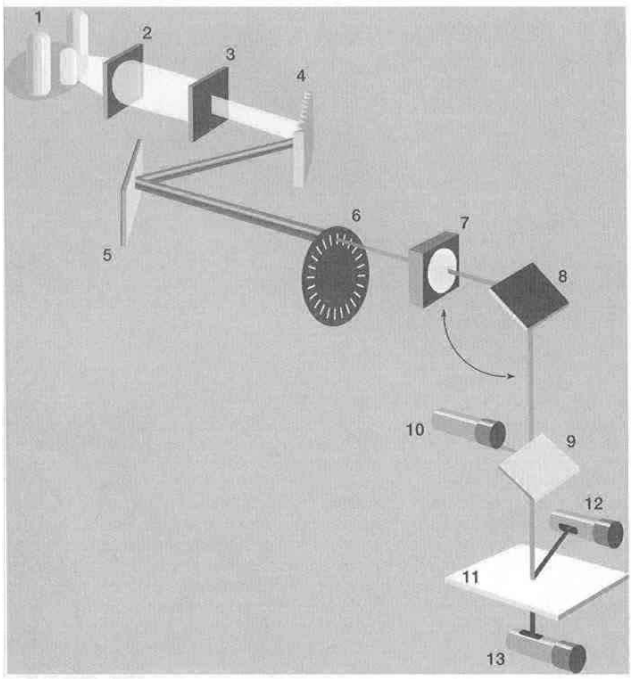

Figure 6. Light path diagram of the TLC Scanner 3 1, Lamp selector; 2, entrance lens system; 3,monochromator entry slit; 4, monochromator grating; 5, mirror; 6, slit aperture disk; 7, lens system;8, mirror; 9, beam splitter; 10, reference photomultiplier; 11, scanning object; 12, measuring photomultiplier;13, photodiode (transmission).

Monochromators or filters (prism or grating) are employed for wavelength selection and a photomultiplier tube or photodiode detector for signal measurement. Measurements are commonly made in the reflectance mode and occasionally in the transmission mode by mounting the plate on a movable stage controlled by stepping motors that is mechanically operated in the x- and y-directions. The plate is scanned with a fixed beam of monochromatic light in the form of an adjustable rectangular slit, the height of which is matched to the width of the largest spot or band [42,46].

Most modern densitometers are designed for automatic scanning of a complete plate. All required for instrumental operatation such as lane changes; spot detection;

and optimization of measuring conditions, calibration, and data reporting are controlled by computer. Computer-controlled densitometer can perform a number of functions: data acquisition by scanning over an entire plate following a preselected geometric pattern with control of all scanning parameters; automated peak searching and optimization of scanning for each fraction located; multiple-wavelength scanning to find, if possible, a common wavelength for all substances to be quantified, to optically resolve fractions incompletely separated by TLC, and to identify fractions by comparison of spectra with those of standards co-chromatographed on the same plate or stored in a spectrum library through pattern recognition techniques; baseline location and correction; computation of peak areas and/or heights of samples and co-developed standards and processing of the analog raw data to quantitative digital results, including calculation of calibration curves by linear or polynomial regression, interpolation of sample concentrations, statistical analysis of reproducibility, and presentation of a complete analysis report; and storage of raw data for later reintegration, calibration, or evaluation with different parameters without rerunning the chromatogram [42,46].

TLC image analysis, digitally enhanced TLC (DE-TLC)

An alternative method for the quantitative processing of thin layer chromatograms is digitally enhanced TLC. DE-TLC is introduced as an inexpensive, new technique for qualitative and quantitative analysis that can be used in any laboratory that cannot afford a commercial densitometer [47].

Recent studies described the use of image analysis methods for quantitatively evaluating the TLC plates. In these studies, the image analysis methods were proved to be very accessible, simple and able to simultaneously evaluate all the samples analyzed by the TLC [48]. TLC-image analysis method has been developed and applied for quantitative assay with good accuracy and precision [49]. DE-TLC does not require any hardware. The DE-TLC equipment consisted of a UV lamp in a cabinet, CCD camera, and image analysis software. This is based on the use of a charge-coupled device (CCD) sensor in camera as a detector that offer more than a million small detectors (pixels) and combination with image analysis software for converting pixel intensity to chromatographic peak. Commercial and free image analysis softwares for TLC image analysis are available in which performances are

based on sensitivity of spot detection, background compensation algorithms, intensity resolution, precision and accuracy of image analysis [42,50,51]. The CCD camera is sufficiently sensitive to detect changes in spot fluorescence intensity from a UV light source. The CCD cameras are detectors containing an array of sensors that can image an area in fraction of seconds or real time [52].

The quantitative evaluation of the TLC plates using the proposed image analysis methods is based on the assumption that the color intensity and the area size of the each individual spot on the plate are a function of the quantity of that particular compound in the corresponding spot. Because the spot color intensity is evaluated by comparing it to the plate color background and the analysis should include the entire TLC plate [47].

The developed TLC plate is illuminated and recorded by a CCD camera under UV light. The TLC plate is positioned in the system, UV cabinet and CCD camera is aligned for optimal pixel resolution of the images. The images are processed using image analysis software or common photo-editing software which gives the peak area of each spot. After that, peak area value is substituted in the equation taken from the standard curve. The result shows the content of active constituent in each sample.

Image analysis software

Another important part of an effective image analyzing system is software. There are at least 20 different versions of software for quantitative evaluation of thin-layer chromatograms with image analyzing systems written by different companies around the world [42]. Such as Photoshop, Sorbfil TLC Video densitometer software, Scion Image software, ImageJ etc.

ImageJ is free computer software, developed at Department of Health and Human Services, National Institutes of Health (NIH) in the United States, especially for handling biological images. It is a public domain image processing and analysis program written in Java which capable to be used with most operating system. Downloadable distributions are available for Windows, Mac OSX and Linux. This software is very easy to operate. ImageJ software contains many built-in algorithms to serve the investigation, sufficient to process the images of TLC plates correctly [53]. It can display, edit, analyze, process, save and print 8–bit, 16–bit and 32–bit images

and can read many image formats including TIFF, GIF, JPEG, BMP, DICOM, FITS [53,54].

ImageJ software can calculate area and pixel value statistics of user-defined selections, can measure distances and angles, can create density histograms and line profile plots. The software supports standard image processing functions such as contrast manipulation, sharpening, smoothing, edge detection and median filtering, geometric transformations such as scaling, rotation and flips. Image can be zoomed up to 32: 1 and down to 1: 32. All analysis and processing functions are available at any magnification factor. The program supports any number of windows (images) simultaneously, limited only by available memory. Spatial calibration is available to provide real world dimensional measurements in units such as millimeters. Density or gray scale calibration is also available [54].

For analyzing the amount of compound on TLC plate by ImageJ software, firstly, the user must convert the spot on TLC plate to peak area. A rectangular tool is used to select interested spot and its area and pixel value statistics are calculated. Density histograms and line profile plots are created to obtain a peak from each spot with peak area calculation.

Method validation

Quantitative methods must be validated to prove the reliability of the obtained results. The International Conference on Harmonization (ICH) developed guidelines for validation of pharmaceutical analyses and adapted for TLC. Basic acceptance criteria for evaluation of validation experiments were proposed for specificity, accuracy (recovery), precision (repeatability and intermediate precision in terms of relative standard deviation (RSD)), specificity, limits of detection, limits of quantification, linearity, and range, and selected parameters for robustness testing. Most published TLC analyses use all or part of the ICH model of requirements for quality assurance of results [55].

ICH has published guidance (Q2R1) for the validation of pharmaceutical analytical methods [56]. Any new method or alteration/improvement of existing methods should be validated in accordance with this guidance to demonstrate the reliability of the obtained results.

Specificity

A method is said to be specific if it provides a response for only a single analyte. This method is to distinguish and quantify the response of the target compounds from the responses of all other compounds. One single spot does not mean that the spot contains only one compound. That is why the measurement of the UV/VIS spectra of all analyte spots is essential to prove the identity and selectivity of the analytical method [57].

Accuracy

ICH defines the accuracy of an analytical procedure as the closeness of agreement between the conventional true value or an accepted reference value and the value found [56]. Recoveries of the analyte observed to be very close to 100% representing the accuracy of the method and also noninterference of excipients [57].

Precision

Repeatability on replication is determined under same conditions, e.g., same analyte, same apparatus, short interval of time, and identical reagents using the same sample [57].

Intermediate precision is determined under different conditions, e.g., different analyte, different days, and different reagents from different sources using the same sample [57].

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value [57].

LOQ is the lowest amount of analyte that can be quantitatively determined in sample with defined precision and accuracy under standard conditions [57].

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. It provides an indication of the procedure's reliability during normal usage [56].

Robustness of the test is examined by introducing small changes in mobile-phase composition, its volume, chamber saturation time, time from spotting to

chromatography, and slight change in the solvent migration distance. The effects on the results usually the peak area are examined. Robustness of the method is determined as percent RSD of peak area among slight variation. [57].

Efficacy evaluation

Antimicrobial activities

Agar diffusion assay

Agar diffusion assay is microbiological screening method that frequently used in preliminary screening for the detection of antimicrobial activity in medicinal plants.

Principle of agar diffusion assay

Microorganisms are spread over an agar surface. A reservoir contains the substance which knows concentration is placed on the agar surface of a suitable solid agar medium. During incubation, the substance is allowed to diffuse through the agar.

If the substance can kill or inhibit the growth of microorganisms the clear zone appears (inhibition zone) around the reservoir. The diameter of the zone of inhibition is measured.

Type of agar diffusion method

There are three types of this method separated by different types of reservoirs. Agar diffusion assay includes agar well diffusion, agar disk diffusion and cylinder diffusion.

In the agar well diffusion assay, a few millimeter diameter holes are punched in the inoculated agar surface with a sterile cork borer or something similar such as a sterile rubber stopper, test tube, marble and filled the sample solutions into the holes. The Petri dishes are incubated. After that, the inhibition zones are measured [58-60].

The agar disk diffusion assay has been widely used method for antimicrobial activity. In this method, small filter paper disks (about 6 mm diameter) are used as the reservoir. The sample solutions to be assayed are dropped on the disk either by use of a pipette or loop, or the disk may be dipped in to the solutions. The disks that contain the solutions are placed on the agar surface previously inoculated agar plates with the test microorganisms. The Petri dishes are incubated and the zones of inhibition are measured [58,59].

In the cylinder diffusion method, the reservoir is the stainless steel, pyrex glass or porcelain cylinder. It is placed on the inoculated agar surface by hand or by the use of automatic dispensing machines. The sample solutions are filled into the cylinders. The plates are incubated. After incubation, the cylinders are removed the inhibition zones are measured [58-60].

Bioautography

Bioautography belongs to biological activity guided fractionation and isolation of active compounds commonly used for the detection of antimicrobial activities. Bioautography is a simple, cheap, time-saving and unsophisticated laboratory technique to detect substances affecting the growth rates of test microorganisms in complex mixtures and matrices. This method is based on the biological activity of the tested substances which can be antibacterial, antifungal, antiprotozoa etc. This detection method can be successfully combined with planar chromatographic techniques, such as thin-layer chromatography (TLC), high- performance thin-layer chromatography (HPTLC), overpressured- layer chromatography (OPLC) and planar electrochromatography (PEC) and can be coupled with MS, IR or NMR techniques to obtain full information about the structure and biological activity of the substances under investigation [58].

TLC-bioautography is commonly used. Both separation and microbial detection are performed on the same TLC plate. The procedure in bioautographic methods is similar to the one used in agar diffusion methods. The difference is that the tested compounds diffuse to inoculated agar medium from the chromatographic layer, which is adsorbent or paper [58].

Bioautography methods are usually divided into three categories: contact bioautography, immersion or agar-overlay bioautography and direct bioautography [58].

Contact bioautography

In the contact bioautography, antimicrobial compounds diffuse from developed TLC plate to the inoculated agar surface. The developed TLC plate are placed face down on the inoculated agar surface and left for some minutes or hours to allow diffusion. Next, the plate is removed or is not removed and the agar layer is

incubated. The inhibition zones are observed on the agar surface where the antimicrobial compounds were in contact with agar surface. This assay is similar to the agar disk diffusion assay [58,59].

Immersion or agar-overlay bioautography

The developed TLC plate is placed face up on the base agar and covered with a seeded agar medium that contains the tested microorganisms. After solidification, the plates are incubated and inhibition zones are observed. Before incubation, the plates are left for a few hours at low temperature to enable better diffusion. This method is combination of contact and direct bioautography [58,59].

Direct bioautography

The developed TLC plate is dipped in the suspension of microorganisms growing in a suitable broth or the suspension is sprayed onto the TLC plate. The TLC plate is incubated. The surface of TLC plate covered with the broth medium becomes a root of nutrients and the tested microorganisms grow directly on it [58,59].

Visualization of bioautography

Inhibition zones are observed as clear zones or are stained with dehydrogenase activity-detecting reagents. The most common are tetrazolium salts. The dehydrogenase of living microorganisms converts tetrazolium salt into intensely colored formazan. These salts are applied onto the bioautogram during incubation. Cream-white zones against a colored background on the TLC plate indicate the presence of antibacterial agents [58,59].

CHAPTER III
MATERIALS AND METHODOLOGY

Chemicals and reagents

Benzene	QRëC, New Zealand
Chloroform	J.T. Baker chemical Co., USA
Chrysazin	Sigma-Aldrich Co., USA
Dimethyl sulfoxide	Merck KGaA, Germany
Ethanol	Liquor Distillery Organization Excise Department, Thailand
Ethyl acetate	RCI Labscan Limited, Thailand
Hydrochloric acid	RCI Labscan Limited, Thailand
Mueller Hinton agar and broth	Himedia Laboratories Pvt. Ltd., India
Petroleum ether	RCI Labscan Limited, Thailand
Sabouraud Dextrose agar and broth	Himedia Laboratories Pvt. Ltd., India
Tetrazolium blue chloride	Sigma-Aldrich Co., USA
Toluene	RCI Labscan Limited, Thailand

All of chemicals and reagents were analytical grade.

Materials

Cover glasses	Menzel-Glaser, Germany
Filter paper No.4	Whatman™, UK
Filter paper No.40 ashless	Whatman™, UK
Microscope slide	Sail Brand, China
TLC silica gel 60 GF_{254}	Merck KGaA, Germany
- 20 x 10 cm, 0.2 mm thickness	

Instruments and equipments

Ashing furnace	Carbolite Limited, UK
Autoclave	ALP Co., Ltd, Japam
Balance readability 0.01 g	Pioneer™ Ohaus Corporation, USA
Balance readability 0.0001 g	Adventurer™ Ohaus Corporation, USA
Canon Power shot A650 IS camera	Canon Marketing (Thailand) Co., Ltd. Thailand
Hot air oven	WTC Binder, Germany

Micropipette	BrandTech Scientific, Inc., Germany
Microscope	Carl Zeiss model Axio Lab, Germany
Rotary vacuum evaporator	BÜCHI Labortechnik AG, Switzerland
Sample Concentrator	Brinkmann, USA
Shaker	Adolf Kuhner AG, Switzerland
Soxhlet apparatus	
Spectrophotometer	PG Instruments Limited, UK
TLC Chamber	CAMAG, Switzerland
TLC-densitometry instrument	CAMAG, Switzerland
- Linomat 5 applicator	
- TLC scanner 3	
Ultrasonic bath	Analytical Lab Science Co., LTD, Thailand
UV fluorescence analysis cabinet	Spectronics Corporation, USA

Computer software

ImageJ software	The National Institute of Mental Health, USA
winCATS software	CAMAG, Switzerland

Methods

Plant materials

Fifteen samples of *X. indica* flowering heads were collected from different sources in Thailand. All samples were authenticated by Ruangrungsi N. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. The samples were dried in hot air oven at 45°C and ground to powders and kept in the closed container.

Macroscopic and microscopic examinations

For macroscopic examinations, the whole plant of *X. indica* was drawn by hand-drawing with scale relative to the real size of plant. The dried flowering head of *X. indica* was evaluated with the organoleptic characters that including shape, size, colour, odour, taste etc.

Microscopic examination of *X. indica* flowering heads was evaluated in ground sample for histological characters. The powders were sieved through 250 micron sieve and mounted onto a slide in water for observation of cell and tissue

structures under microscope with 10X, 20X, and 40X objective lens magnifications and 10X eyepiece lens. Pictures were taken with a digital camera. The histological characters were drawn in proportional scale related to the original size.

Determination of loss on drying

Weighed exactly 3 g of the ground sample using digital balance (readability 0.0001 g) and placed in a pre-weighed crucible. Heated at 105°C for about 6-8 hours until constant weight was obtained, cooled in a desiccator and weighed. The loss of weight was calculated in a percentage of the dried sample. Each sample was tested in triplicate.

Determination of total ash

Ignited the crucible with dried sample aforementioned in an incinerator. The temperature was gradually ramped and held at 500ºC until carbon free ash (white grey ash) was obtained. The crucible was cooled in a desiccator, weighed immediately and calculated the content of total ash in a percentage with reference to the dried sample. Each sample was tested in triplicate.

Determination of acid-insoluble ash

The crucible containing the total ash was added with 25 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 crucible. The insoluble matter was collected on an ashless filter-paper No.40 and washed with hot water until the filtrate was neutral. Transferred the filter paper containing the insoluble matter to the original crucible, evaporated to dryness, ignited at a 500ºC to get a constant weight of ash. Allowed the residue to cool in a desiccator and weighted immediately, calculated the content of acid-insoluble ash in a percentage with reference to the dried sample. Each sample was tested in triplicate.

Determination of water-soluble extractive value

Five grams of the ground sample were exactly weighed and macerated with 70 ml of distilled water in a closed conical flask, shaked frequently during 6 hours and allowed to stand for 18 hours. Filtered, rinsed the marc and adjusted to 100 ml of final volume. Transferred 20 ml of the filtrate to a pre-weighed small beaker and evaporated to dryness on a water bath, dried at 105ºC to constant weight. Cooled in a

desiccator and weighed immediately. The content of water extractable matter was calculated in a percentage with reference to the dried sample. Each sample was tested in triplicate.

Determination of ethanol-soluble extractive value

Five grams of the ground sample were exactly weighed and macerated with 70 ml of 95% ethanol in a closed conical flask, shaked frequently during 6 hours and allowed to stand for 18 hours. Filtered rapidly, taking precautions against loss of solvent, rinsed the marc and adjusted to 100 ml of final volume. Transferred 20 ml of the filtrate to a pre-weighed small beaker and evaporated to dryness on a water bath, dried at 105ºC to constant weight. Cooled in a desiccator and weighed immediately. The content of ethanol extractable matter was calculated in a percentage with reference to the dried sample. Each sample was tested in triplicate.

Determination of water content

Fifty grams of the ground sample were distilled in 200 ml of water-saturated toluene with azeotropic apparatus. Sample was boiled until no more water distilled over. After the toluene and the water layers in receiving tube were separated completely, the volume of water distilled over was read and calculated the content of water in a percentage with reference to the dried sample. Each sample was tested in triplicate.

Determination of volatile oil

One hundred grams of the ground sample were distilled in 600 ml of water with Clevenger apparatus. Sample was boiled until the volatile oil completely distilled. The volume of volatile oil was read and calculated the content of volatile oil in a percentage with reference to the dried sample. Each sample was tested in triplicate.

Thin layer chromatographic fingerprint

Transferred another 20 ml of the ethanolic filtrate mentioned above and evaporated to dryness. Re-dissolved in 1 ml of ethanol. Spotted 3 µl onto Silica gel 60 GF_{254} TLC plate and developed for about 8.0 cm using a mixture of petroleum ether and ethyl acetate (8:1) as mobile phase. After development, the plate was dried and

visualized under UV light at 254 nm, 365 nm in UV cabinet. TLC plate was finally dipped in 5% potassium hydroxide staining reagent.

Quantitative analysis of chrysazin in *Xyris indica*

Preparation of chrysazin standard solution

The stock solution of chrysazin (0.5 mg/ml) was prepared in 95 % ethanol and diluted to obtain the series of standard solutions with concentration of 15, 30, 45, 60 and 75 μg/ml.

Preparation of benzene extracts of *Xyris indica* flowering heads

To determine the content of chrysazin in 15 samples of *X. indica* flowering heads, the ground samples (5 g) were exhaustively extracted with benzene by soxhlet apparatus. The extract was filtered. Then, the solvent was evaporated by rotary evaporator. The yield was recorded. Each extract was dissolved in 95 % ethanol to obtain a concentration of 5.0 mg/ml. For further assayed by TLC-densitometry.

TLC-densitometry of chrysazin

Three microliters of 15 benzene extracts and 5 standard solutions (15- 75 μg/ml) were applied as 5.00 mm band length onto the silica gel 60 GF_{254} TLC plate by Linomat 5 applicator. A distance between each band was 8.9 mm. The slit dimension was 4.00 x 0.30 mm. The plate was developed to a distance of 8.0 cm in a TLC chamber that contained a mixture of petroleum ether and ethyl acetate (8:1) as mobile phase. After development, the plate was air dried. Developed TLC plate was scanned under wavelength of maximum absorbance at 430 nm for quantitative analysis of the band on TLC plates by TLC scanner 3. After scanning, the peak area of each band was carried out. The content of chrysazin was determined by comparing peak area to the calibration curve obtained from the same TLC plate. All of instruments were operated by winCATS software. The quantitative analysis was performed in triplicate.

TLC image analysis of chrysazin by ImageJ software

Developed TLC plate from above was photographed under UV 365 nm by a digital camera and stored as JPEG files. The content of chrysazin was quantitated by using ImageJ software. The image file was opened with ImageJ software and

smoothened with smooth function 5 times. Then a rectangular tool was used to crop the interested spot. After the first spot was cropped, number one bottom on keyboard was pressed. Number two bottom was pressed when the next spot was cropped and number 3 bottom was pressed when the last spot was cropped. The result from a rectangular tool showed peaks in each spot. The straight line was selected for drawing the line under the peak. The peak area was given from a wand tool by clicking in the peak which was wanted to know its area. The content of chrysazin was determined by comparing peak area to the calibration curve obtained from the same TLC plate.

Method validation

Linearity

Regression line and correlation coefficient of the calibration curve were established by Microsoft Excel 2007. Each test was done in triplicate.

Specificity

Specificity was evaluated by peak identity. For identity checking, the UV/VIS spectrum of the chrysazin peak from the samples was compared to the spectrum of standard chrysazin, which developed on the same plate. The developed TLC plate was scanned spectrum in the range of 300-600 nm by TLC scanner 3.

Accuracy

The accuracy of this method was examined as % recovery by the addition of known amount of standard chrysazin into the sample. Three different levels of standard were added to the sample. Three examinations were performed at each level. Each test was done in triplicate. The percentage recovery was determined by following formula.

$$\textbf{\% Recovery} = [C1/(C2+C3)] \times 100$$

C_1 = the amount of chrysazin found in spiked sample

C_2 = the amount of chrysazin found in un-spiked sample

C_3 = the amount of standard chrysazin added to the sample

Precision

The precision of this method was evaluated by repeatability and intermediate precision. Repeatability was evaluated by analyzing sample solutions that contained three different concentrations of chrysazin on the same day. Intermediate precision was evaluated by analyzing sample solutions that contained three different concentrations of chrysazin on three different days. Each test was done in triplicate. The contents of chrysazin were determined and the coefficient of variation was calculated as % relative standard deviation (%RSD) by following formula.

$$\% \text{ RSD} = \text{SD} \times 100/\text{Mean}$$

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ were evaluated from the calibration curves of chrysazin using following formula. Each test was done in triplicate.

$$\text{LOD} = 3.3\ \sigma/S$$

$$\text{LOQ} = 10\ \sigma/S$$

σ = the standard deviation of y-intercepts

S = the slope of the calibration curve

Robustness

Robustness test was examined by introducing small changes in mobile-phase composition. Eight mg/ml of sample was applied 3 μl/spot onto TLC plate. Mobile phases having different compositions of petroleum ether: ethyl acetate (8:1 v/v, 8.1:0.9 v/v, 8.2:0.8 v/v, 7.9:1.1 v/v and 7.8:1.2 v/v) were used and TLC plates were developed. The % RSD of chrysazin peak area among mobile phase variations was calculated.

Efficacy evaluation

Antimicrobial activities

Microorganisms

Thirteen microorganisms included gram positive bacteria, gram negative bacteria and fungi were shown in Table 2.

Table 2. The microorganisms

Microorganisms	
Gram positive bacteria	*Basillus cereus* ATCC11778[3] *Bacillus subtilis* ATCC6633[1] *Micrococcus luteus* ATCC9341[3] *Staphylococcus aureus* ATCC6538P[1] *Staphylococcus epidermidis* (Isolates) [2]
Gram negative bacteria	*Escherichia coli* ATCC25922[1] *Enterobacter aerogenes* ATCC13048[3] *Pseudomonas aeruginosa* ATCC9027[1] *Salmonella typhi* (Isolates) [3] *Salmonella typhimurium* ATCC13311[2] *Shigella spp.* (Isolates) [2]
Fungi	*Candida albicans* ATCC10230[1] *Saccharomyces cerevisiae* ATCC9763[1]

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

[2] Department of Microbiology, Faculty of Sciences, Chulalongkorn University

[3] Department of Microbiology, Faculty of Sciences and Technology, Suan Sunandha Rajabhat University

Preparation of inoculums

Bacteria were cultivated on Mueller Hinton agar (MHA) at 37°C for 18-24 hours. Fungi were cultivated on Sabouraud Dextrose agar (SDA) at 24-48 hours. Suspended 4-5 well-isolated colonies in 0.85 % normal saline and adjusted the

turbidity of this suspension to get the absorbance of 0.08-0.10 by spectrophotometry at 625 nm with 1-cm light path. This turbidity was equivalent to 0.5 McFarland standard or 1 x 10^8 CFU/ml [59].

Agar-overlay bioautography

Developed TLC plate of chrysazin from benzene extract of *X. indica* flowering heads was performed. The extract concentration was 100 mg/ml in 95% ethanol and the volume applied was 10 µl onto the Silica gel 60 GF_{254} TLC plate. The mobile phase was petroleum ether and ethyl acetate (8:1).

Standard chrysazin was dissolved in chloroform to obtain a concentration of 60 mg/ml (maximum solubility). These amounts of standard chrysazin (5, 10, 20 µl) was applied onto Silica gel 60 GF_{254} TLC plate, left dried for complete removal of chloroform.

Fifteen millimeter of sterile nutrient agar (MHA for bacteria and SDA for fungi) was poured into a Petri dish as base agar and allowed to set. TLC plate of benzene extract and chrysazin standard were placed on the nutrient agar base and 6 ml of seeded agar medium that contained 100 µl of selected microorganism was poured over it. The inoculated plates were allowed to solidify and incubated at 37 ºC, 18-24 hours for bacteria and 24-48 hours for fungi. The plates were further stained with 0.05 % tetrazolium blue chloride and further incubated at 37 ºC until the color of surface agar changed. Zone of inhibition was recorded as clear zone against a blue or violet background [56,57,59].

Data analysis

The results of pharmacognostic evaluation were expressed as grand mean ± pooled SD. The contents of chrysazin between TLC image analysis and TLC-densitometry were compared by paired t-test statistical analysis.

CHAPTER IV
RESULTS

Pharmacognostic specification

The macroscopic characteristics of *Xyris indica* described that this plant is a perennial herb to 30-60 cm high which is grass-like. Stems are tufted and erect. Dark green leaves are narrowly linear, thin, and glabrous with a conspicuous sheath at the base, entire at margin, acute to obtuse at apex. The leaf size is 0.4-0.5 × 13-40 cm. Peduncle is 31-53 cm long. The small yellow flowers are borne singly in the axils of scale-like bracts. Bracts are densely overlapping and form a compact head or spike, mostly 0.5-1.4 × 0.5-2.2 cm. The yellowish brown bracts are suborbicular or ovate, obtuse to truncate at base, membranous and golden-yellow along margin, entire or emarginated at apex, 5-8 × 5-7 mm. There are 3 yellow sepals. 2 lateral sepals are boat-shaped, 0.8-1.4 × 5-7mm. Median sepal is cap- shaped, 2-2.5 × 4-6 mm, enclosing the petals. There are 3 yellow petals, obovate, serrate at apex, 3-4 × 3-4.5 mm. For stamen, there are 3 fertile stamens with anthers 2 lobed, 2-4 mm and 3 sterile stamens (staminodes) with hairy, 2-3 mm. Ovary is obovoid, 1.5 x 1 mm. 3 styles is 2-3 mm long with capitate stigmas. Seeds are ovoid or ellipsoid, 0.3-0.5 mm long. The picture of whole plant of *X. indica* was drawn and shown in Figure 7.

The organoleptic characteristics of *X. indica* dried flowering heads and flowering heads powder were evaluated with shape, size, colour, odour, taste. The colour of dried flowering heads was shown in brown bract and yellow flowers. Shape and size of dried flowering heads including component inside and outside were nearly fresh flowering heads. The flowering heads powder was brown in colour with slightly characteristic odour and slightly bitter, astringent taste. Dried flowering heads of *X. indica* was used as crude drug (Figure 8).

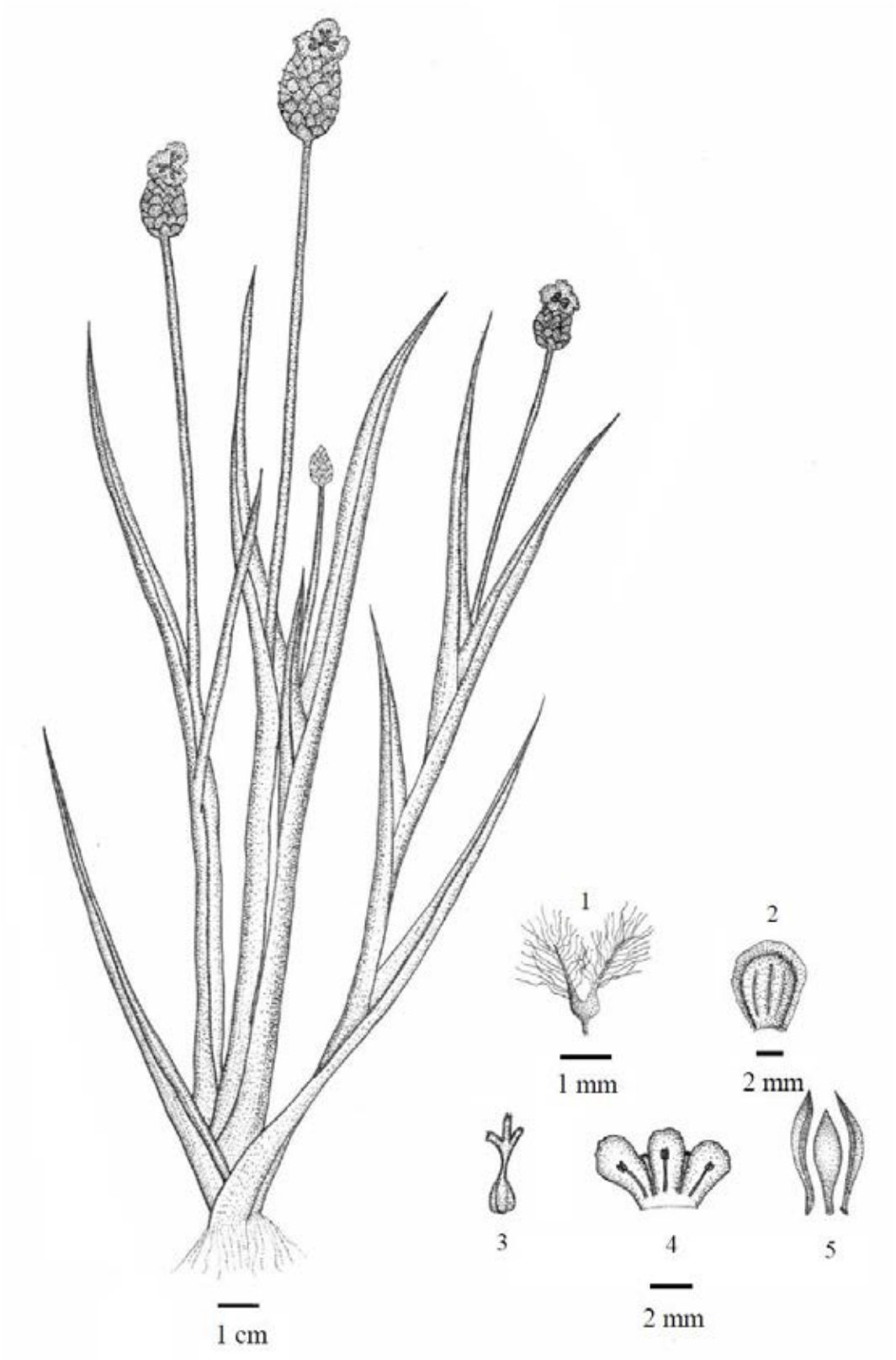

Figure 7. The whole plant of *Xyris indica* L. 1. staminode 2. bract 3. pistil 4. corolla laid open 5. sepals

Figure 8. Crude drug of *Xyris indica* L. flowering heads

The histological characteristics consisted of parenchyma, fragment of fiber, sclereid, fragment of corolla, seeds, pollen grain and staminode (Figure 39).

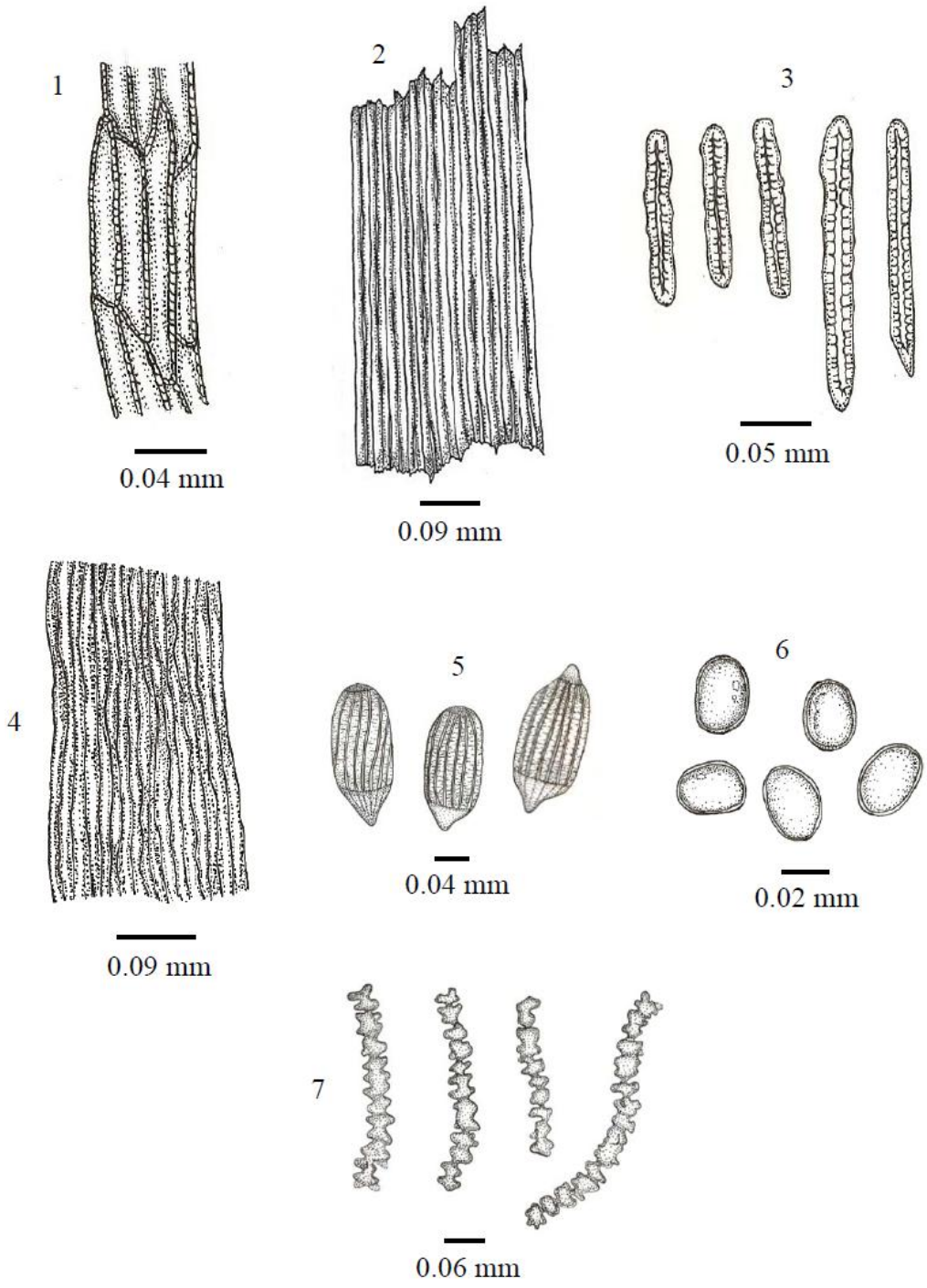

Figure 9. Histological characteristics of the powder of *Xyris indica* L. flowering heads 1. parenchyma of bract 2. fragment of fibers of bract 3. sclereid of bract 4. fragment of corolla 5. seeds 6. pollen grains 7. staminodes

Thin layer chromatographic fingerprint of ethanolic extract of *X. indica* flowering heads was shown in Figure 10.

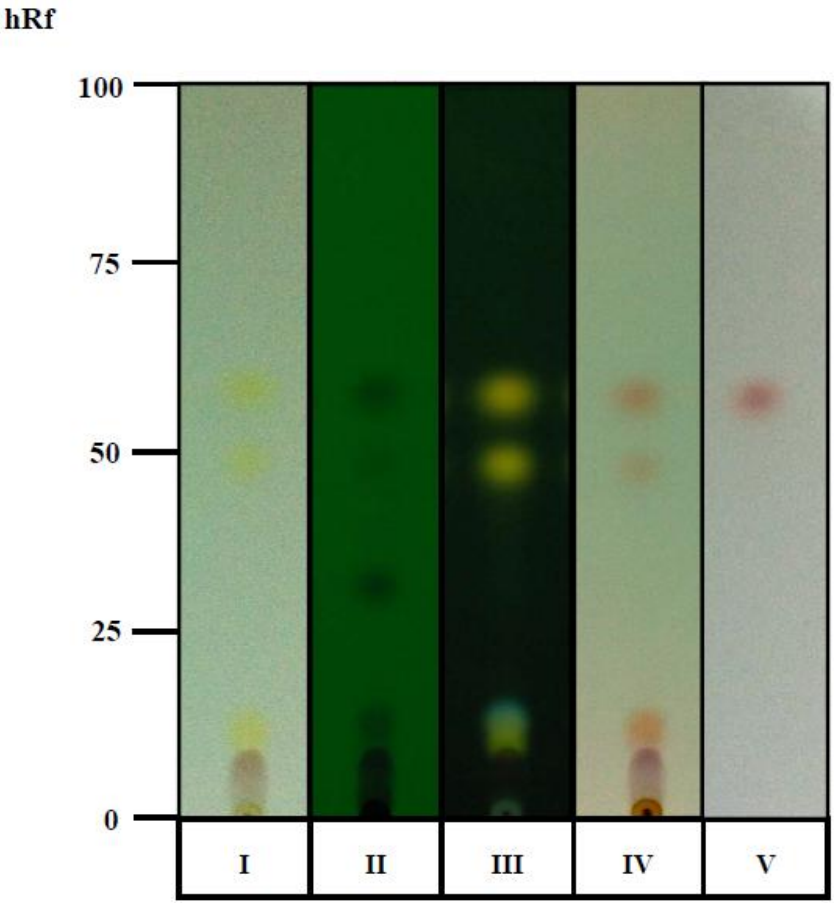

Figure 10. TLC fingerprint of ethanolic extract of *Xyris indica* flowering heads (I-IV) and chrysazin standard (V)

Adsorbent Silica gel 60 GF254

Solvent system Petroleum ether: ethyl acetate 8:1

Detection I = under daylight

II = under UV light 254 nm

III = under UV light 365 nm

IV, V = staining with 5% potassium hydroxide in methanol

The constant numbers due to the quality of *X. indica* flowering heads were shown in Table 3. The loss on drying, total ash, acid insoluble ash and water content should be not more than 6.899, 2.497, 0.409 and 11.121 % of dry weight respectively while water-soluble extractive and ethanol-soluble extractive values should be not less than 6.592 and 4.030 % of dry weight respectively.

Table 3. The constant numbers due to the quality of *Xyris indica* flowering heads

Content (% by weight)	Mean ± SD	Range (Mean ± 3SD)
Loss on drying	6.899 ± 0.165	6.403 - 7.395
Total ash	2.497 ± 0.033	2.399 - 2.595
Acid-insoluble ash	0.409 ± 0.027	0.329 - 0.490
Water-soluble extractive	6.592 ± 0.474	5.170 - 8.014
Ethanol-soluble extractive	4.030 ± 0.486	2.573 - 5.487
Water content	11.121 ± 1.132	7.725 - 14.518
Volatile oil content	0	0

*The parameters were shown as grand mean ± pooled SD. Samples were from 15 different sources in Thailand. Each sample was tested in triplicate.

Benzene extraction of *Xyris indica* flowering heads

The percent yield of benzene extracts of *X. indica* flowering heads were 2.26 - 6.91% by weight and the average was 3.55±1.34 % by weight (Table 4).

Table 4. The percent yield of benzene extracts of *Xyris indica* flowering heads

Source	weight of sample (g)	weight of extractive value (g)	% yield
1	5.0062	0.1533	3.0622
2	5.0085	0.2212	4.4165
3	5.0063	0.1720	3.4357
4	5.0041	0.3094	6.1829
5	5.0032	0.1582	3.1620
6	5.0053	0.1916	3.8279
7	5.0056	0.3461	6.9143
8	5.0090	0.1315	2.6253
9	5.0085	0.1628	3.2505

Source	weight of sample (g)	weight of extractive value (g)	% yield
10	5.0086	0.1132	2.2601
11	5.0088	0.1270	2.5355
12	5.0049	0.1543	3.0830
13	5.0069	0.1428	2.8521
14	5.0080	0.1379	2.7536
15	5.0080	0.1411	2.8175
		Average	**3.5453±1.3361**

Specificity

The UV/VIS spectrum of chrysazin separated from samples showed identical absorption pattern with standard chrysazin supported by the wavelength of maximum absorbance at 430 nm (Figure 66).

Chrysazin quantitation by TLC-densitometry

Calibration curve

The calibration curve of chrysazin by TLC densitometric method was linear with the regression equation of y = 98.67x - 147.7 and its correlation coefficient (R^2) of 0.9997. The linearity range of chrysazin was 15.00-75.00 µg/ml.

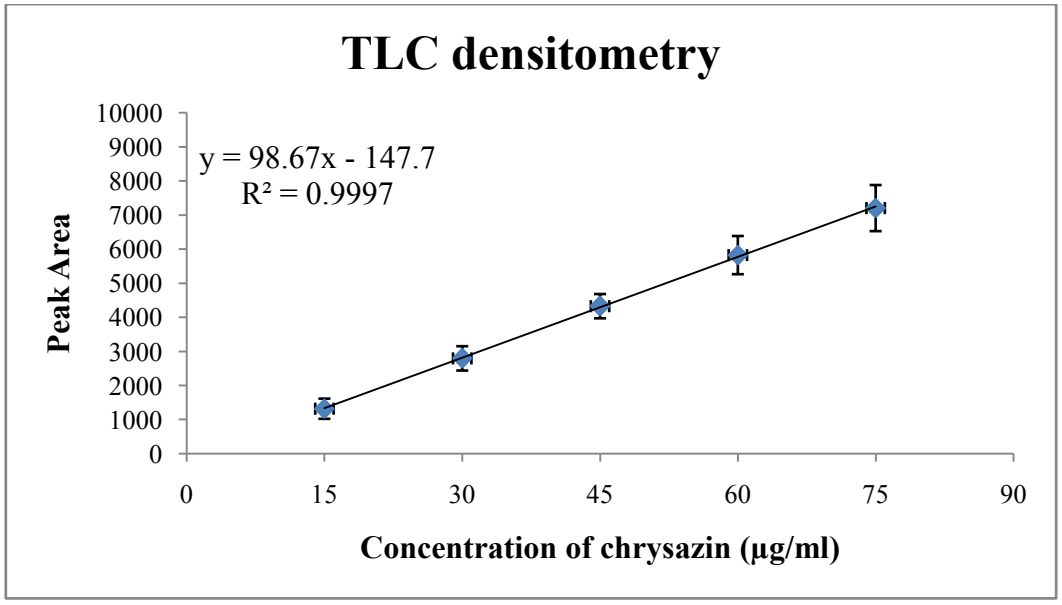

Figure 11. The calibration curve of chrysazin by TLC-densitometry

Accuracy

The accuracy of chrysazin content by TLC densitometric method was performed using recovery studies. The samples were spiked with known amount of standard chrysazin at three levels. The percent recoveries were in the range of 90.67-99.16 % (Table 8).

Table 5. Recovery of chrysazin in *Xyris indica* flowering heads by TLC-densitometry (n = 3)

Chrysazin added (µg/ml)	Chrysazin found in spike sample (µg/ml)	% Recovery
0	26.029±0.797	-
10	35.708±0.990	99.16
25	46.694±0.408	91.51
45	64.398±0.489	90.67

Precision

Repeatability and intermediate precision by TLC-densitometry method were performed in triplicate with sample solutions that contained three different concentrations of chrysazin. The repeatability and intermediate precision were in range of 0.76 – 2.77 %RSD and 8.40 – 14.28 %RSD, respectively (Table 6).

Table 6. Repeatability and intermediate precision of chrysazin in *Xyris indica* flowering heads by TLC-densitometry

Chrysazin content (µg/ml)	Repeatability (%RSD) (n = 3)	Intermediate precision (%RSD) (n = 9)
10	2.77	8.40
25	0.87	11.72
45	0.76	14.28

LOD and LOQ

LOD and LOQ in TLC-densitometry were evaluated based on the standard deviation of y-intercepts and the slope of the calibration curve. The slope and the standard deviation of y-intercepts were calculated from 3 calibration curve. The slope value and the standard deviation of y-intercepts were 98.65 and 163.97, respectively. The LOD and LOQ values for TLC-densitometry were 5.48 µg and 16.62 µg, respectively.

Robustness

The peak area of chrysazin in *X. indica* flowering heads by each mobile phase was shown in Table 7. Mean and SD of peak areas was calculated. The robustness of this method was 4.44 %RSD.

Table 7. Robustness of chrysazin in *Xyris indica* flowering heads by TLC-densitometry

Mobile phase composition (petroleum ether: ethyl acetate v/v)	**Peak area**
8.0:1.0	4818.4
8.1:0.9	4813.9
8.2:0.8	5319.4
7.9:1.1	5014.9
7.8:1.2	4819.0
mean±SD	**4957.12±219.90**

The content of chrysazin in *Xyris indica* flowering heads

The chrysazin content in benzene extracts of *X. indica* flowering heads from all samples were determined in triplicate by TLC-densitometry and calculated as grams of chrysazin per 100 grams of the crude drug.

Table 8. The content of chrysazin in *Xyris indica* flowering heads by TLC-densitometry

Source	Chrysazin in the benzene extract (mg /g)		Yield of benzene extract (g/100 g of dried crude drug)	Chrysazin in *Xyris indica* flowering heads (g/100 g of dried crude drug)	
	Mean*	SD		Mean	SD
1	4.6761	0.0617	3.0622	0.0143	0.0002
2	5.2127	0.2632	4.4165	0.0230	0.0012
3	5.1198	0.1528	3.4357	0.0176	0.0005
4	6.1690	0.2910	6.1829	0.0381	0.0018
5	6.5891	0.0899	3.1620	0.0208	0.0003
6	3.8202	0.1889	3.8279	0.0146	0.0007
7	5.6341	0.1971	6.9143	0.0390	0.0014
8	4.8159	0.2708	2.6253	0.0126	0.0007
9	5.2651	0.2892	3.2505	0.0171	0.0009
10	6.5290	0.5560	2.2601	0.0148	0.0013
11	10.2437	0.5035	2.5355	0.0260	0.0013
12	6.9560	0.5400	3.0830	0.0214	0.0017
13	8.4143	0.3758	2.8521	0.0240	0.0011
14	8.6529	0.2957	2.7536	0.0238	0.0008
15	9.9142	0.0733	2.8175	0.0279	0.0002
Average**	6.5341±0.3172		3.5453±1.3361	0.0223±0.0011	

* n=3 ** grand mean ± pooled SD

Chrysazin quantitation by TLC image analysis using ImageJ software

Calibration curve

The calibration curve of chrysazin by TLC image analysis was linear with the regression equation of y = 480.2x + 586.7 and its correlation coefficient (R^2) of 0.9986. The linearity range of chrysazin was 15.00-75.00 µg/ml.

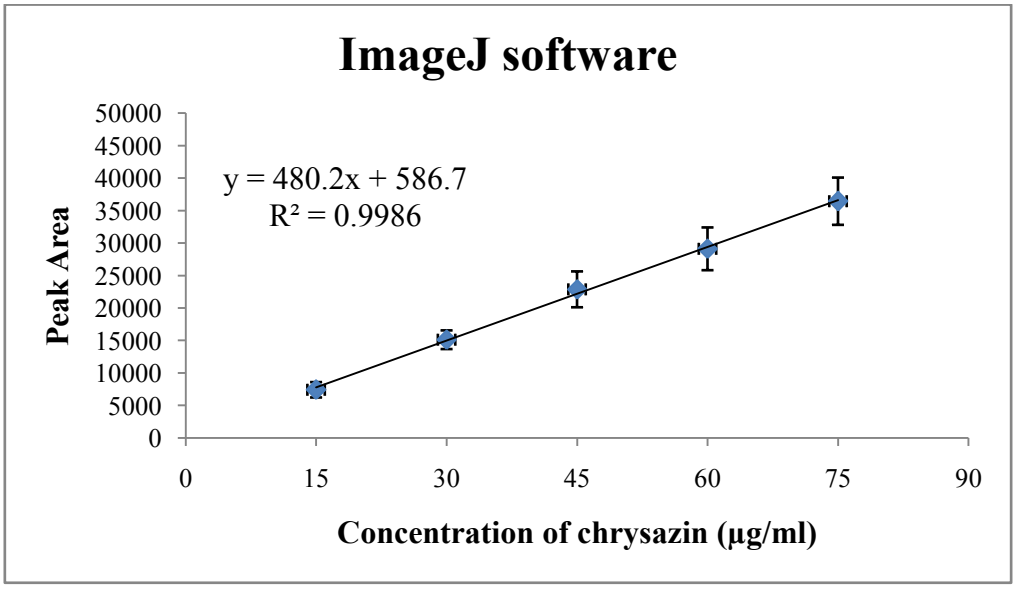

Figure 12. The calibration curve of chrysazin by TLC image analysis

Accuracy

The accuracy of chrysazin content by TLC image analysis method was performed using recovery studies. The samples were spiked with known concentration of standard chrysazin at three levels. The percent recoveries were in the range of 91.87-96.00 % (Table 9).

Table 9. Recovery of chrysazin in *Xyris indica* flowering heads by TLC image analysis (n = 3)

Chrysazin added (µg/ml)	Chrysazin found in spike sample (µg/ml)	% Recovery
0	25.808±0.152	-
10	34.375±1.052	96.00
25	47.296±0.947	93.09
45	65.050±0.808	91.87

Precision

Repeatability and intermediate precision by TLC image analysis method were performed in triplicate with sample solutions that contained three different concentrations of chrysazin. The repeatability and intermediate precision were in range of 0.78 – 3.10 %RSD and 2.46 – 3.95 %RSD, respectively (Table 10).

Table 10. Repeatability and intermediate precision of chrysazin in *Xyris indica* flowering heads by TLC image analysis

Chrysazin content (µg/ml)	Repeatability (%RSD) (n = 3)	Intermediate precision (%RSD) (n = 9)
10	3.06	3.95
25	0.78	3.65
45	1.02	2.46

LOD and LOQ

LOD and LOQ in TLC image analysis were evaluated based on the standard deviation of y-intercepts and the slope of the calibration curve. The slope and the standard deviation of y-intercepts were calculated from 3 calibration curve. The slope value and the standard deviation of y-intercepts were 480.20 and 858.94, respectively. The LOD and LOQ values for TLC image analysis were 5.90 µg and 17.89 µg, respectively.

Robustness

The peak area of chrysazin in *X. indica* flowering head by each mobile phase was shown in Table 11. Mean and SD of peak areas was calculated. The robustness of this method was 3.60 %RSD.

Table 11. Robustness of chrysazin in *Xyris indica* flowering heads by TLC image analysis

Mobile phase composition (petroleum ether: ethyl acetate v/v)	Peak area
8.0:1.0	27083.59
8.1:0.9	25833.82
8.2:0.8	27773.54
7.9:1.1	27264.23
7.8:1.2	28503.69
mean±SD	**27291.77±983.69**

The content of chrysazin in *Xyris indica* flowering heads

The chrysazin content in benzene extracts of *X. indica* flowering heads from all samples were determined in triplicate by image analysis and calculated as grams of chrysazin per 100 grams of the crude drug.

Table 12. The content of chrysazin in *Xyris indica* flowering heads by TLC image analysis

Source	Chrysazin in the benzene extract (mg /g)		Yield of benzene extract (g/100 g of dried crude drug)	Chrysazin in *Xyris indica* flowering heads (g/100 g of dried crude drug)	
	Mean*	SD		Mean	SD
1	4.4750	0.2384	3.0622	0.0137	0.0007
2	5.4506	0.2242	4.4165	0.0241	0.0010
3	5.3323	0.4182	3.4357	0.0183	0.0014
4	6.0822	0.0693	6.1829	0.0376	0.0004
5	6.4822	0.3459	3.1620	0.0205	0.0011
6	3.8254	0.0799	3.8279	0.0146	0.0003
7	5.4480	0.2102	6.9143	0.0377	0.0015
8	4.7073	0.0877	2.6253	0.0124	0.0002
9	5.1106	0.0920	3.2505	0.0166	0.0003
10	6.7297	0.1740	2.2601	0.0152	0.0004
11	9.8296	0.0875	2.5355	0.0249	0.0002
12	6.6584	0.1714	3.0830	0.0205	0.0005
13	7.7139	0.1388	2.8521	0.0220	0.0004
14	8.6815	0.0400	2.7536	0.0239	0.0001
15	9.4145	0.1328	2.8175	0.0265	0.0004
Average**	6.3961±0.1965		3.5453±1.3361	0.0219±0.0007	

* n=3 ** grand mean ± pooled SD

Comparison of chrysazin contents between TLC-densitometry and TLC image analysis

Comparison of chrysazin contents between TLC-densitometry and TLC image analysis were statistically tested using paired T-test statistical analysis. The result was shown that the chrysazin contents by both methods were not significantly different ($P = 0.064$) (Table 13).

Table 13. Comparison of chrysazin contents between TLC-densitometry and TLC image analysis

Source	% Chrysazin content	
	TLC-densitometry	**TLC image analysis**
1	0.0143	0.0137
2	0.0230	0.0241
3	0.0176	0.0183
4	0.0381	0.0376
5	0.0208	0.0205
6	0.0146	0.0146
7	0.0390	0.0377
8	0.0126	0.0124
9	0.0171	0.0166
10	0.0148	0.0152
11	0.0260	0.0249
12	0.0214	0.0205
13	0.0240	0.0220
14	0.0238	0.0239
15	0.0279	0.0265
Average	0.0223±0.0011	0.0219±0.0007

Antimicrobial activities

Agar-overlay bioautography

The developed TLC plate of benzene extract of *X. indica* flowering heads showed two spot of compounds. The above spot is chrysazin. (Figure 13). Agar-overlay bioautography was performed in triplicate.

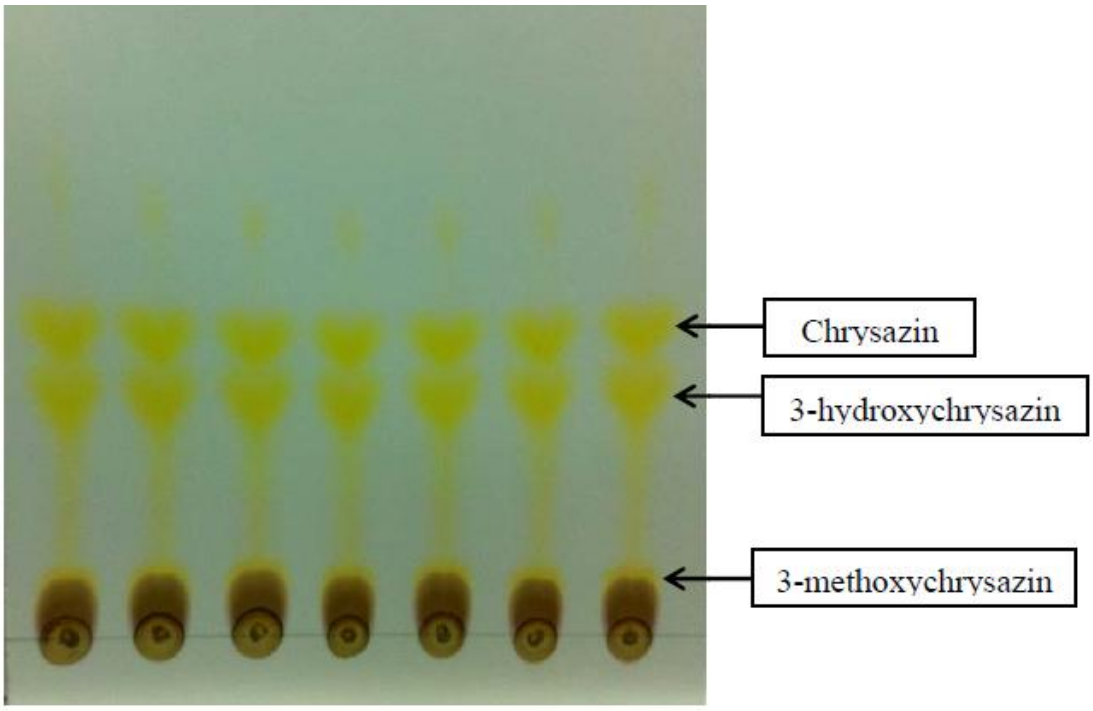

Figure 13. The developed TLC plate of benzene extract of *Xyris indica* flowering heads for performing agar-overlay bioautography

The chrysazin spot that was separated from benzene extract revealed antibacterial activities against *Bacillus subtilis*. The spot of standard chrysazin on TLC plate also showed similar result (Figure 14).

The origin spot on TLC plate of benzene extract showed inhibition zone against *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Micrococcus luteus*. The result showed no inhibition zones against gram negative bacteria and fungi tested in this study (Figure 14). The results of antimicrobial assay were shown in Table 14.

Table 14. Antimicrobial potential of benzene extract of *Xyris indica* flowering heads and standard chrysazin by agar-overlay bioautography

Microorganisms	Inhibition Zone		
	Origin spot of benzene extract	Chrysazin in benzene extract	Standard chrysazin
Basillus cereus	-	-	-
Bacillus subtilis	+	+	+
Micrococcus luteus	+	-	-
Staphylococcus aureus	+	-	-
Staphylococcus epidermidis	+	-	-
Escherichia coli	-	-	-
Enterobacter aerogenes	-	-	-
Pseudomonas aeruginosa	-	-	-
Salmonella typhi	-	-	-
Salmonella typhimurium	-	-	-
Shigella spp.	-	-	-
Candida albicans	-	-	-
Saccharomyces cerevisiae	-	-	-

Each sample was tested in triplicate. - = no activity. + = activity

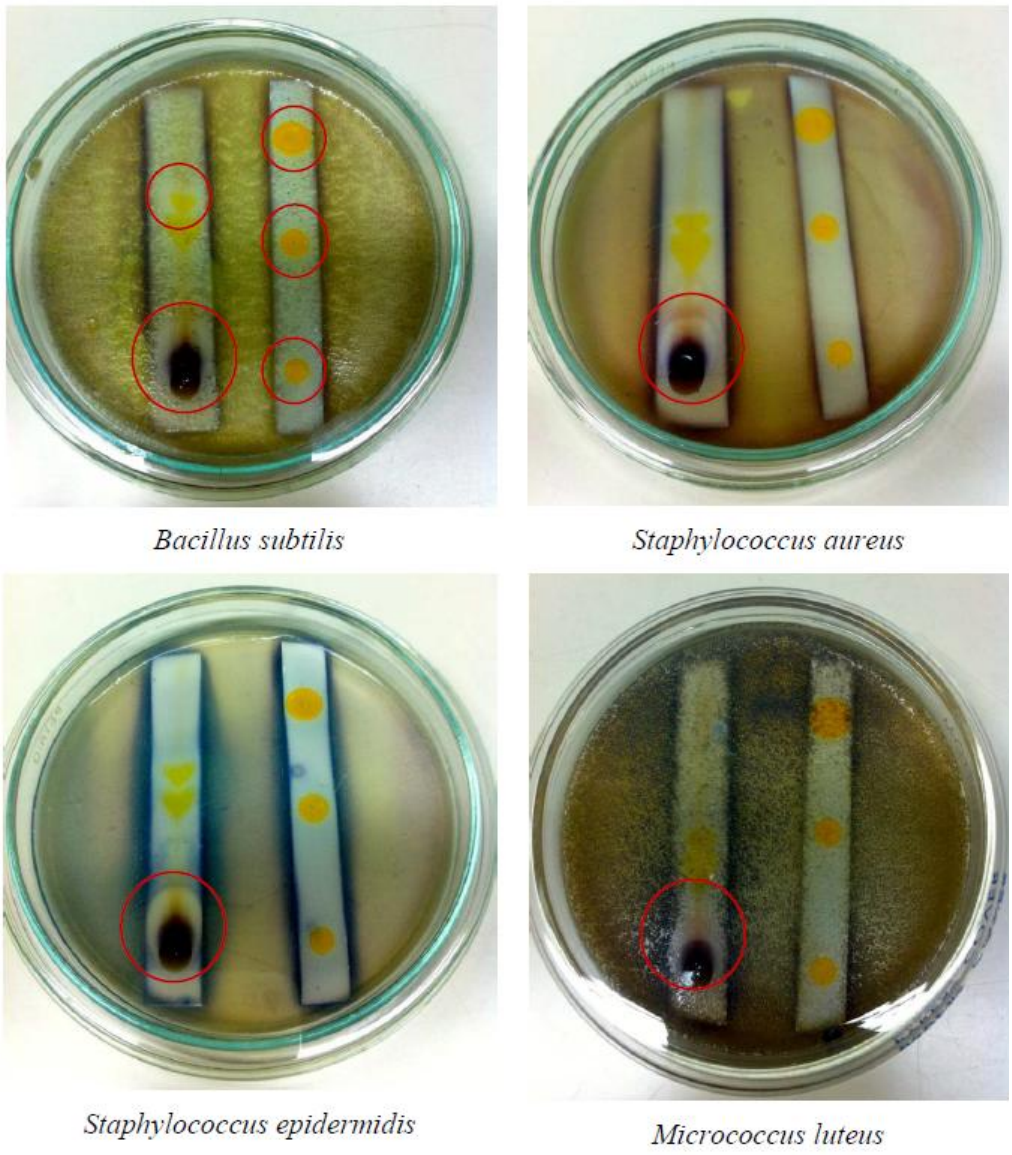

Figure 14. The clear zone of benzene extract (left strip on Petri dish) and chrysazin standard (right strip on Petri dish) after staining with tetrazolium blue chloride

CHAPTER V
DISCUSSION AND CONCLUSION

The quality control methods are important tool in traditional medicines which serve as useful information for identification, authentication and standardization of herbal medicine [62]. The safety and efficacy of herbal medicine are dependent on the standardization and quality of plant materials [63]. So, it's necessary for focusing on quality control and development of herbal medicines standardization which include important processes namely, macroscopic and microscopic examinations, physio-chemical parameters, fingerprint profiles and quantification of marker compound. After performing these standardization methods, all of the results can be published in an herbal pharmacopoeia.

The present study deals with the pharmacognostic evaluation and chrysazin content of *X. indica* flowering heads. It started with macroscopic and microscopic examinations. The microscopic characteristics of powdered *X. indica* flowering heads revealed that fragment of corolla, seeds, pollen grain and staminode were remarkable diagnostic characteristics of this plant part. Fragment of corolla, pollen grain and staminode were found only in flower. This procedure was determined for genuine identity and primary screening test for impurities of plant materials. It should be performed before any tests are undertaken [8]. This first step provides the information that can help to identify and authenticate plant materials. Correct identification is the right starting point of quality control. It can be help to certify which plant is the required species and the correct part of the plant is being taken that make for assuring quality, safety and efficacy of herbal medicines [64].

The physico-chemical analysis is an important parameter that used to establish numerical standard for plant materials. The constant numbers that revealed in this study could be useful for quality and purity of plant materials as well as detecting adulteration. Excessive moisture content in plant materials could encourage bacteria, fungi or yeast growth which leads to spoilage and makes possible for the enzymatic activity which causes compound destruction. So, limit of moisture content should be set for plant materials. The general requirement of moisture content in plant materials is not more than 13% w/w [65]. It was found that all plant materials determined in this

study showed acceptable quality of loss on drying and water content. A high ash value is indicative of contamination, substitution, adulteration, or carelessness in preparing the plant materials. The total ash and acid-insoluble ash were low in all samples. It indicated that the inorganic matter and non- physiological matter is less in *X. indica* flowering heads. Extractive values are useful to evaluate the chemical compounds present in plant materials and also helpful in estimation of specific compound soluble in a particular solvent. Water-soluble extractive and ethanol-soluble extractive of this plant showed low content of polar compounds.

Thin layer chromatographic fingerprint of ethanolic extract of *X. indica* flowering revealed clearly separated spot of chrysazin as the major compound, appearing dark spot under UV 254 nm and yellow fluorescent spot under UV 365 nm. The hRf value was 59 coinciding with the hRf value of standard chrysazin which developed on the same TLC plate. It was similar to previously reported that performed with the same mobile phase and obtained the hRf value of 63 [13]. The compound turned into pink spot with 5% potassium hydroxide. This staining reagent proved that chrysazin is anthraquinone compound. The developed TLC plate showed the pattern of phytochemical characteristic components that can be used to identify the authenticity of plant materials.

TLC was selected for quantitative analysis of chrysazin. It is appropriate chromatographic technique for separation and quantification of compound in the samples. The mobile phase is crucial factor for separation of target compound. Many trials for optimal solvent system were performed following the literature reviews for separating chrysazin. The mixture of petroleum ether and ethyl acetate (8:1) showed good separation for chrysazin on developed TLC plate. The yellow fluorescence spot of chrysazin was clearly detected under UV 365 nm and separated from other compounds in the samples. This mobile phase was also used in TLC fingerprint.

TLC-densitometry is widely used for quantitative analysis of major compound in medicinal plants which is convenient and easy to perform with automated equipments. TLC-densitometry is instrumental TLC. Each of the steps in the process has been automated with sample applicator, development chamber and scanning densitometer which controlled by software [45]. Unfortunately, all of equipments are expensive which some laboratories cannot afford.

To develop alternative TLC method for analyzing chrysazin content, TLC-densitometry and TLC image analysis using ImageJ software were performed to evaluate chrysazin content in *X. indica* flowering heads from 15 different sources in Thailand and compared the results between both methods. This study is the first time for quantitative analysis of chrysazin in *X. indica* flowering heads with both methods.

TLC-densitometry and TLC image analysis for quantitative analysis were validated in terms of specificity, linearity, accuracy, precision, LOD, LOQ and robustness to confirm that the analytical procedure employed reliable and accurate information.

The specificity of the TLC method was confirmed by comparing UV/VIS spectrum of the chrysazin peak in the sample with standard chrysazin peak. The result from scanning wavelength ranged 300-600 nm showed the identical absorption spectra with maximum absorbance at 430 nm. This wavelength is optimal wavelength for scanning developed TLC plate by scanning densitometer in this study that quantified chrysazin accurately.

For linearity, the calibration curves between peak area and concentration of both methods were plotted and showed very good linearity relationships with correlation coefficient (R^2) more than 0.99 in concentration range of 15.0-75.0 μg/ml.

The accuracy was examined from percent recovery by spiking known amount of chrysazin in a sample. The recovery value of TLC-densitometry was in the range of 90.67-99.16 % and TLC image analysis was in the range of 91.87-96.00 %. These results exhibited an acceptable accuracy of both methods.

The precision of these methods was studied using three concentration of standard chrysazin at 10, 25 and 45 μg/ml. The results showed that repeatability and intermediate precision of the methods were satisfactory as % RSD less than 15 in all cases [66]. However, the precision between both methods showed in different %RSD. TLC image analysis showed better value than TLC-densitometry. This might be caused by different operation of peak area between both methods. Peak area of TLC-densitometry automatically obtains by scanning densitometer that controlled under winCATS software while peak area of TLC image analysis manually obtains by man made.

Moreover, The LOD and LOQ values of TLC-densitometry were 5.48 and 16.62 µg, respectively and TLC image analysis were 5.90 and 17.89 µg, respectively. These values from both methods showed similar results which displayed sufficient sensitivity of the methods.

The robustness studied by changing composition of mobile phase (petroleum ether: ethyl acetate 8:1, 8.1:0.9, 8.2:0.8, 7.9:1.1, 7.8:1.2 v/v) was 4.44 % RSD of peak area from TLC-densitometry. For TLC image analysis, the robustness was determined under the same condition which provided lower result with 3.60 %RSD. Some parameters such as retardation factor (Rf) and peak purity can be affected by the changes in chromatographic conditions. The result of robustness indicated that changing mobile phase composition was not affected in both methods.

Chrysazin was chosen as marker for quantitative analysis of *X. indica* flowering heads. The chrysazin contents of *X. indica* flowering heads from 15 different sources in Thailand analysed by TLC-densitometry were between 0.0126-0.0390 g/100 g of dried crude drug and the grand average chrysazin content among all samples was 0.0223 ± 0.0011 g/100 g of dried crude drug. The content by TLC image analysis were between 0.0124-0.0377 g/100 g of dried crude drug and the grand average chrysazin content among all samples was 0.0219 ± 0.0007 g/100 g of dried crude drug. For TLC-densitometry, the developed TLC plates were scanned under wavelength of maximum absorbance at 430 nm which closed to the previous study. Maximum UV absorption spectrum of chrysazin was 426 nm [13].

The previous report of chrysazin isolation from *X. indica* showed that for 1 kg of dried flowering heads extracted with chloroform by soxhlet apparatus and purified by silica gel column chromatography, 974 mg of chrysazin was isolated as dark orange colour [13,16]. For other species, dried leaves and stems of *X. semifuscata* by means of steam distillation followed by column chromatography on silica gel reported 50 mg of chrysazin as dark orange crystals from 30 g of plant materials [28]. Chrysazin also occurs naturally in *Cassia*, *Aloe*, *Rheum* and *Rhamnus* species [36].

The chrysazin content of *X. indica* flowering heads from 15 different sources in Thailand that obtained from TLC-densitometry and TLC image analysis were compared statistically using paired t-test statistical analysis. It was indicated that the chrysazin content from both methods were not significantly different with $P > 0.05$.

Thus, the result from method validation and paired t-test statistical analysis indicated that TLC image analysis is efficient, reliable and suitable technique for using in quantitative analysis of chrysazin in *X. indica*. Moreover, this study suggests that TLC image analysis method can be use as alternative method for any laboratory due to its advantages which is easy to perform, fast, inexpensive instruments. Today the digital camera with charge-coupled device image sensor becomes more widely used because it is much faster and efficient than scanning densitometer [38]. Image analysis using ImageJ software was not required sophisticated instrument and easily applicable. The software is easy to operate as well [53]. In addition, it has enabled the simple and cost effective use of TLC as a quantitative analysis. The combined technique, digitally enhanced-thin layer chromatography, is applicable to determine accurate amount of compound in sample solution [67].

Besides, ImageJ software, other image analysis softwares can also be used in TLC quantitative analysis. For example, Photoshop 7.0 and Scion image software were chosen to determine the amount of three curcuminoid in *Curcuma longa*. The techniques were validated and showed to be accurate and reliable method [68,69]. Some analysis software was written for this work such as TLC Analyzer etc. [47].

The results of antimicrobial assay revealed the area of origin spot including 3-methoxychrysazin spot on TLC plate of benzene extract of *X. indica* flowering heads inhibited the growth of 4 tested gram positive bacteria, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Micrococcus luteus*. This suggested that the antimicrobial activities of benzene extract were selective against gram positive bacteria. The previous study also found the consistent information, the petroleum ether extract of this plant were active against 3 tested gram positive bacteria and no clear zone against gram negative bacteria. Moreover, the ethanol extract displayed inhibition zone on *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Micrococcus luteus* [32] which similar to the benzene extract of this study. Some of plant extracts from *Xyris* species affected against *Staphylococcus aureus* and *Micrococcus luteus* as well [34,35]. The spot of standard chrysazin and separated chrysazin spot from benzene extract of *X. indica* flowering heads inhibited the growth of *Bacillus subtilis*. The previous study found nearby result namely three isolated anthraquinones including chrysazin, 3-methoxychrysazin and

3-hydroxychrysazin) of this plant showed only marginal or no antibacterial activity at the tested concentration [16].

All of the results that were taken from agar-overlay bioautography and from the previous studies [32,34,35] indicated that the crude extracts of *X. indica* flowering heads and crude extracts of some *Xyris* species showed better antimicrobial activities than single compound that was isolated from *X. indica* such as chrysazin. It might be caused that the crude extract contained many active compounds that could be lead to additive or synergistic interactions and obtained better effect than single compound.

The limitation of antimicrobial assay is solubility of standard chrysazin. Chrysazin has marginal solubility in miscible solvents used in antimicrobial assay including dimethyl sulfoxide (DMSO). Chrysazin is good soluble in chloroform which is toxic to microorganisms. Thus, diffusion method cannot perform for screening antimicrobial testing. Agar-overlay bioautography was selected because it can solve the problem about toxicity of chloroform on microorganisms by applying the chrysazin solution on TLC and then left dried for complete evaporation of chloroform. However, chloroform dissolved chrysazin only with maximum concentration of 60 mg/ml. In addition, this method also had limitation about the volume and concentration of sample solution loaded on TLC plate before development step. Maximum volume of sample solution was 10 µl/spot. Maximum concentration of sample solution was 100 mg/ml. Using overload concentration and volume affected unclear separated spot and spot tailing or called retardation on developed TLC plate. hRf value decreased as well.

In conclusion, this research provides pharmacognostic specification and chrysazin content of *X. indica* flowering heads that can be used for basic quality control and standardization of plant material. Besides, it provides scientific evidences in antibacterial potential of *X. indica* flowering heads in Thailand.

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APPENDIX

Table 15. Loss on drying of *Xyris indica* flowering heads form 15 sources

Source	No.	Amount (% by weight)	Mean	SD
1	1	7.255	7.248	0.031
	2	7.275		
	3	7.214		
2	1	6.855	6.870	0.015
	2	6.885		
	3	6.869		
3	1	6.683	6.645	0.046
	2	6.658		
	3	6.594		
4	1	5.859	5.849	0.078
	2	5.921		
	3	5.767		
5	1	6.815	6.781	0.062
	2	6.819		
	3	6.709		
6	1	6.735	6.729	0.026
	2	6.751		
	3	6.701		
7	1	6.687	6.734	0.041
	2	6.754		
	3	6.761		
8	1	7.133	7.758	0.541
	2	8.044		
	3	8.095		
9	1	7.065	6.921	0.130
	2	6.886		
	3	6.811		
10	1	6.922	6.905	0.204
	2	6.694		
	3	7.100		
11	1	6.925	7.016	0.079
	2	7.052		
	3	7.070		
12	1	6.951	6.993	0.043
	2	7.038		
	3	6.990		
13	1	7.012	7.045	0.086
	2	7.143		
	3	6.980		
14	1	7.035	7.004	0.161
	2	7.148		
	3	6.831		
15	1	7.007	6.989	0.042
	2	6.941		
	3	7.018		
		Grand mean	6.899	
		Pooled SD	0.165	

Table 16. Total ash of *Xyris indica* flowering heads form 15 sources

Source	No.	Amount (% by weight)	Mean	SD
1	1	2.205	2.184	0.037
	2	2.207		
	3	2.141		
2	1	2.681	2.695	0.018
	2	2.715		
	3	2.689		
3	1	2.395	2.400	0.024
	2	2.378		
	3	2.426		
4	1	3.493	3.506	0.033
	2	3.543		
	3	3.483		
5	1	2.303	2.358	0.083
	2	2.317		
	3	2.453		
6	1	2.354	2.368	0.020
	2	2.358		
	3	2.391		
7	1	2.846	2.840	0.006
	2	2.842		
	3	2.833		
8	1	1.905	1.894	0.046
	2	1.933		
	3	1.843		
9	1	2.290	2.288	0.022
	2	2.265		
	3	2.309		
10	1	2.341	2.345	0.034
	2	2.313		
	3	2.380		
11	1	2.467	2.489	0.026
	2	2.518		
	3	2.482		
12	1	2.544	2.523	0.025
	2	2.531		
	3	2.495		
13	1	2.572	2.570	0.010
	2	2.579		
	3	2.558		
14	1	2.481	2.475	0.008
	2	2.466		
	3	2.479		
15	1	2.527	2.519	0.014
	2	2.528		
	3	2.503		
		Grand mean	2.497	
		Pooled SD	0.033	

Table 17. Acid-insoluble ash of *Xyris indica* flowering heads form 15 sources

Source	No.	Amount (% by weight)	Mean	SD
1	1	0.359	0.362	0.006
	2	0.369		
	3	0.359		
2	1	0.346	0.315	0.028
	2	0.290		
	3	0.309		
3	1	0.333	0.345	0.011
	2	0.350		
	3	0.352		
4	1	0.267	0.310	0.038
	2	0.326		
	3	0.336		
5	1	0.339	0.389	0.081
	2	0.346		
	3	0.483		
6	1	0.439	0.438	0.015
	2	0.422		
	3	0.453		
7	1	0.405	0.392	0.013
	2	0.380		
	3	0.392		
8	1	0.455	0.458	0.017
	2	0.477		
	3	0.443		
9	1	0.382	0.377	0.006
	2	0.370		
	3	0.379		
10	1	0.383	0.376	0.009
	2	0.366		
	3	0.379		
11	1	0.456	0.463	0.010
	2	0.458		
	3	0.475		
12	1	0.476	0.470	0.013
	2	0.455		
	3	0.478		
13	1	0.506	0.494	0.011
	2	0.483		
	3	0.492		
14	1	0.490	0.481	0.012
	2	0.486		
	3	0.466		
15	1	0.495	0.469	0.024
	2	0.462		
	3	0.450		
		Grand mean	0.409	
		Pooled SD	0.027	

Table 18. Water-soluble extractive of *Xyris indica* flowering heads form 15 sources

Source	No.	Amount (% by weight)	Mean	SD
1	1	6.079	5.818	0.278
	2	5.526		
	3	5.849		
2	1	7.840	7.872	0.474
	2	7.416		
	3	8.361		
3	1	5.736	5.461	0.354
	2	5.062		
	3	5.585		
4	1	12.172	12.371	0.251
	2	12.286		
	3	12.654		
5	1	6.166	5.774	0.372
	2	5.729		
	3	5.428		
6	1	5.890	5.191	0.809
	2	4.304		
	3	5.379		
7	1	6.285	6.718	0.461
	2	6.667		
	3	7.202		
8	1	3.104	3.237	0.245
	2	3.088		
	3	3.520		
9	1	6.569	6.297	0.267
	2	6.287		
	3	6.035		
10	1	6.689	6.362	0.313
	2	6.064		
	3	6.334		
11	1	8.498	7.374	0.973
	2	6.810		
	3	6.813		
12	1	6.997	6.846	0.407
	2	7.155		
	3	6.385		
13	1	6.734	6.990	0.223
	2	7.099		
	3	7.137		
14	1	7.033	6.285	0.652
	2	5.837		
	3	5.984		
15	1	6.378	6.286	0.239
	2	6.465		
	3	6.015		
		Grand mean	6.592	
		Pooled SD	0.474	

Table 19. Ethanol-soluble extractive of *Xyris indica* flowering heads form 15 sources

Source	No.	Amount (% by weight)	Mean	SD
1	1	4.236	4.343	0.096
	2	4.421		
	3	4.372		
2	1	6.025	5.903	0.108
	2	5.862		
	3	5.820		
3	1	4.330	4.371	0.220
	2	4.609		
	3	4.175		
4	1	3.868	4.444	1.751
	2	3.053		
	3	6.410		
5	1	4.582	4.456	0.109
	2	4.402		
	3	4.384		
6	1	4.711	4.574	0.119
	2	4.500		
	3	4.511		
7	1	4.924	5.173	0.217
	2	5.270		
	3	5.324		
8	1	3.393	3.180	0.187
	2	3.990		
	3	3.047		
9	1	3.978	4.112	0.167
	2	4.059		
	3	4.299		
10	1	3.586	3.641	0.078
	2	3.730		
	3	3.608		
11	1	3.875	3.662	0.185
	2	3.535		
	3	3.576		
12	1	3.303	3.144	0.231
	2	2.879		
	3	3.249		
13	1	3.147	3.150	0.007
	2	3.145		
	3	3.157		
14	1	2.874	2.953	0.073
	2	3.019		
	3	2.965		
15	1	3.687	3.350	0.410
	2	3.469		
	3	2.894		
		Grand mean	4.030	
		Pooled SD	0.486	

Table 20. Water content of *Xyris indica* flowering heads form 15 sources

Source	No.	Amount (% by weight)	Mean	SD
1	1	13.324	12.886	0.380
	2	12.667		
	3	12.667		
2	1	9.333	10.217	1.537
	2	11.992		
	3	9.327		
3	1	14.657	11.883	2.408
	2	10.660		
	3	10.333		
4	1	7.995	7.552	0.771
	2	8.000		
	3	6.662		
5	1	11.992	11.328	1.156
	2	12.000		
	3	9.993		
6	1	12.667	11.331	1.157
	2	10.660		
	3	10.667		
7	1	10.000	9.663	0.574
	2	9.990		
	3	9.000		
8	1	10.000	11.222	1.171
	2	11.333		
	3	12.333		
9	1	12.658	12.658	0
	2	12.658		
	3	12.658		
10	1	11.330	11.554	1.019
	2	10.667		
	3	12.667		
11	1	10.667	10.218	0.771
	2	10.660		
	3	09.327		
12	1	11.988	11.992	0.007
	2	12.000		
	3	11.988		
13	1	11.000	10.993	0.006
	2	10.989		
	3	10.989		
14	1	13.000	10.997	1.735
	2	9.990		
	3	10.000		
15	1	11.000	12.325	1.147
	2	12.987		
	3	12.987		
		Grand mean	11.121	
		Pooled SD	1.132	

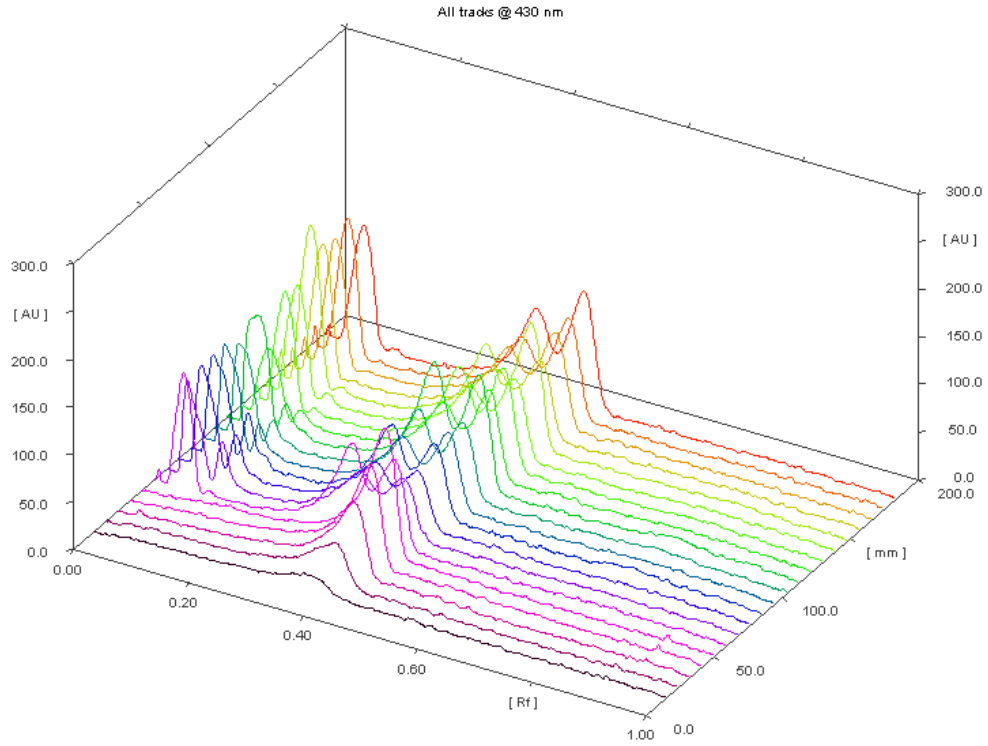


Figure 15. 3D TLC-Densitometry chromatogram (Plate A-Sample No. 1-15)

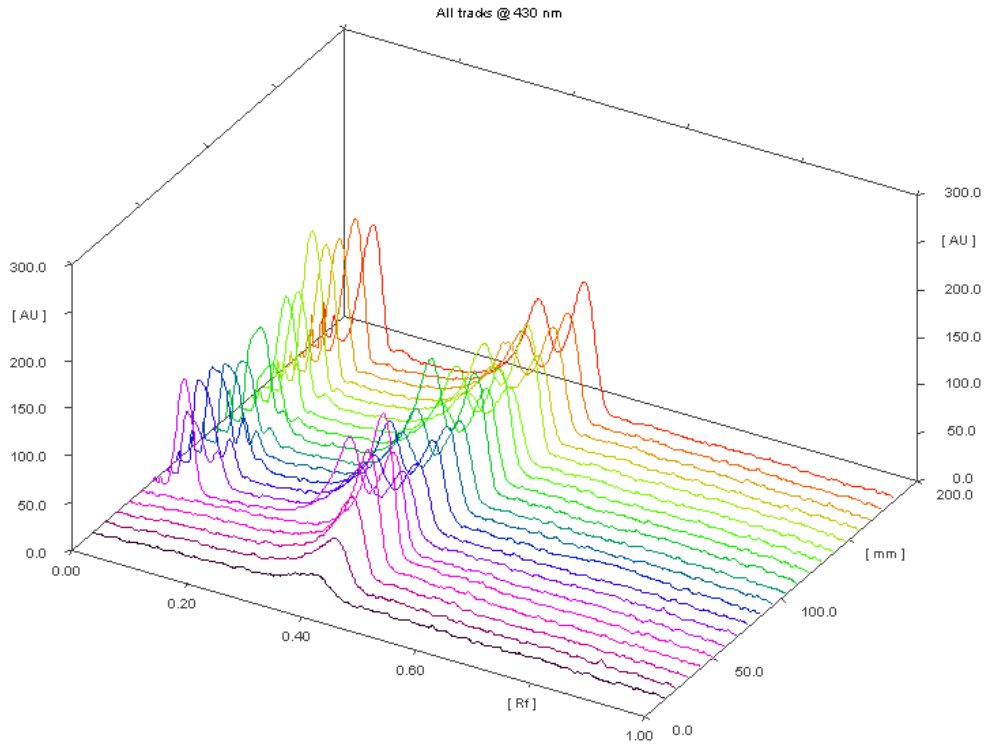


Figure 16. 3D TLC-Densitometry chromatogram (Plate B-Sample No. 1-15)

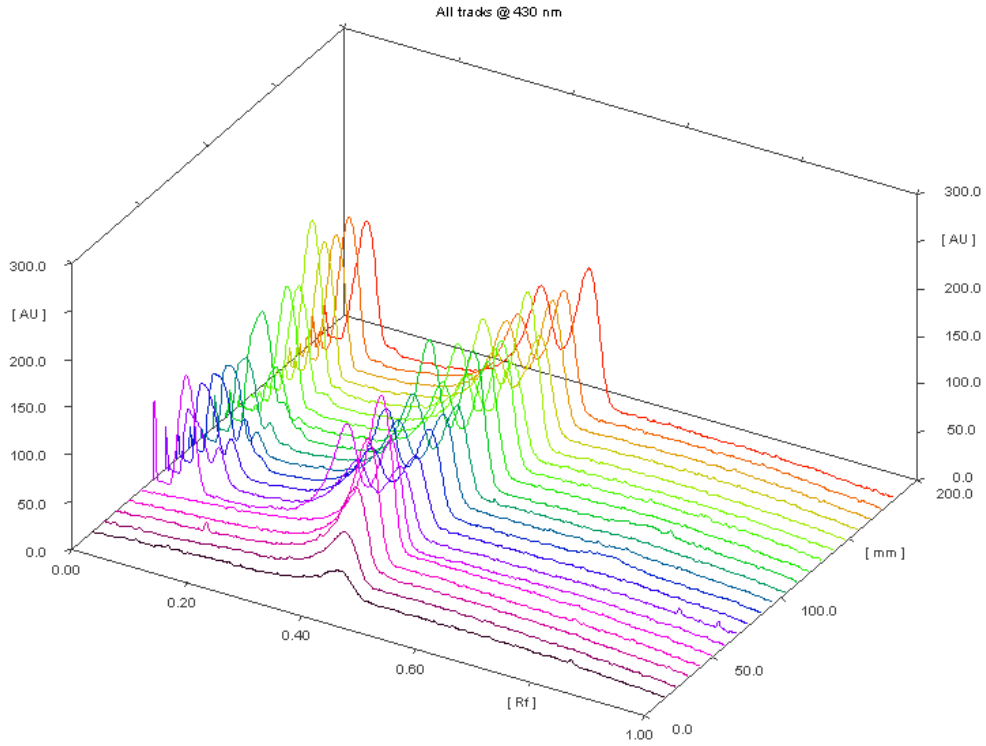


Figure 17. 3D TLC-Densitometry chromatogram (Plate C-Sample No. 1-15)

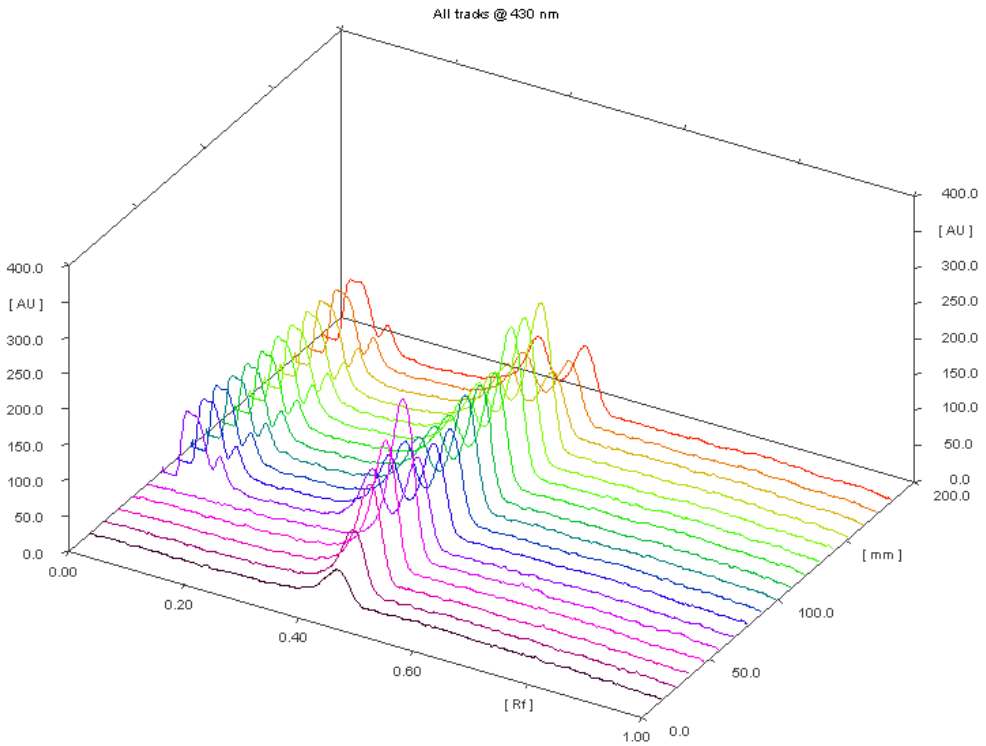


Figure 18. 3D TLC-Densitometry chromatogram (Plate D-Accuracy)

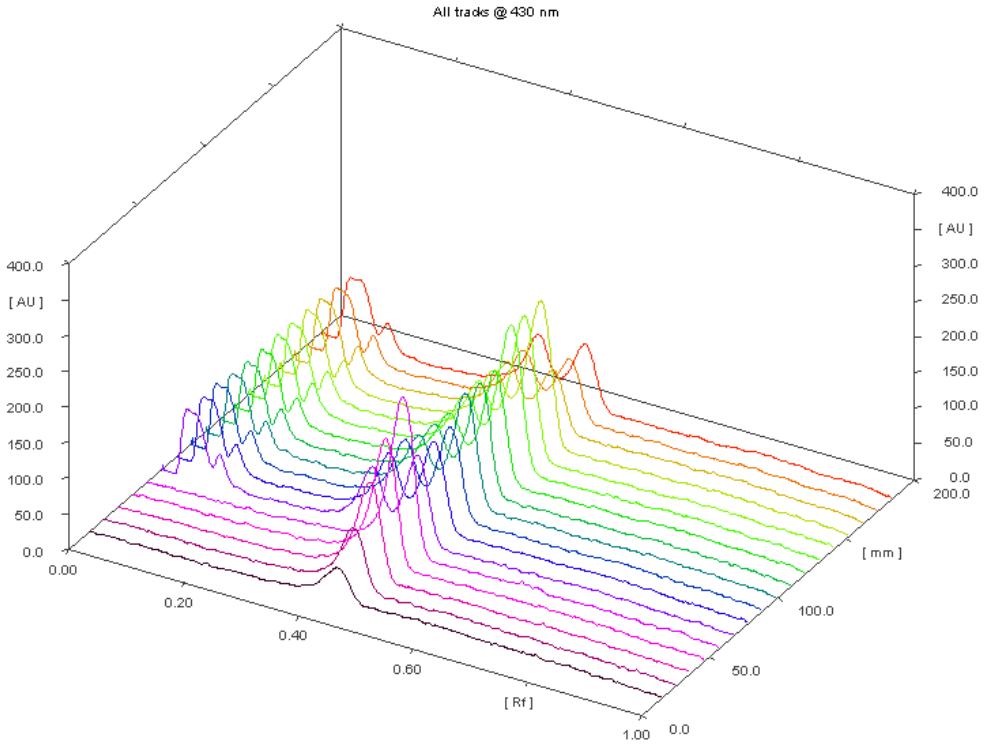


Figure 19. 3D TLC-Densitometry chromatogram (Plate E-Precision 1)

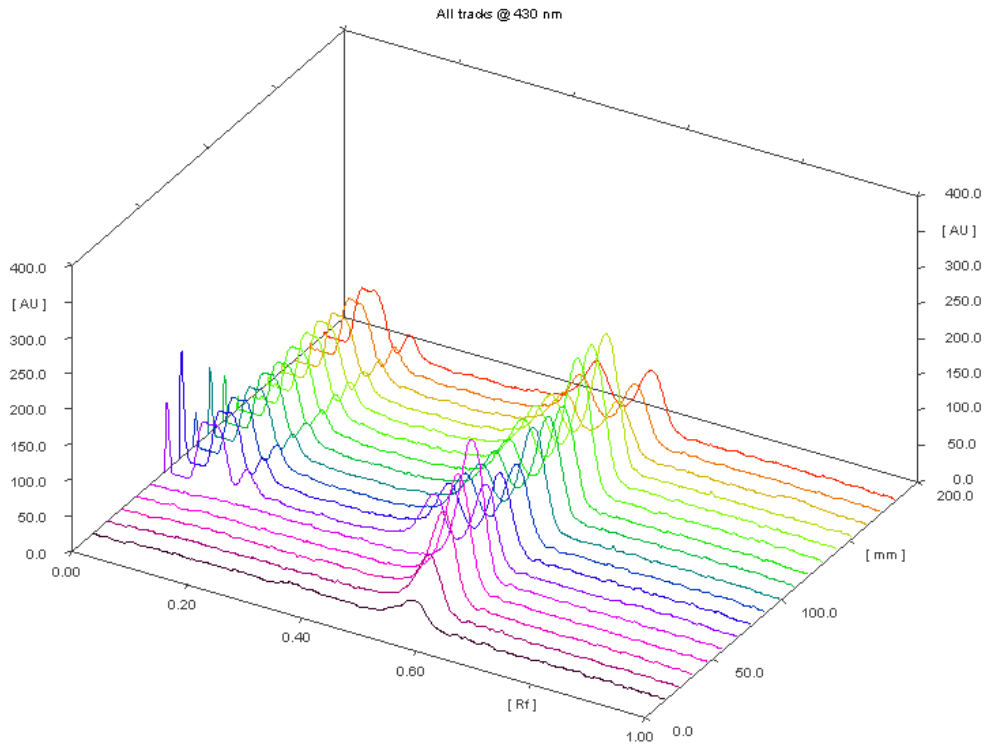


Figure 20. 3D TLC-Densitometry chromatogram (Plate F-Precision 2)

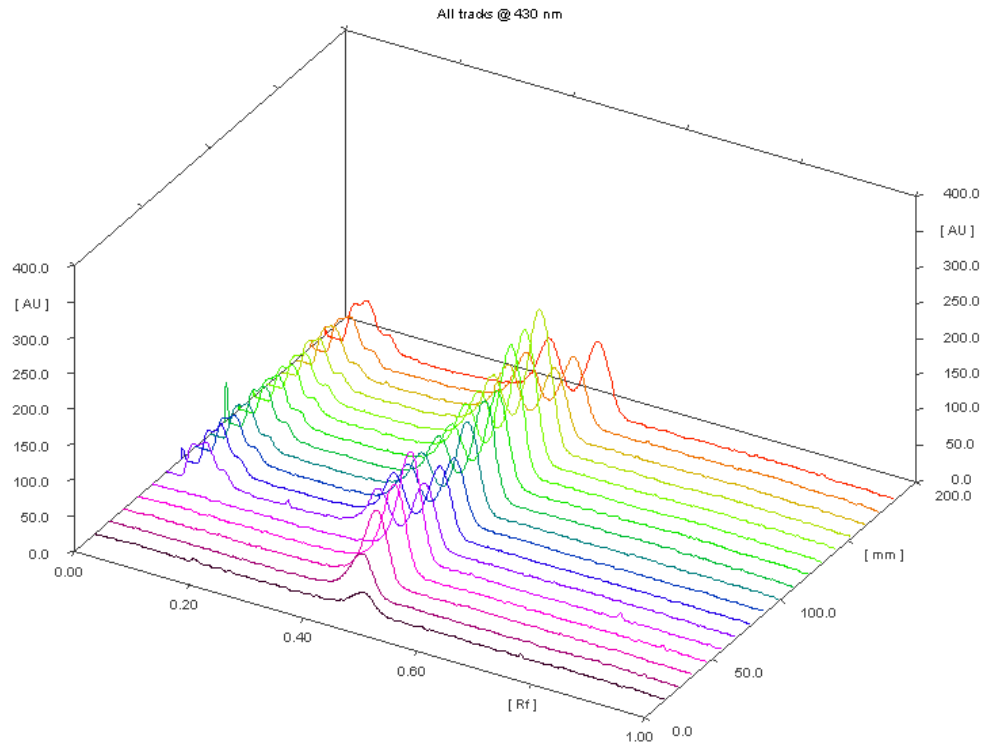


Figure 21. 3D TLC-Densitometry chromatogram (Plate G-Precision 3)

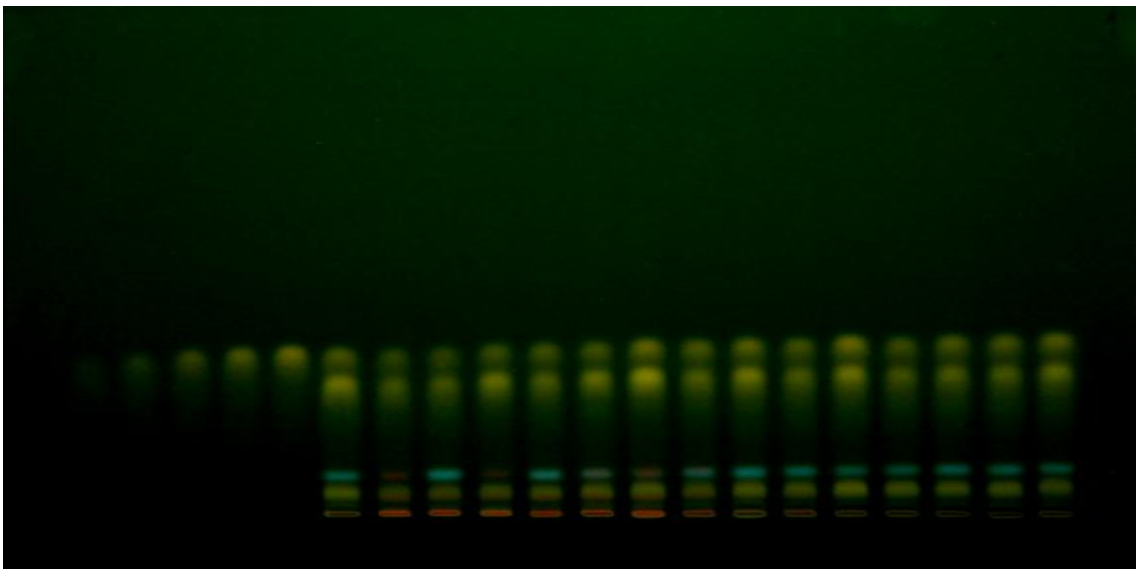


Figure 22. The TLC plates (Plate A-Sample No. 1-15) developed with petroleum ether: ethyl acetate (8:1) visual under 365 nm original image

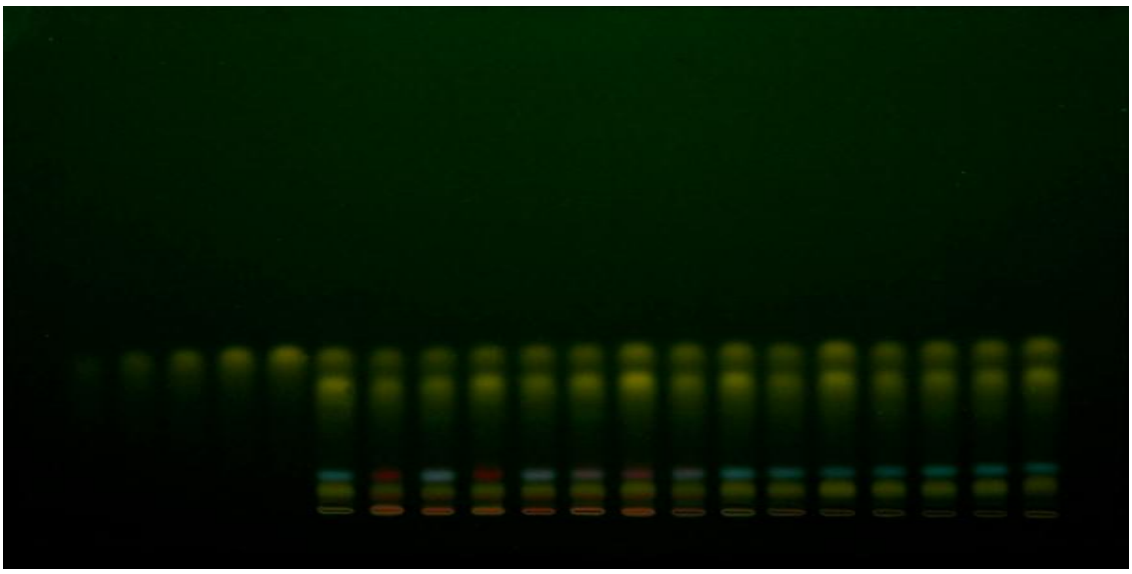


Figure 23. The TLC plates (Plate B-Sample No. 1-15) developed with petroleum ether: ethyl acetate (8:1) visual under 365 nm original image

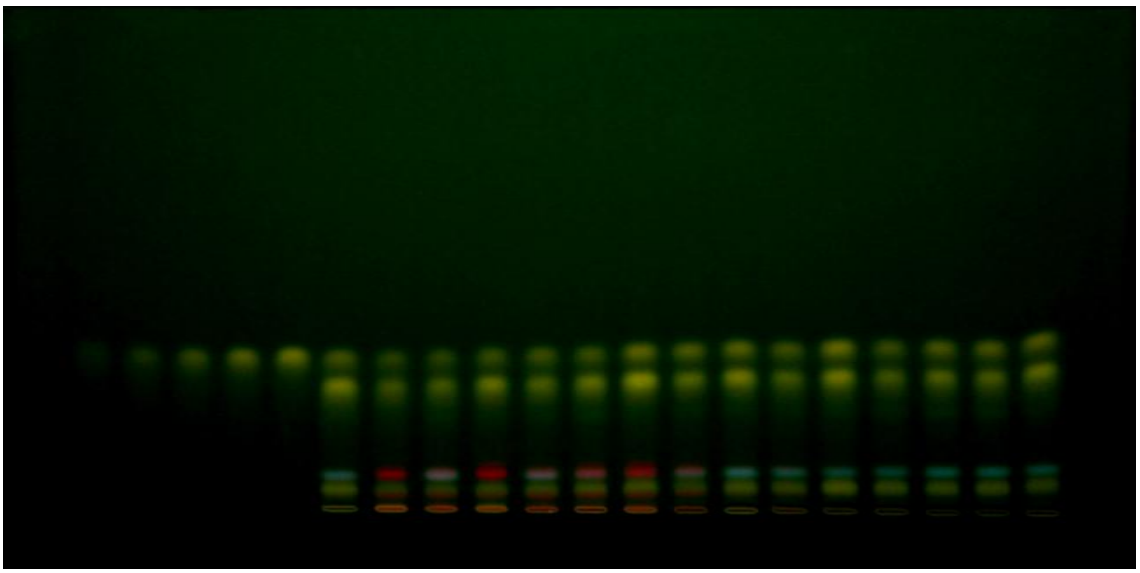


Figure 24. The TLC plates (Plate C-Sample No. 1-15) developed with petroleum ether: ethyl acetate (8:1) visual under 365 nm original image

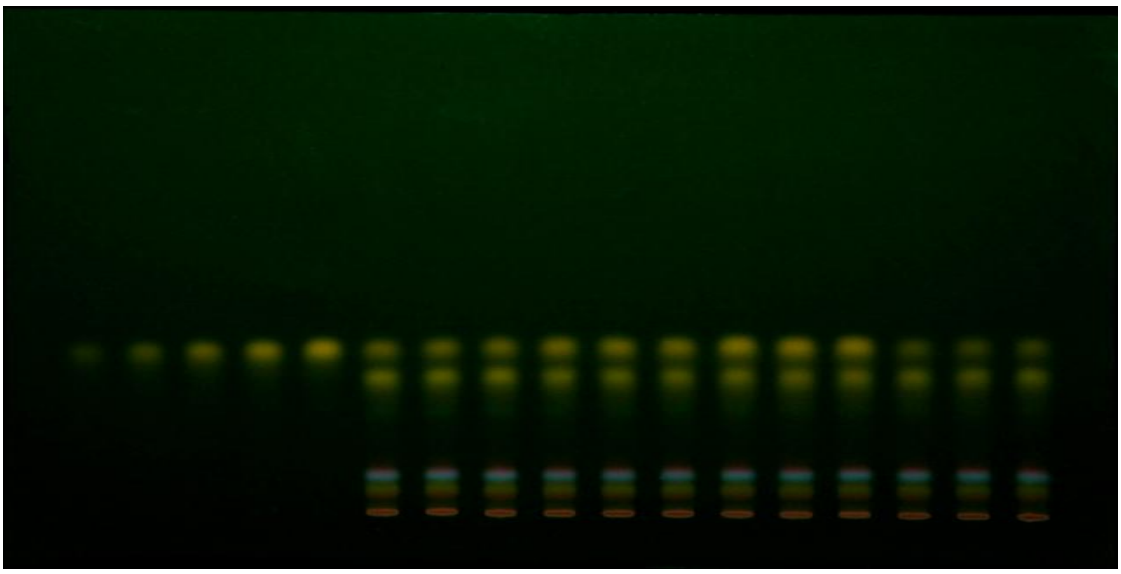


Figure 25. The TLC plates (Plate D-Accuracy) developed with petroleum ether: ethyl acetate (8:1) visual under 365 nm original image

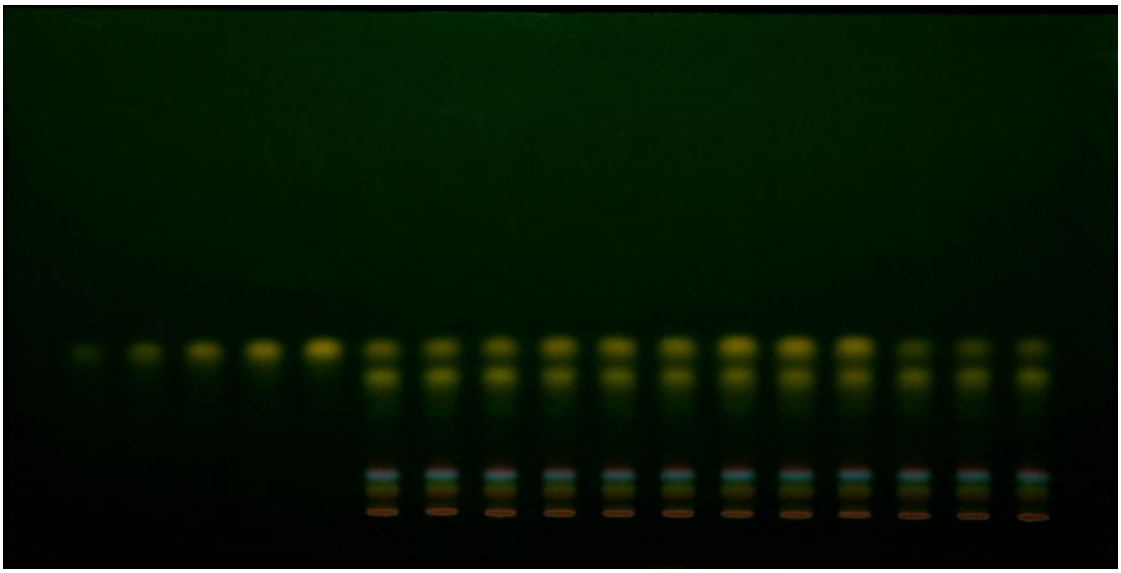


Figure 26. The TLC plates (Plate E- Precision 1) developed with petroleum ether: ethyl acetate (8:1) visual under 365 nm original image

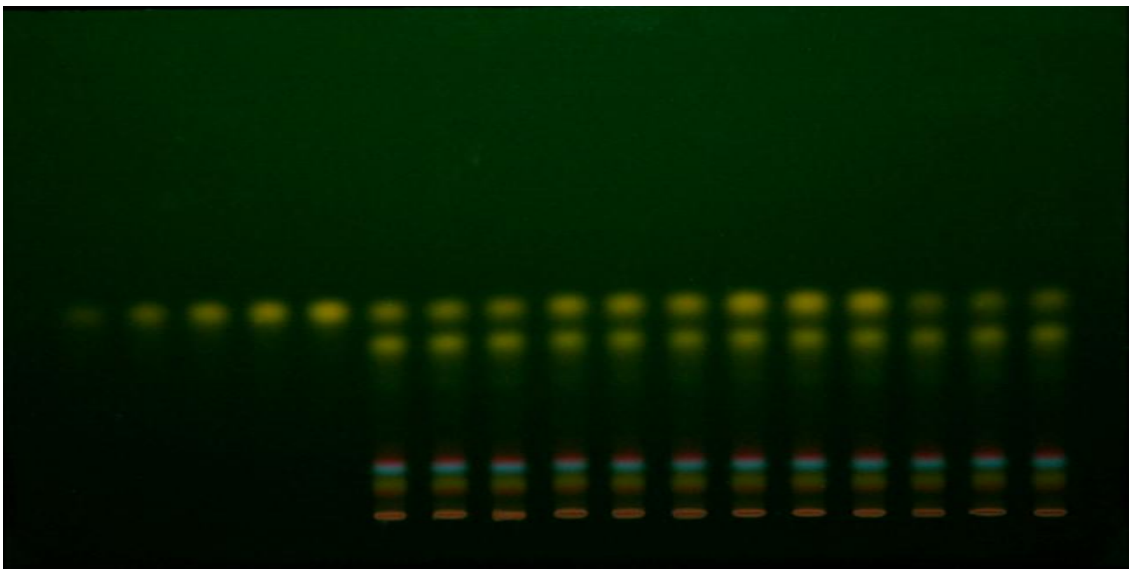


Figure 27. The TLC plates (Plate F- Precision 2) developed with petroleum ether: ethyl acetate (8:1) visual under 365 nm original image

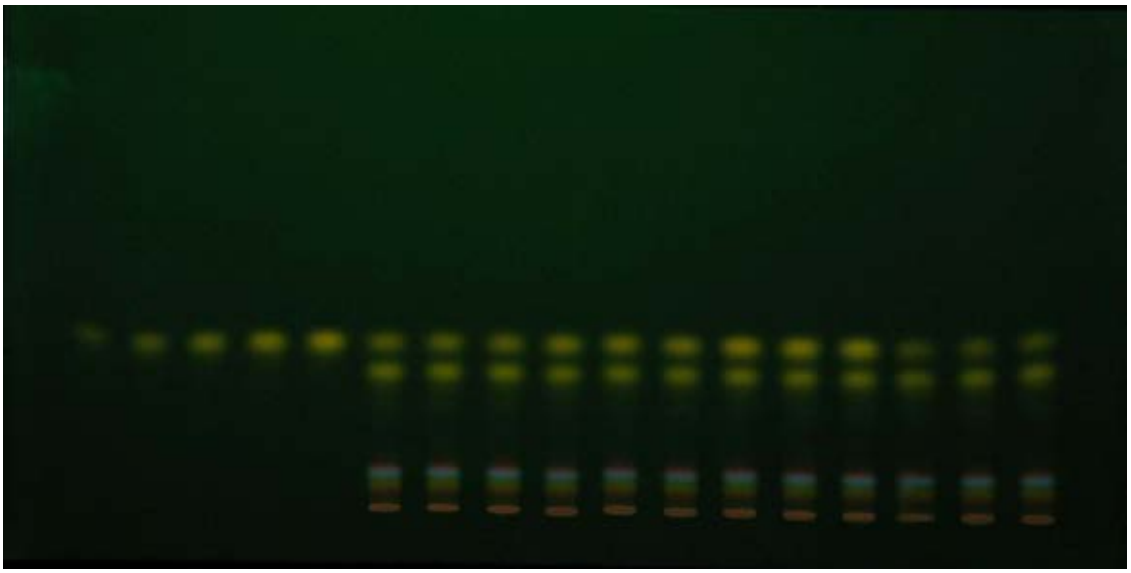
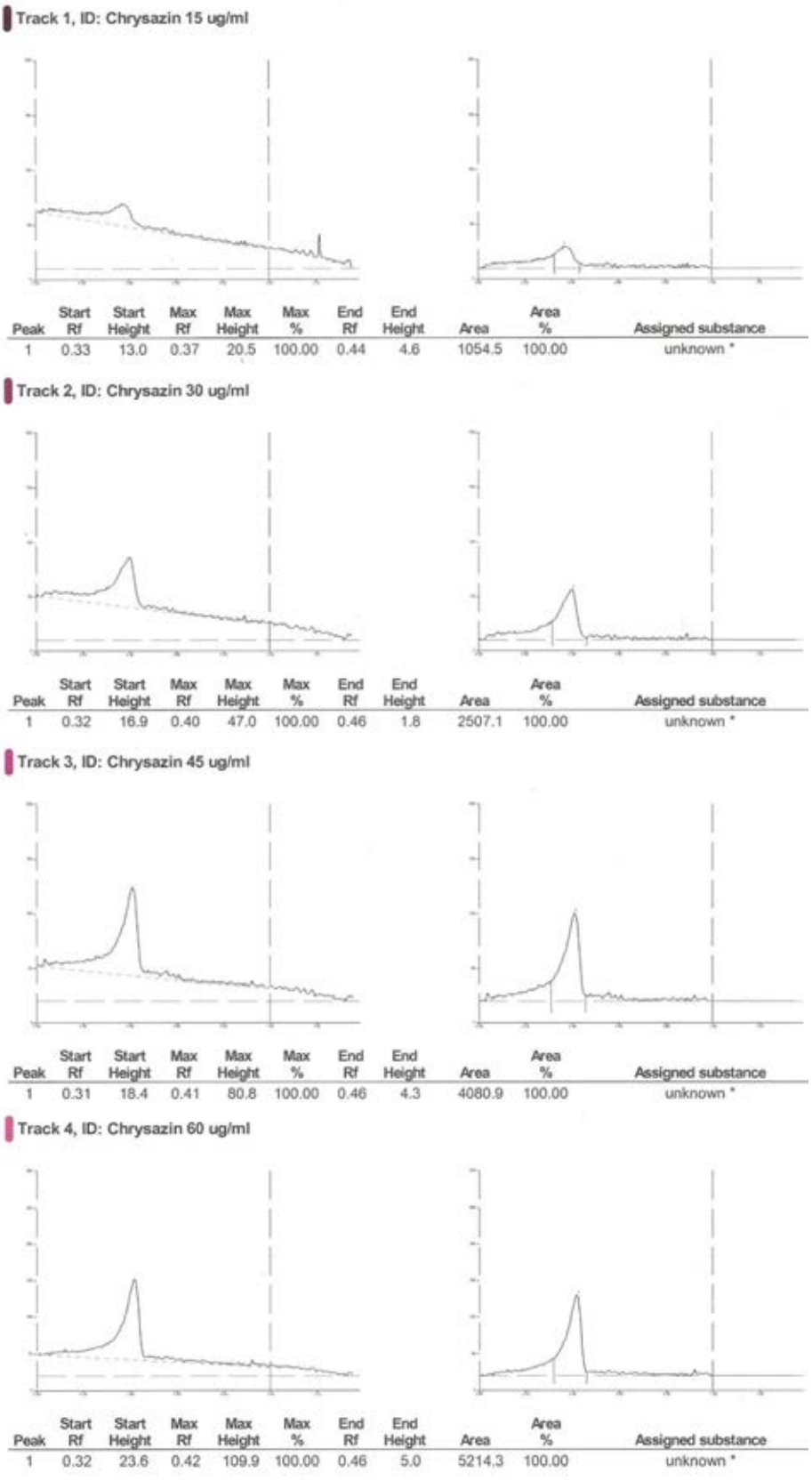


Figure 28. The TLC plates (Plate G- Precision 3) developed with petroleum ether: ethyl acetate (8:1) visual under 365 nm original image



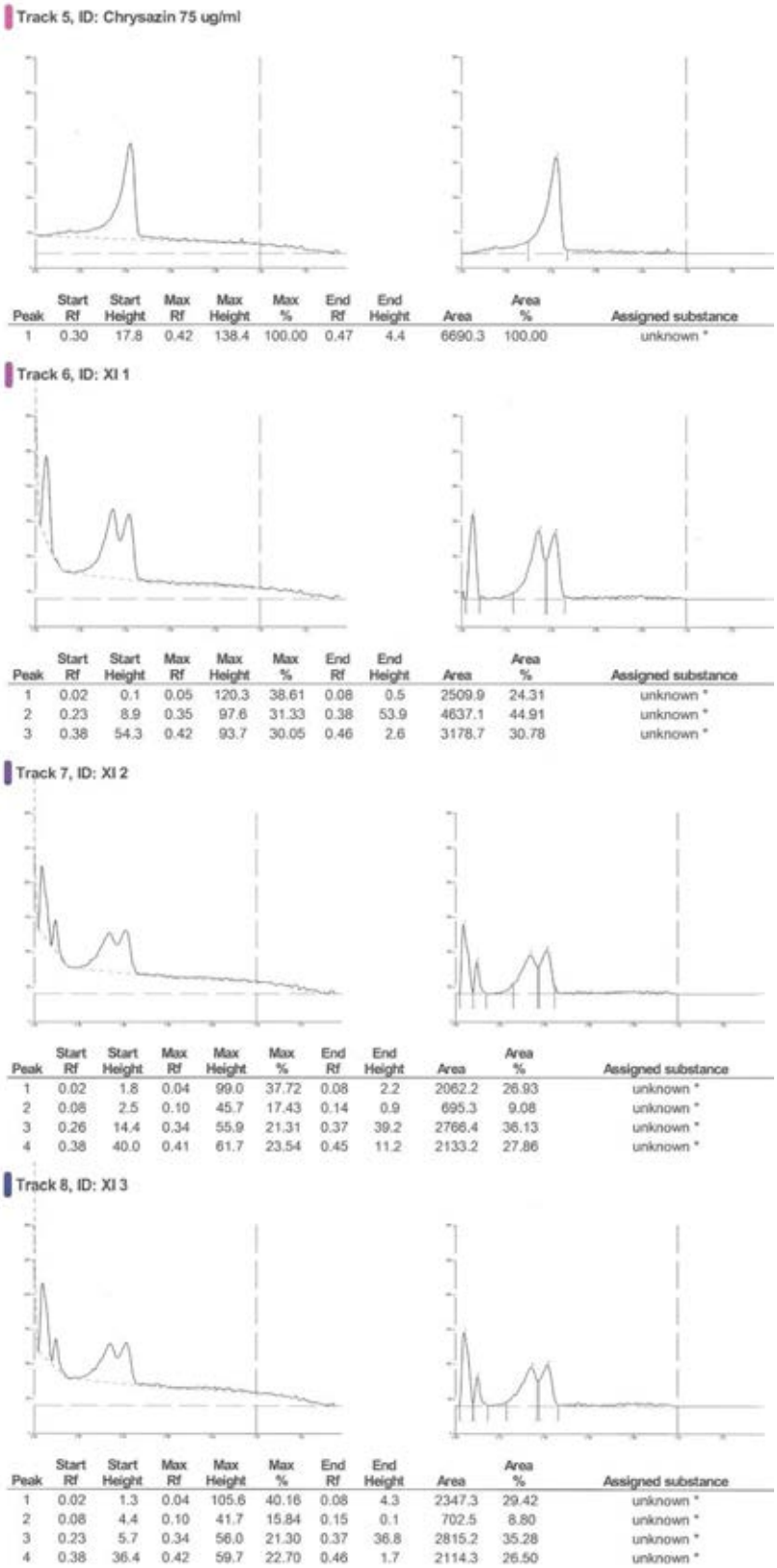
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.33	13.0	0.37	20.5	100.00	0.44	4.6	1054.5	100.00	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.32	16.9	0.40	47.0	100.00	0.46	1.8	2507.1	100.00	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.31	18.4	0.41	80.8	100.00	0.46	4.3	4080.9	100.00	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.32	23.6	0.42	109.9	100.00	0.46	5.0	5214.3	100.00	unknown *

Figure 29. TLC-densitometry chromatogram (Plate A-Sample No. 1-15)



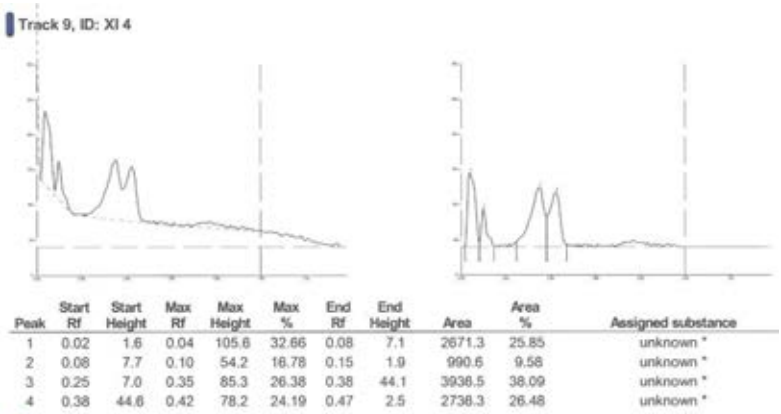
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.30	17.8	0.42	138.4	100.00	0.47	4.4	6690.3	100.00	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	0.1	0.05	120.3	38.61	0.08	0.5	2509.9	24.31	unknown *
2	0.23	8.9	0.35	97.6	31.33	0.38	53.9	4637.1	44.91	unknown *
3	0.38	54.3	0.42	93.7	30.05	0.46	2.6	3178.7	30.78	unknown *

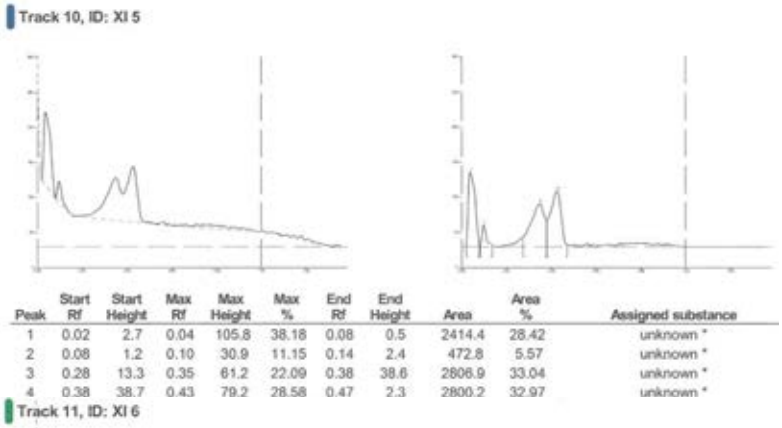
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	1.8	0.04	99.0	37.72	0.08	2.2	2062.2	26.93	unknown *
2	0.08	2.5	0.10	45.7	17.43	0.14	0.9	695.3	9.08	unknown *
3	0.26	14.4	0.34	55.9	21.31	0.37	39.2	2766.4	36.13	unknown *
4	0.38	40.0	0.41	61.7	23.54	0.45	11.2	2133.2	27.86	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	1.3	0.04	105.6	40.16	0.08	4.3	2347.3	29.42	unknown *
2	0.08	4.4	0.10	41.7	15.84	0.15	0.1	702.5	8.80	unknown *
3	0.23	5.7	0.34	56.0	21.30	0.37	36.8	2815.2	35.28	unknown *
4	0.38	36.4	0.42	59.7	22.70	0.46	1.7	2114.3	26.50	unknown *

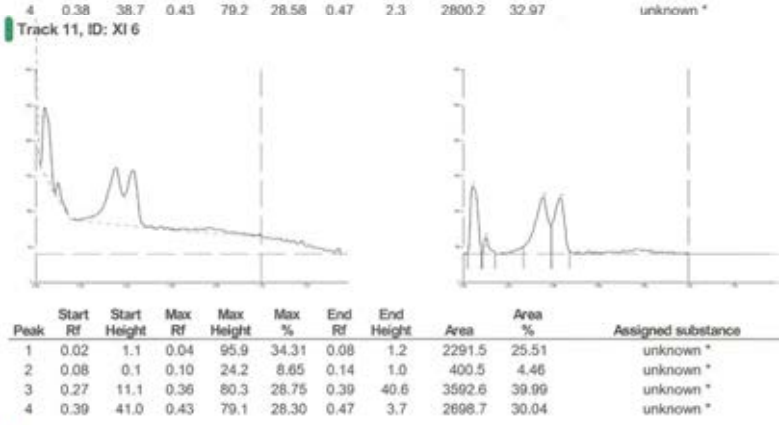
Figure 29 (cont.). TLC-densitometry chromatogram (Plate A-Sample No. 1-15)



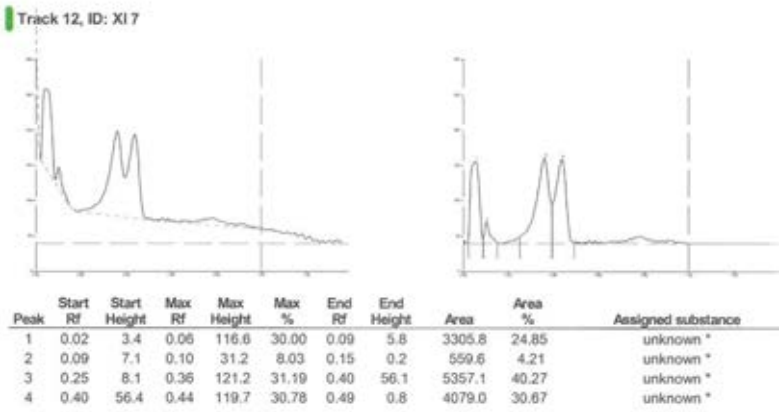
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	1.6	0.04	105.6	32.66	0.08	7.1	2671.3	25.85	unknown *
2	0.08	7.7	0.10	54.2	16.78	0.15	1.9	990.6	9.58	unknown *
3	0.25	7.0	0.35	85.3	26.38	0.38	44.1	3936.5	38.09	unknown *
4	0.38	44.6	0.42	78.2	24.19	0.47	2.5	2736.3	26.48	unknown *



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	2.7	0.04	105.8	38.18	0.08	0.5	2414.4	28.42	unknown *
2	0.08	1.2	0.10	30.9	11.15	0.14	2.4	472.8	5.57	unknown *
3	0.28	13.3	0.35	61.2	22.09	0.38	38.6	2806.9	33.04	unknown *
4	0.38	38.7	0.43	79.2	28.58	0.47	2.3	2800.2	32.97	unknown *

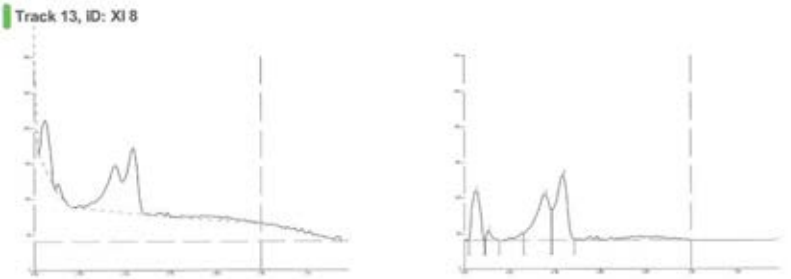


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	1.1	0.04	95.9	34.31	0.08	1.2	2291.5	25.51	unknown *
2	0.08	0.1	0.10	24.2	8.65	0.14	1.0	400.5	4.46	unknown *
3	0.27	11.1	0.36	80.3	28.75	0.39	40.6	3592.6	39.99	unknown *
4	0.39	41.0	0.43	79.1	28.30	0.47	3.7	2698.7	30.04	unknown *



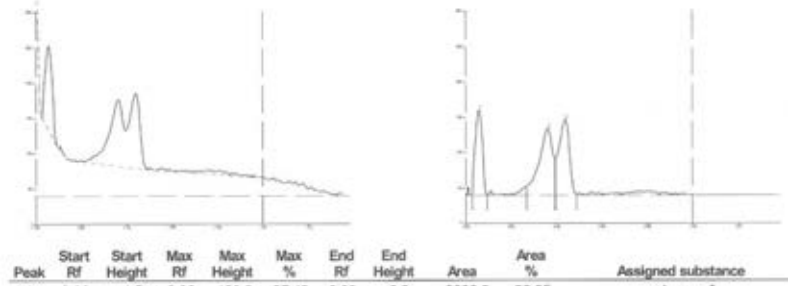
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	3.4	0.06	116.6	30.00	0.09	5.8	3305.8	24.85	unknown *
2	0.09	7.1	0.10	31.2	8.03	0.15	0.2	559.6	4.21	unknown *
3	0.25	8.1	0.36	121.2	31.19	0.40	56.1	5357.1	40.27	unknown *
4	0.40	56.4	0.44	119.7	30.78	0.49	0.8	4079.0	30.67	unknown *

Figure 29 (cont.). TLC-densitometry chromatogram (Plate A-Sample No. 1-15)



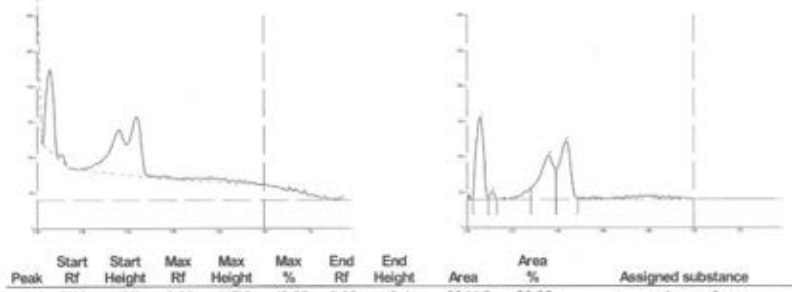
Track 13, ID: XI 8

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	1.0	0.05	70.3	29.01	0.09	0.3	1871.4	21.83	unknown *
2	0.09	0.4	0.11	14.2	5.86	0.15	0.5	238.3	2.78	unknown *
3	0.26	10.1	0.36	65.4	27.00	0.39	43.5	3075.1	35.88	unknown *
4	0.39	43.5	0.44	92.4	38.13	0.49	1.0	3386.1	39.51	unknown *



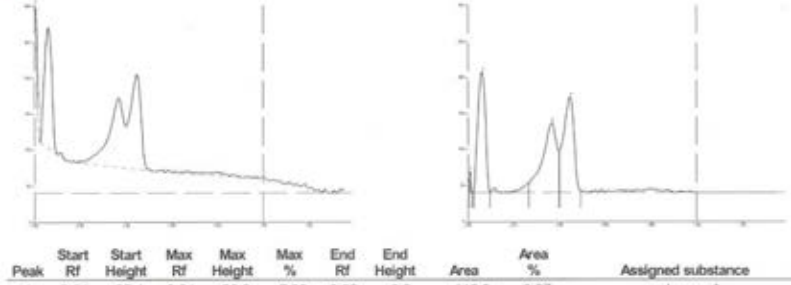
Track 14, ID: XI 9

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.5	0.06	120.6	37.42	0.09	0.3	2830.8	26.05	unknown *
2	0.27	11.3	0.36	94.7	29.39	0.40	51.7	4290.6	39.48	unknown *
3	0.40	51.8	0.44	107.0	33.19	0.49	0.9	3747.2	34.48	unknown *



Track 15, ID: XI 10

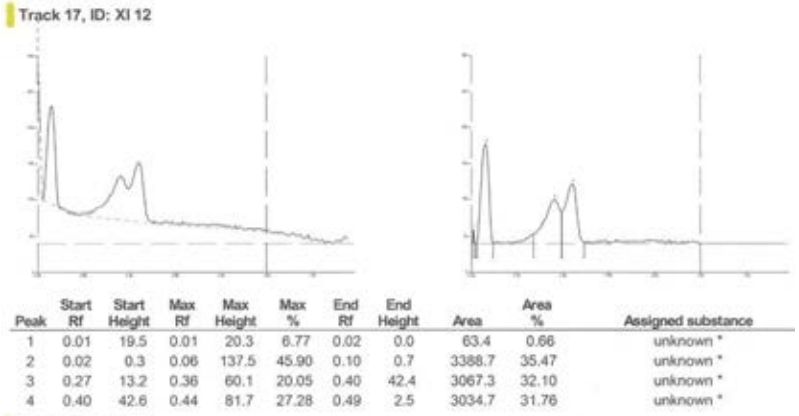
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	2.0	0.06	115.9	42.68	0.09	2.4	2841.9	32.00	unknown *
2	0.10	2.5	0.11	10.8	3.98	0.13	0.0	155.6	1.75	unknown *
3	0.28	15.2	0.36	62.4	22.97	0.39	42.0	2911.6	32.79	unknown *
4	0.40	42.1	0.44	82.5	30.38	0.49	0.7	2971.2	33.46	unknown *



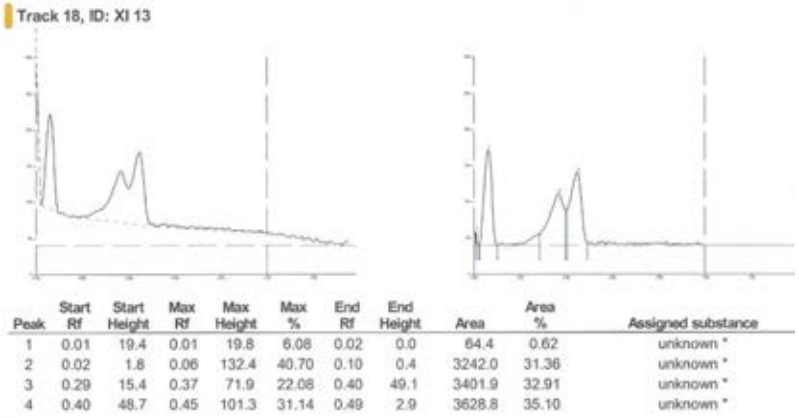
Track 16, ID: XI 11

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	25.1	0.01	30.0	7.00	0.02	0.0	118.3	0.87	unknown *
2	0.02	0.5	0.06	168.4	39.28	0.10	0.4	4161.6	30.71	unknown *
3	0.27	13.5	0.37	97.0	22.62	0.40	58.4	4609.1	34.01	unknown *
4	0.40	58.8	0.45	133.2	31.09	0.49	2.5	4662.2	34.40	unknown *

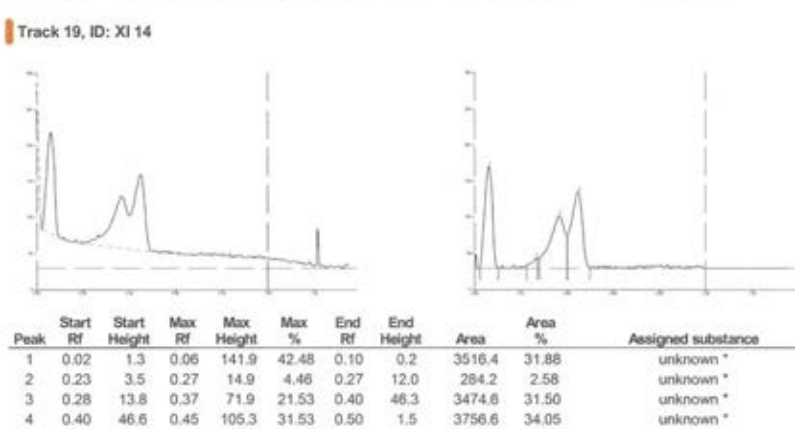
Figure 29 (cont.). TLC-densitometry chromatogram (Plate A-Sample No. 1-15)



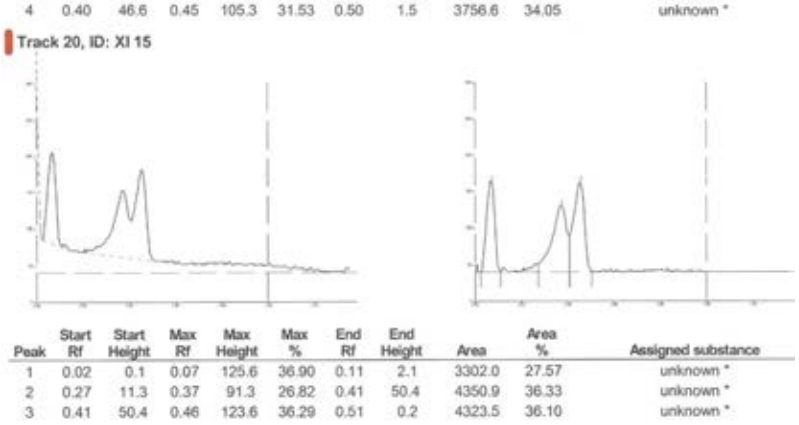
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	19.5	0.01	20.3	6.77	0.02	0.0	63.4	0.66	unknown *
2	0.02	0.3	0.06	137.5	45.90	0.10	0.7	3388.7	35.47	unknown *
3	0.27	13.2	0.36	60.1	20.05	0.40	42.4	3067.3	32.10	unknown *
4	0.40	42.6	0.44	81.7	27.28	0.49	2.5	3034.7	31.76	unknown *



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	19.4	0.01	19.8	6.08	0.02	0.0	64.4	0.62	unknown *
2	0.02	1.8	0.06	132.4	40.70	0.10	0.4	3242.0	31.36	unknown *
3	0.29	15.4	0.37	71.9	22.08	0.40	49.1	3401.9	32.91	unknown *
4	0.40	48.7	0.45	101.3	31.14	0.49	2.9	3628.8	35.10	unknown *



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	1.3	0.06	141.9	42.48	0.10	0.2	3516.4	31.88	unknown *
2	0.23	3.5	0.27	14.9	4.46	0.27	12.0	284.2	2.58	unknown *
3	0.28	13.8	0.37	71.9	21.53	0.40	46.3	3474.6	31.50	unknown *
4	0.40	46.6	0.45	105.3	31.53	0.50	1.5	3756.6	34.05	unknown *



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	0.1	0.07	125.6	36.90	0.11	2.1	3302.0	27.57	unknown *
2	0.27	11.3	0.37	91.3	26.82	0.41	50.4	4350.9	36.33	unknown *
3	0.41	50.4	0.46	123.6	36.29	0.51	0.2	4323.5	36.10	unknown *

Figure 29 (cont.). TLC-densitometry chromatogram (Plate A-Sample No. 1-15)

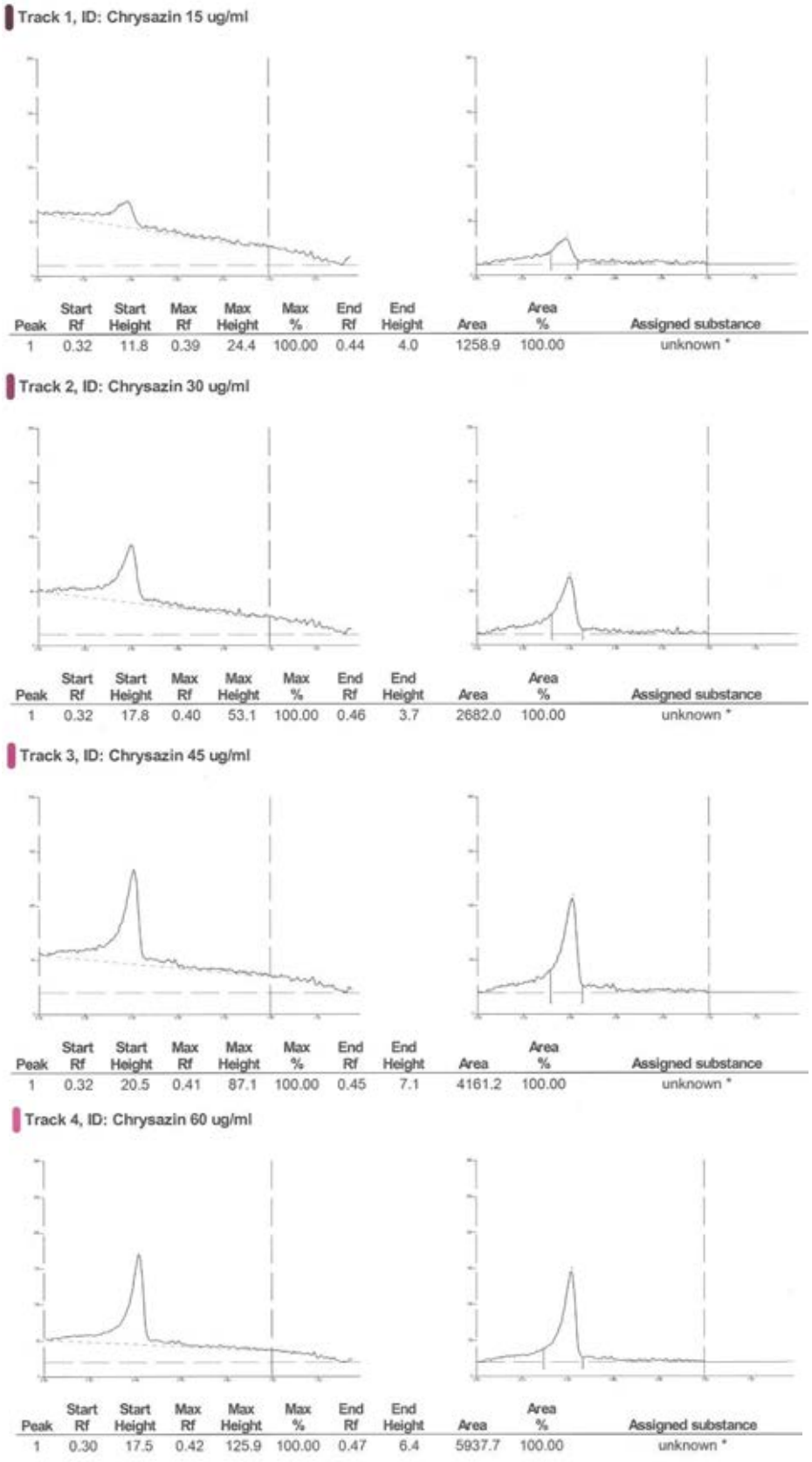
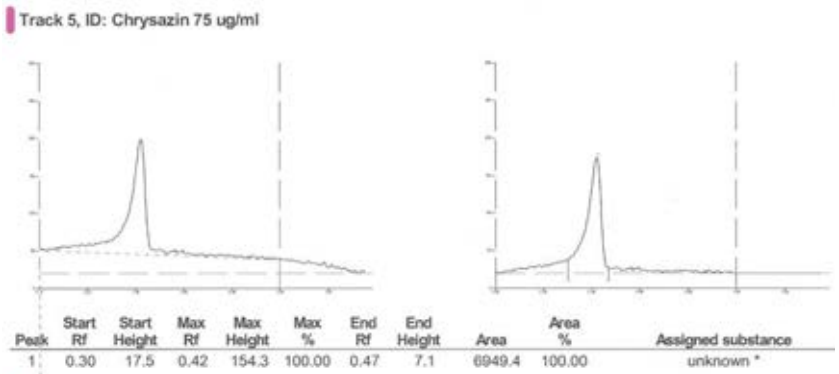
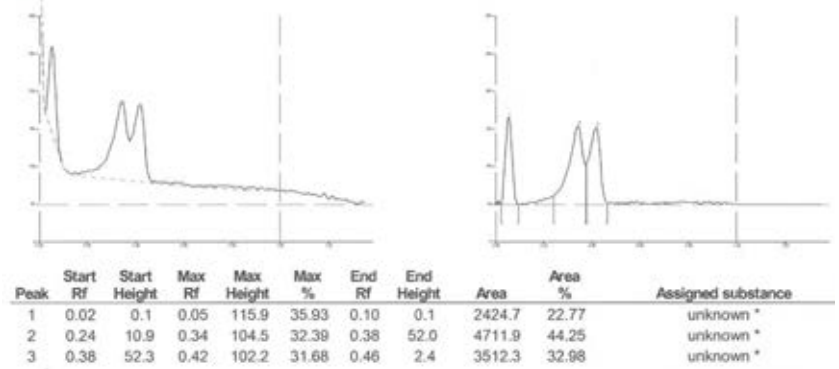


Figure 30. TLC-densitometry chromatogram (Plate B-Sample No. 1-15)



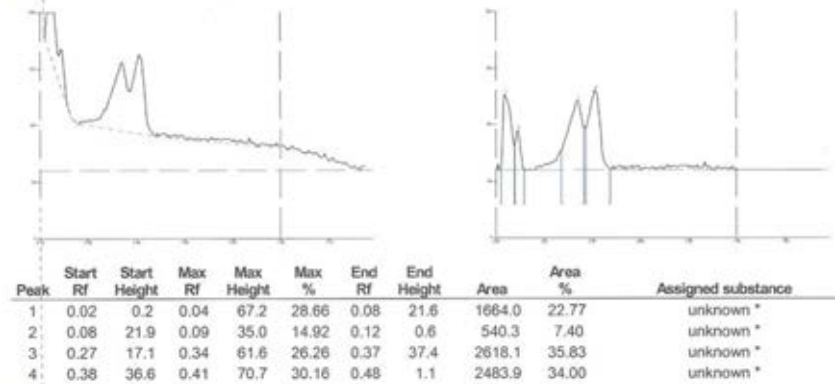
Track 5, ID: Chrysazin 75 ug/ml

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.30	17.5	0.42	154.3	100.00	0.47	7.1	6949.4	100.00	unknown *



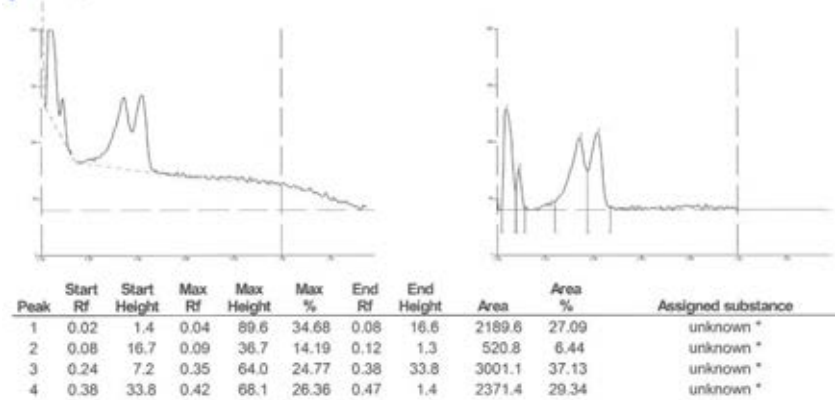
Track 6, ID: XI 1

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	0.1	0.05	115.9	35.93	0.10	0.1	2424.7	22.77	unknown *
2	0.24	10.9	0.34	104.5	32.39	0.38	52.0	4711.9	44.25	unknown *
3	0.38	52.3	0.42	102.2	31.68	0.46	2.4	3512.3	32.98	unknown *



Track 7, ID: XI 2

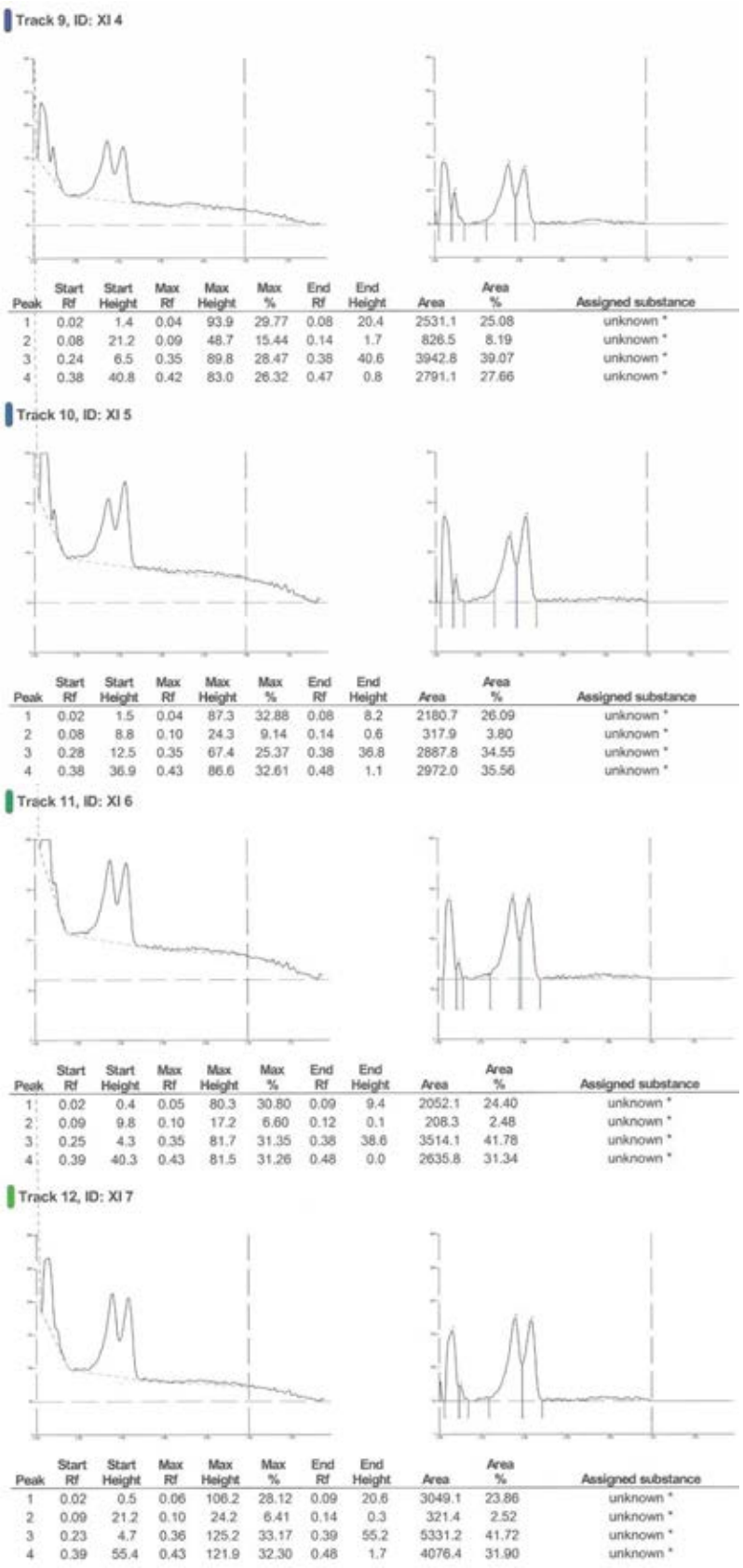
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	0.2	0.04	67.2	28.66	0.08	21.6	1664.0	22.77	unknown *
2	0.08	21.9	0.09	35.0	14.92	0.12	0.6	540.3	7.40	unknown *
3	0.27	17.1	0.34	61.6	26.26	0.37	37.4	2618.1	35.83	unknown *
4	0.38	36.6	0.41	70.7	30.16	0.48	1.1	2483.9	34.00	unknown *



Track 8, ID: XI 3

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	1.4	0.04	89.6	34.68	0.08	16.6	2189.6	27.09	unknown *
2	0.08	16.7	0.09	36.7	14.19	0.12	1.3	520.8	6.44	unknown *
3	0.24	7.2	0.35	64.0	24.77	0.38	33.8	3001.1	37.13	unknown *
4	0.38	33.8	0.42	68.1	26.36	0.47	1.4	2371.4	29.34	unknown *

Figure 30 (cont.). TLC-densitometry chromatogram (Plate B-Sample No. 1-15)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	1.4	0.04	93.9	29.77	0.08	20.4	2531.1	25.08	unknown *
2	0.08	21.2	0.09	48.7	15.44	0.14	1.7	826.5	8.19	unknown *
3	0.24	6.5	0.35	89.8	28.47	0.38	40.6	3942.8	39.07	unknown *
4	0.38	40.8	0.42	83.0	26.32	0.47	0.8	2791.1	27.66	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	1.5	0.04	87.3	32.88	0.08	8.2	2180.7	26.09	unknown *
2	0.08	8.8	0.10	24.3	9.14	0.14	0.6	317.9	3.80	unknown *
3	0.28	12.5	0.35	67.4	25.37	0.38	36.8	2887.8	34.55	unknown *
4	0.38	36.9	0.43	86.6	32.61	0.48	1.1	2972.0	35.56	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	0.4	0.05	80.3	30.80	0.09	9.4	2052.1	24.40	unknown *
2	0.09	9.8	0.10	17.2	6.60	0.12	0.1	208.3	2.48	unknown *
3	0.25	4.3	0.35	81.7	31.35	0.38	38.6	3514.1	41.78	unknown *
4	0.39	40.3	0.43	81.5	31.26	0.48	0.0	2635.8	31.34	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	0.5	0.06	106.2	28.12	0.09	20.6	3049.1	23.86	unknown *
2	0.09	21.2	0.10	24.2	6.41	0.14	0.3	321.4	2.52	unknown *
3	0.23	4.7	0.36	125.2	33.17	0.39	55.2	5331.2	41.72	unknown *
4	0.39	55.4	0.43	121.9	32.30	0.48	1.7	4076.4	31.90	unknown *

Figure 30 (cont.). TLC-densitometry chromatogram (Plate B-Sample No. 1-15)

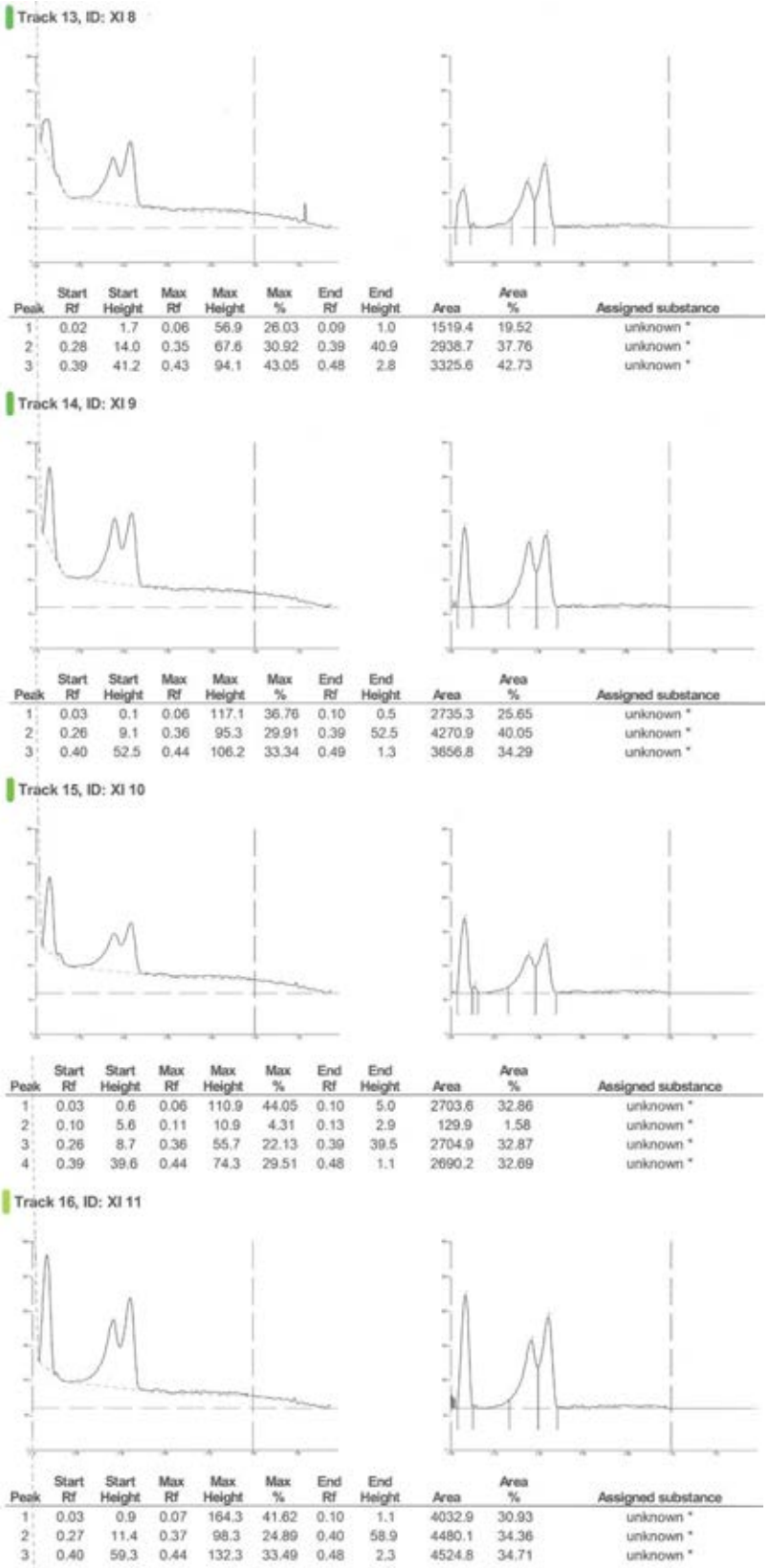
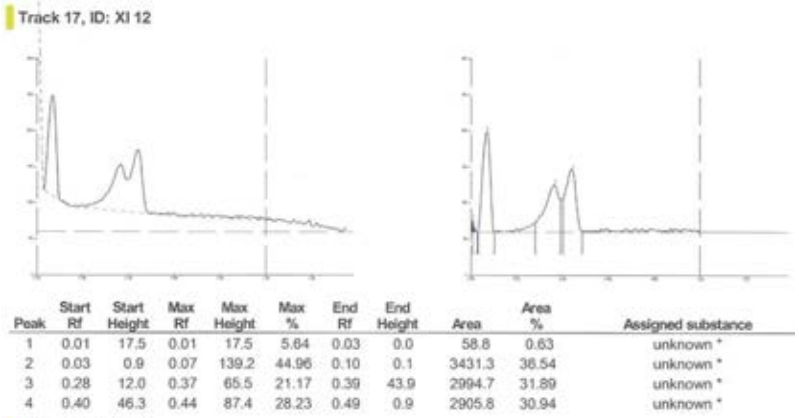
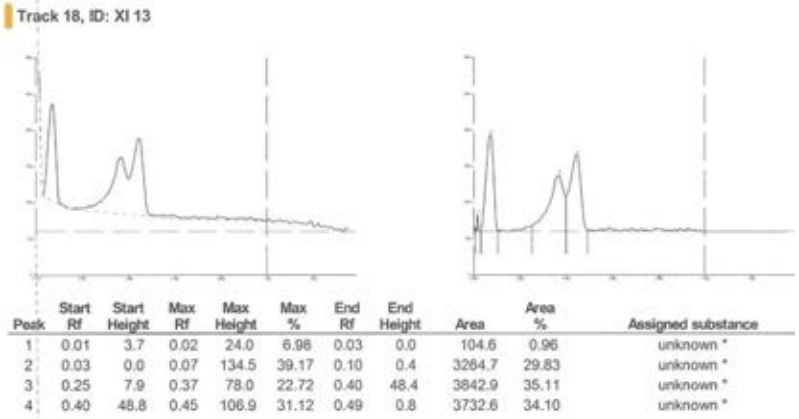


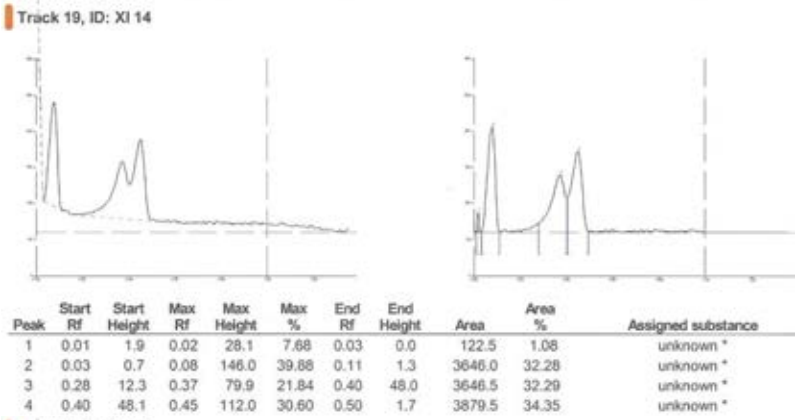
Figure 30 (cont.). TLC-densitometry chromatogram (Plate B-Sample No. 1-15)



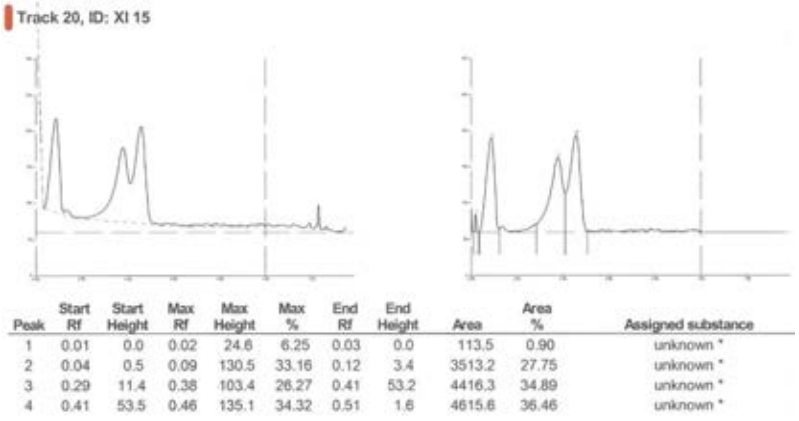
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	17.5	0.01	17.5	5.64	0.03	0.0	58.8	0.63	unknown *
2	0.03	0.9	0.07	139.2	44.96	0.10	0.1	3431.3	36.54	unknown *
3	0.28	12.0	0.37	65.5	21.17	0.39	43.9	2994.7	31.89	unknown *
4	0.40	46.3	0.44	87.4	28.23	0.49	0.9	2905.8	30.94	unknown *



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	3.7	0.02	24.0	6.98	0.03	0.0	104.6	0.96	unknown *
2	0.03	0.0	0.07	134.5	39.17	0.10	0.4	3264.7	29.83	unknown *
3	0.25	7.9	0.37	78.0	22.72	0.40	48.4	3842.9	35.11	unknown *
4	0.40	48.8	0.45	106.9	31.12	0.49	0.8	3732.6	34.10	unknown *

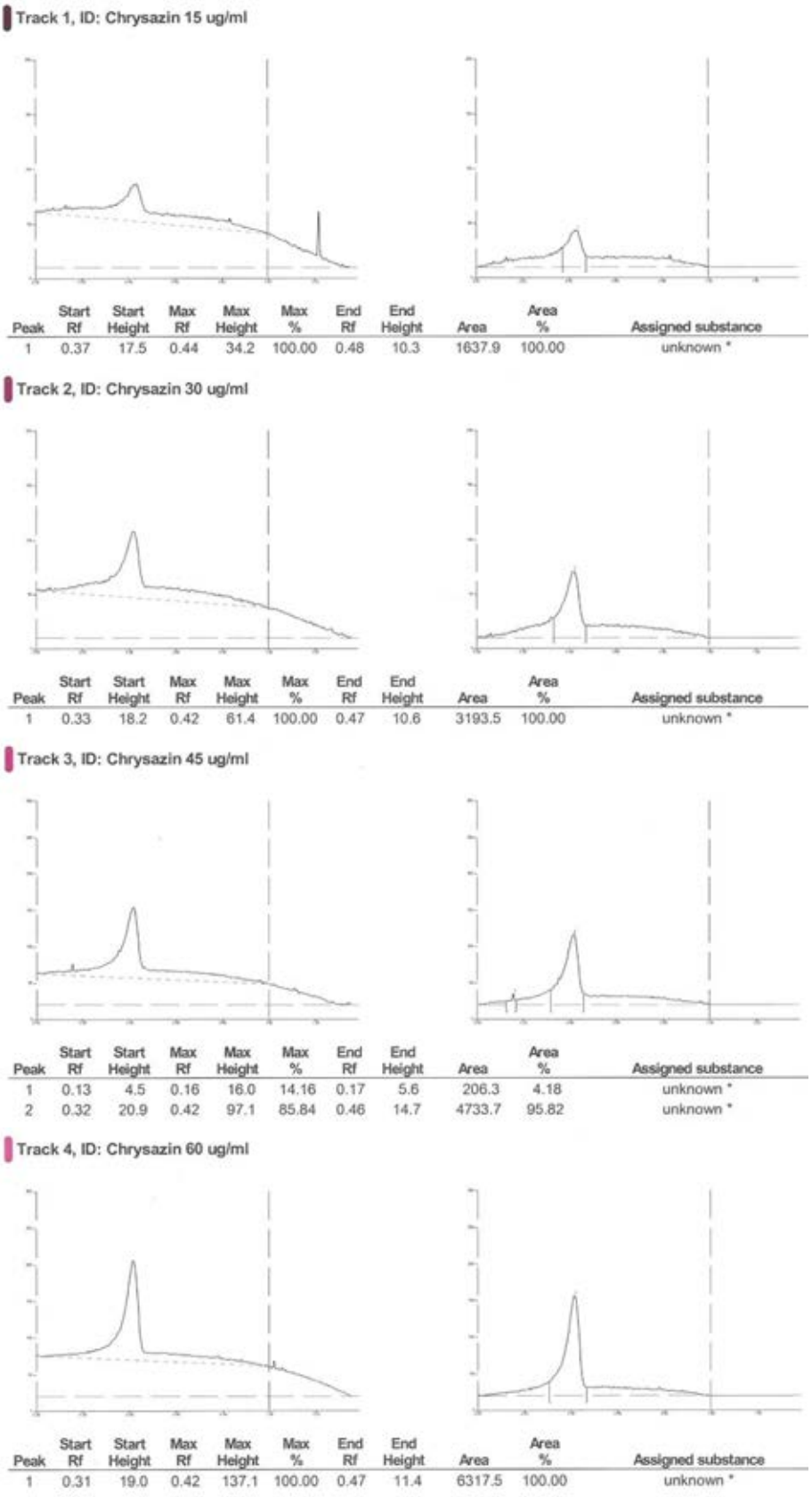


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	1.9	0.02	28.1	7.68	0.03	0.0	122.5	1.08	unknown *
2	0.03	0.7	0.08	146.0	39.88	0.11	1.3	3646.0	32.28	unknown *
3	0.28	12.3	0.37	79.9	21.84	0.40	48.0	3646.5	32.29	unknown *
4	0.40	48.1	0.45	112.0	30.60	0.50	1.7	3879.5	34.35	unknown *



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	0.0	0.02	24.6	6.25	0.03	0.0	113.5	0.90	unknown *
2	0.04	0.5	0.09	130.5	33.16	0.12	3.4	3513.2	27.75	unknown *
3	0.29	11.4	0.38	103.4	26.27	0.41	53.2	4416.3	34.89	unknown *
4	0.41	53.5	0.46	135.1	34.32	0.51	1.6	4615.6	36.46	unknown *

Figure 30 (cont.). TLC-densitometry chromatogram (Plate B-Sample No. 1-15)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.37	17.5	0.44	34.2	100.00	0.48	10.3	1637.9	100.00	unknown *

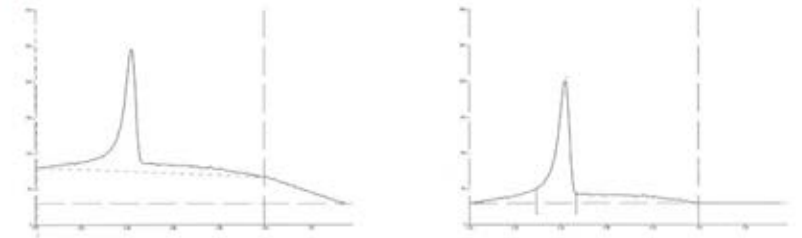
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.33	18.2	0.42	61.4	100.00	0.47	10.6	3193.5	100.00	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.13	4.5	0.16	16.0	14.16	0.17	5.6	206.3	4.18	unknown *
2	0.32	20.9	0.42	97.1	85.84	0.46	14.7	4733.7	95.82	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.31	19.0	0.42	137.1	100.00	0.47	11.4	6317.5	100.00	unknown *

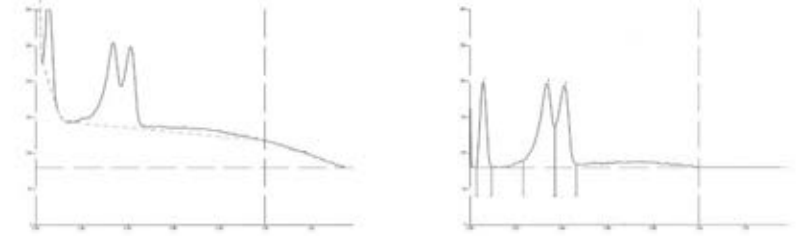
Figure 31. TLC-densitometry chromatogram (Plate C-Sample No. 1-15)

Track 5, ID: Chrysazin 75 ug/ml



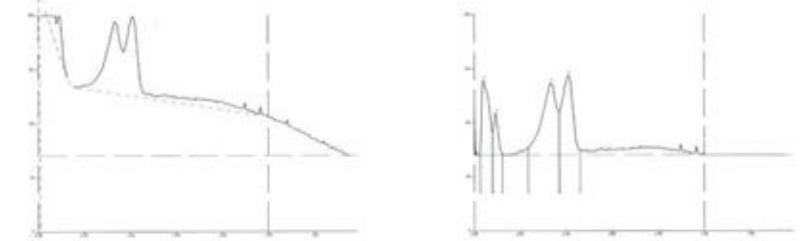
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.29	19.6	0.42	171.6	100.00	0.47	13.3	7969.7	100.00	unknown *

Track 6, ID: XI 1



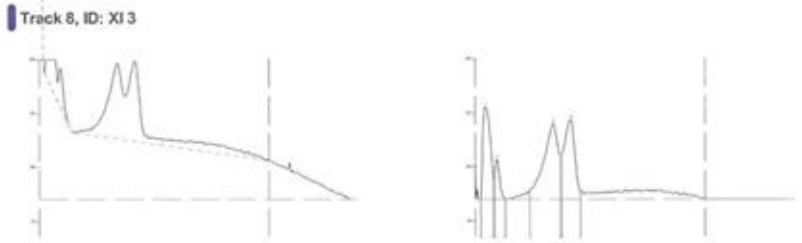
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.5	0.06	119.4	34.08	0.09	0.4	2438.6	21.14	unknown *
2	0.23	8.8	0.34	116.9	33.39	0.37	57.4	5161.6	44.74	unknown *
3	0.37	57.5	0.42	113.9	32.53	0.47	4.1	3936.0	34.12	unknown *

Track 7, ID: XI 2



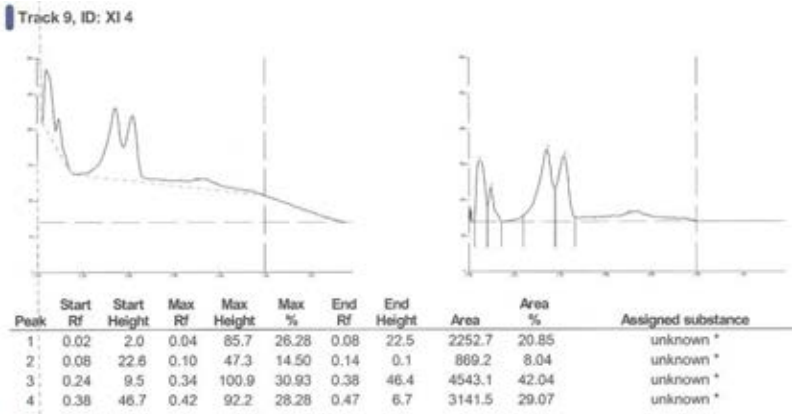
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	3.0	0.04	69.6	27.89	0.08	20.5	1676.2	20.57	unknown *
2	0.08	21.1	0.09	38.9	15.57	0.12	1.8	580.1	7.12	unknown *
3	0.24	6.8	0.33	66.9	26.80	0.37	39.9	3247.0	39.85	unknown *
4	0.37	40.2	0.41	74.3	29.74	0.46	4.0	2644.5	32.46	unknown *

Track 8, ID: XI 3



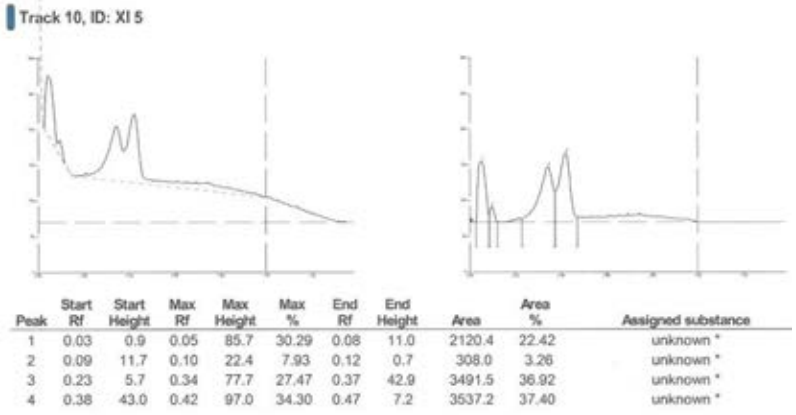
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	1.7	0.04	86.1	32.07	0.08	18.7	2139.4	24.50	unknown *
2	0.08	19.5	0.10	37.2	13.88	0.13	0.2	569.2	6.52	unknown *
3	0.24	8.0	0.34	70.6	26.30	0.37	40.5	3382.1	38.74	unknown *
4	0.38	40.6	0.42	74.5	27.75	0.46	6.6	2640.5	30.24	unknown *

Figure 31 (cont.). TLC-densitometry chromatogram (Plate C-Sample No. 1-15)



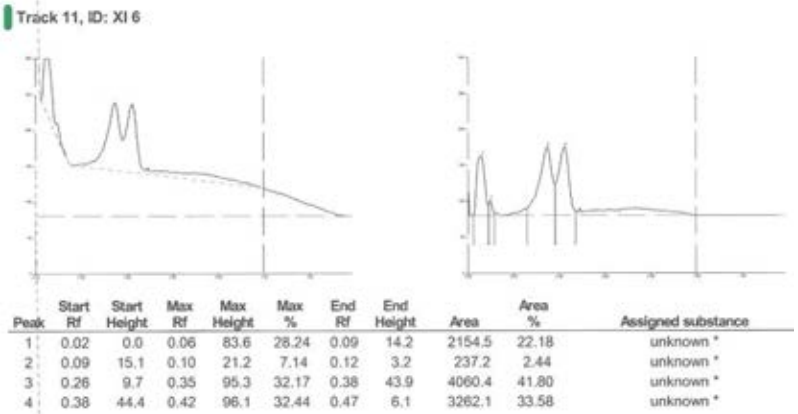
Track 9, ID: XI 4

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	2.0	0.04	85.7	26.28	0.08	22.5	2252.7	20.85	unknown *
2	0.08	22.6	0.10	47.3	14.50	0.14	0.1	869.2	8.04	unknown *
3	0.24	9.5	0.34	100.9	30.93	0.38	46.4	4543.1	42.04	unknown *
4	0.38	46.7	0.42	92.2	28.28	0.47	6.7	3141.5	29.07	unknown *



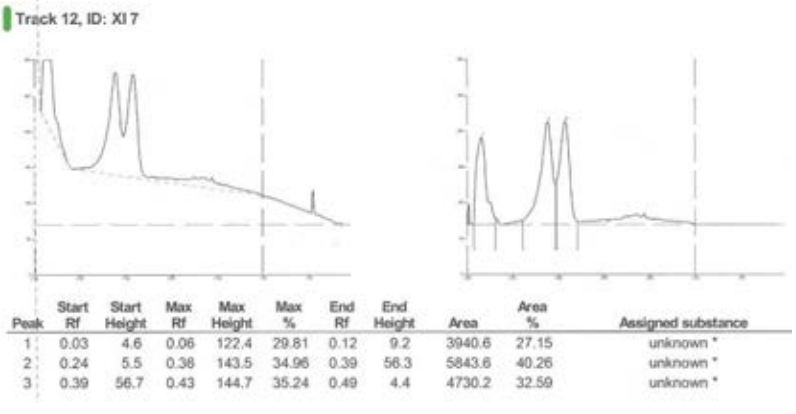
Track 10, ID: XI 5

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.9	0.05	85.7	30.29	0.08	11.0	2120.4	22.42	unknown *
2	0.09	11.7	0.10	22.4	7.93	0.12	0.7	308.0	3.26	unknown *
3	0.23	5.7	0.34	77.7	27.47	0.37	42.9	3491.5	36.92	unknown *
4	0.38	43.0	0.42	97.0	34.30	0.47	7.2	3537.2	37.40	unknown *



Track 11, ID: XI 6

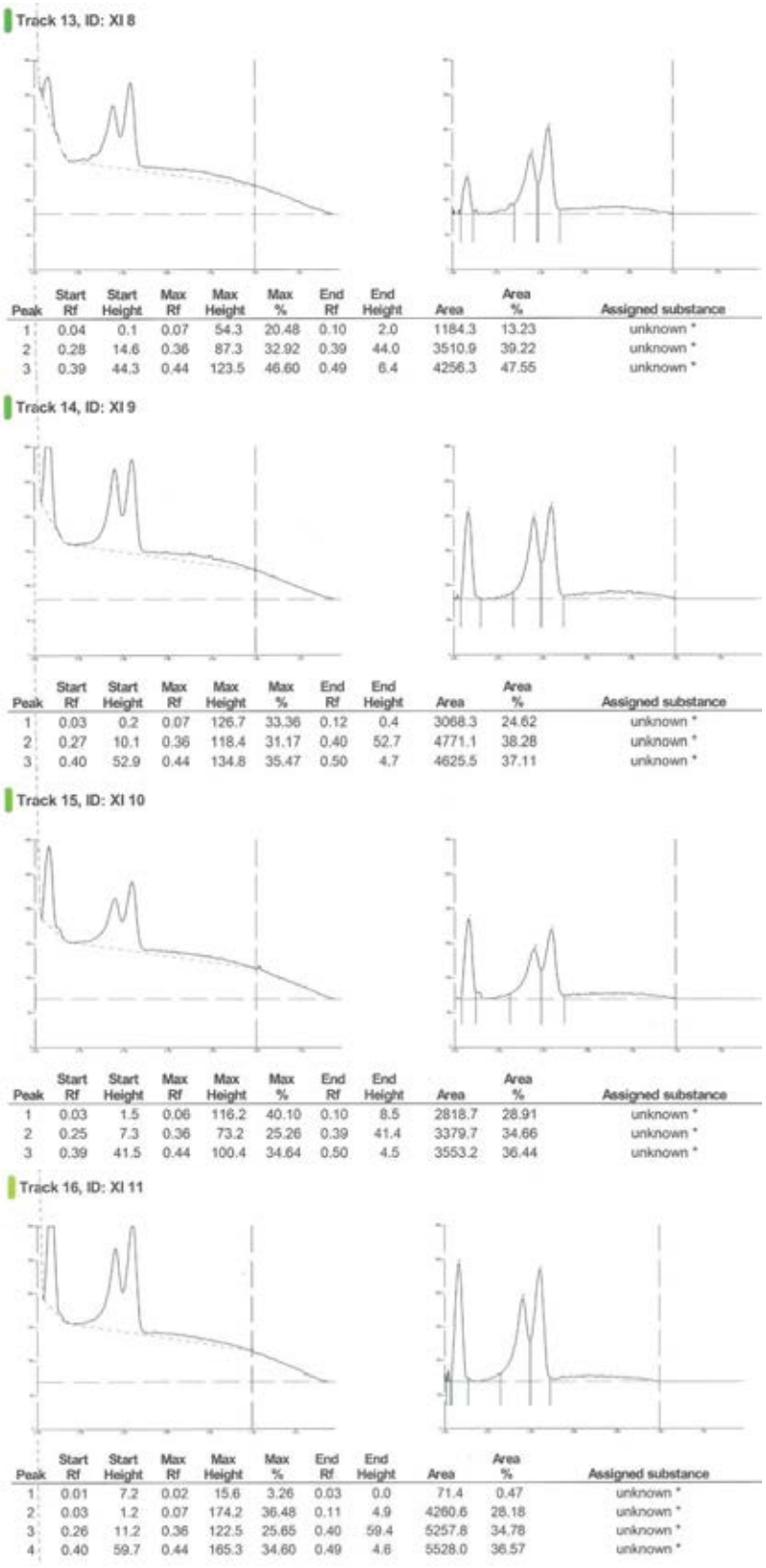
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	0.0	0.06	83.6	28.24	0.09	14.2	2154.5	22.18	unknown *
2	0.09	15.1	0.10	21.2	7.14	0.12	3.2	237.2	2.44	unknown *
3	0.26	9.7	0.35	95.3	32.17	0.38	43.9	4060.4	41.80	unknown *
4	0.38	44.4	0.42	96.1	32.44	0.47	6.1	3262.1	33.58	unknown *



Track 12, ID: XI 7

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	4.6	0.06	122.4	29.81	0.12	9.2	3940.6	27.15	unknown *
2	0.24	5.5	0.38	143.5	34.96	0.39	56.3	5843.6	40.26	unknown *
3	0.39	56.7	0.43	144.7	35.24	0.49	4.4	4730.2	32.59	unknown *

Figure 31 (cont.). TLC-densitometry chromatogram (Plate C-Sample No. 1-15)



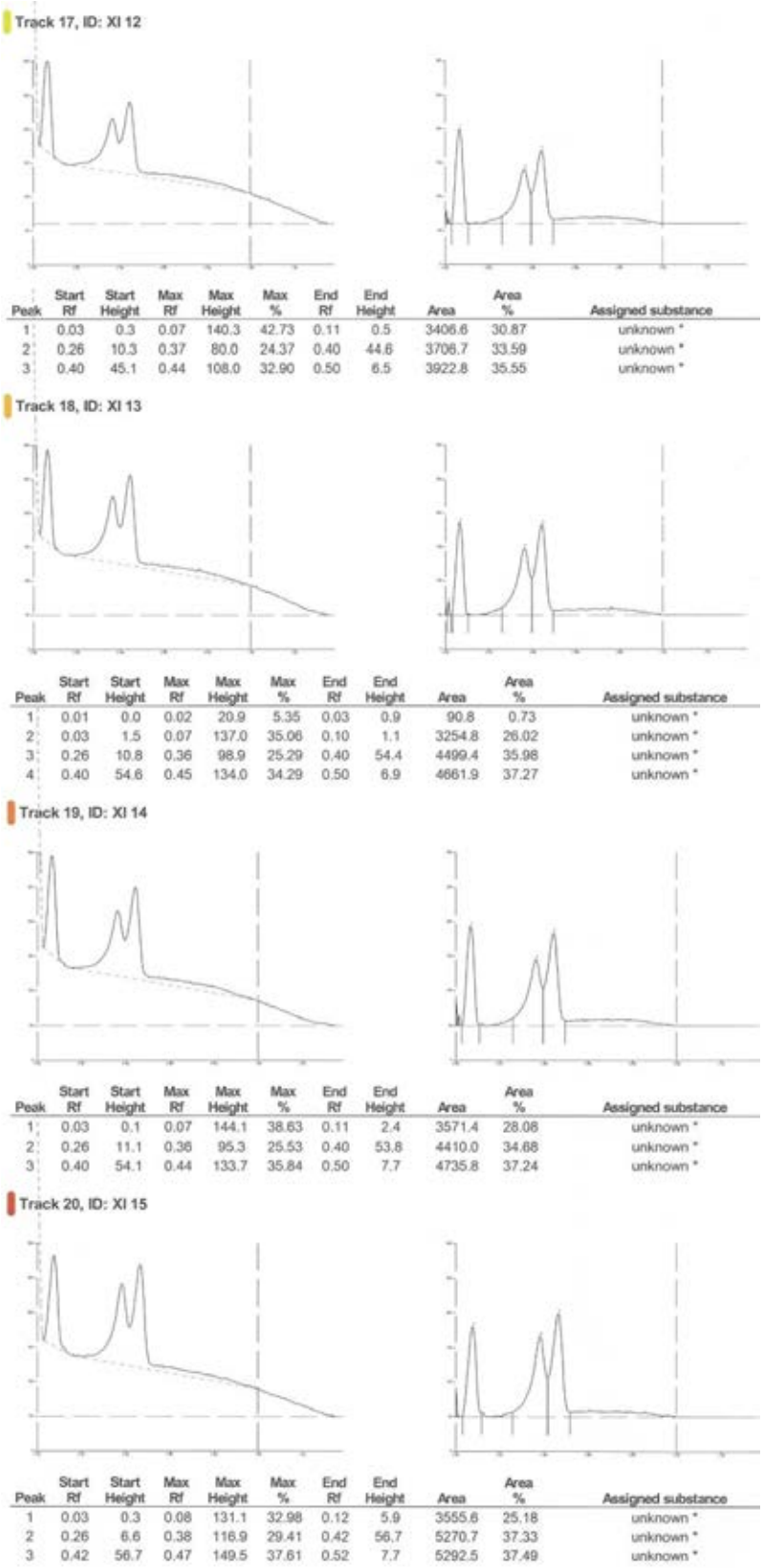
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	0.1	0.07	54.3	20.48	0.10	2.0	1184.3	13.23	unknown *
2	0.28	14.6	0.36	87.3	32.92	0.39	44.0	3510.9	39.22	unknown *
3	0.39	44.3	0.44	123.5	46.60	0.49	6.4	4256.3	47.55	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.2	0.07	126.7	33.36	0.12	0.4	3068.3	24.62	unknown *
2	0.27	10.1	0.36	118.4	31.17	0.40	52.7	4771.1	38.28	unknown *
3	0.40	52.9	0.44	134.8	35.47	0.50	4.7	4625.5	37.11	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.5	0.06	116.2	40.10	0.10	8.5	2818.7	28.91	unknown *
2	0.25	7.3	0.36	73.2	25.26	0.39	41.4	3379.7	34.66	unknown *
3	0.39	41.5	0.44	100.4	34.64	0.50	4.5	3553.2	36.44	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	7.2	0.02	15.6	3.26	0.03	0.0	71.4	0.47	unknown *
2	0.03	1.2	0.07	174.2	36.48	0.11	4.9	4260.6	28.18	unknown *
3	0.26	11.2	0.36	122.5	25.65	0.40	59.4	5257.8	34.78	unknown *
4	0.40	59.7	0.44	165.3	34.60	0.49	4.6	5528.0	36.57	unknown *

Figure 31 (cont.). TLC-densitometry chromatogram (Plate C-Sample No. 1-15)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.3	0.07	140.3	42.73	0.11	0.5	3406.6	30.87	unknown *
2	0.26	10.3	0.37	80.0	24.37	0.40	44.6	3706.7	33.59	unknown *
3	0.40	45.1	0.44	108.0	32.90	0.50	6.5	3922.8	35.55	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	0.0	0.02	20.9	5.35	0.03	0.9	90.8	0.73	unknown *
2	0.03	1.5	0.07	137.0	35.06	0.10	1.1	3254.8	26.02	unknown *
3	0.26	10.8	0.36	98.9	25.29	0.40	54.4	4499.4	35.98	unknown *
4	0.40	54.6	0.45	134.0	34.29	0.50	6.9	4661.9	37.27	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.1	0.07	144.1	38.63	0.11	2.4	3571.4	28.08	unknown *
2	0.26	11.1	0.36	95.3	25.53	0.40	53.8	4410.0	34.68	unknown *
3	0.40	54.1	0.44	133.7	35.84	0.50	7.7	4735.8	37.24	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.3	0.08	131.1	32.98	0.12	5.9	3555.6	25.18	unknown *
2	0.26	6.6	0.38	116.9	29.41	0.42	56.7	5270.7	37.33	unknown *
3	0.42	56.7	0.47	149.5	37.61	0.52	7.7	5292.5	37.49	unknown *

Figure 31 (cont.). TLC-densitometry chromatogram (Plate C-Sample No. 1-15)

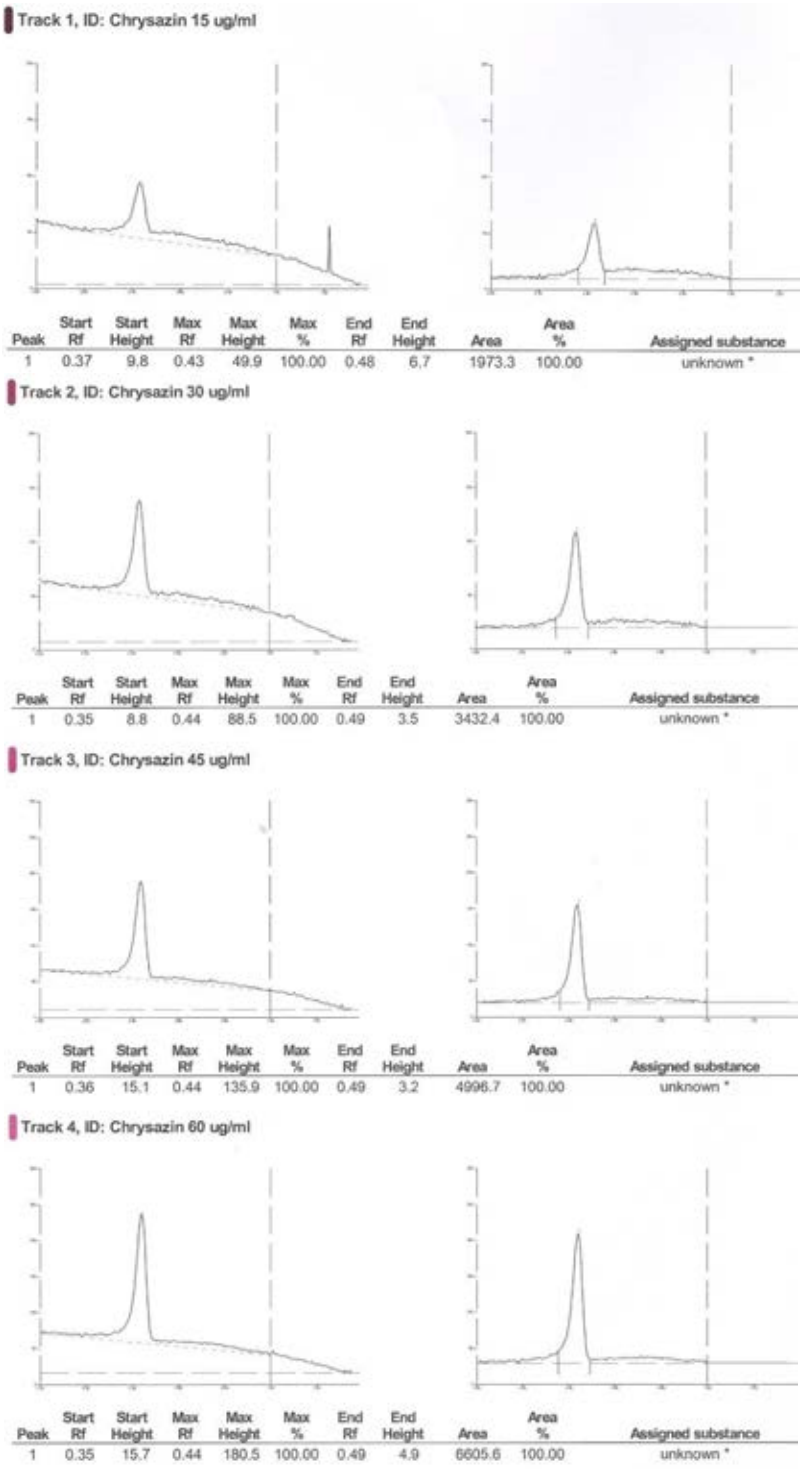
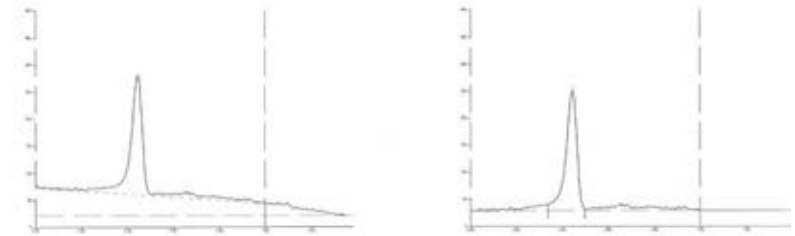


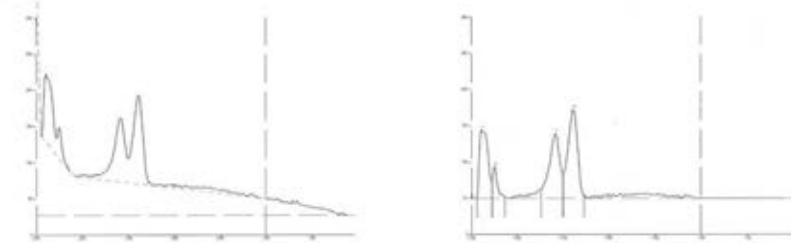
Figure 32. TLC-densitometry chromatogram (Plate D-Accuracy)

Track 5, ID: Chrysazin 75 ug/ml



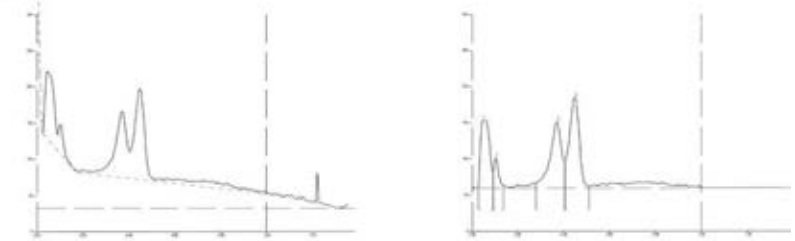
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.34	11.1	0.45	221.1	100.00	0.50	3.9	8114.4	100.00	unknown *

Track 6, ID: Spike sample 10 ug/ml



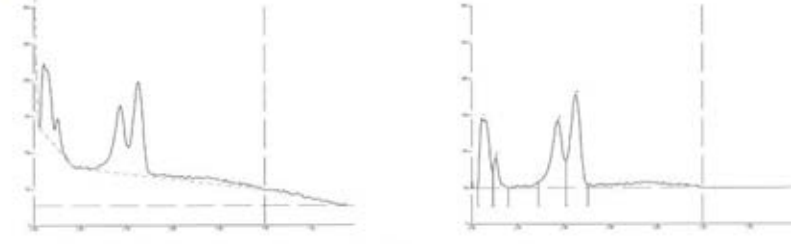
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	0.7	0.04	94.8	27.04	0.09	23.8	2704.0	25.79	unknown *
2	0.09	24.6	0.10	44.1	12.57	0.15	1.7	692.2	6.60	unknown *
3	0.30	12.4	0.37	88.7	25.30	0.40	34.3	3134.6	29.90	unknown *
4	0.40	34.3	0.45	123.0	35.08	0.49	3.1	3952.3	37.70	unknown *

Track 7, ID: Spike sample 10 ug/ml



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.6	0.05	94.6	26.83	0.09	21.9	2740.0	25.07	unknown *
2	0.09	22.6	0.10	41.5	11.77	0.14	2.8	664.9	6.08	unknown *
3	0.28	6.2	0.37	91.4	25.89	0.40	37.1	3430.5	31.39	unknown *
4	0.41	37.2	0.45	125.3	35.51	0.51	1.8	4094.8	37.46	unknown *

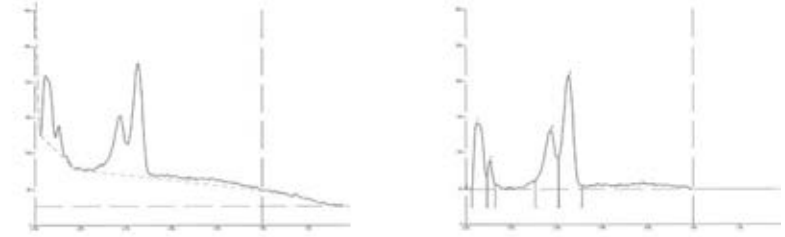
Track 8, ID: Spike sample 10 ug/ml



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	2.0	0.05	96.2	26.63	0.09	22.7	2802.5	25.12	unknown *
2	0.09	23.9	0.10	42.7	11.83	0.16	0.2	721.0	6.46	unknown *
3	0.29	8.6	0.38	93.4	25.85	0.41	37.1	3484.1	31.23	unknown *
4	0.41	37.1	0.45	129.0	35.69	0.50	4.2	4150.0	37.19	unknown *

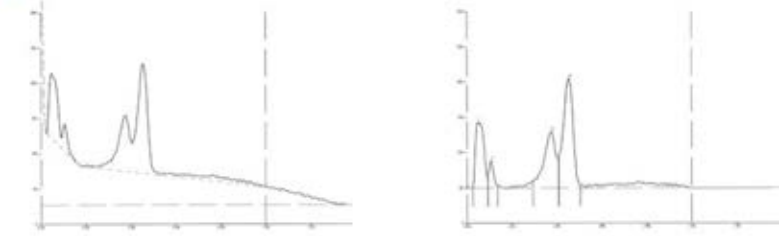
Figure 32 (cont.). TLC-densitometry chromatogram (Plate D-Accuracy)

Track 9, ID: Spike sample 25 ug/ml



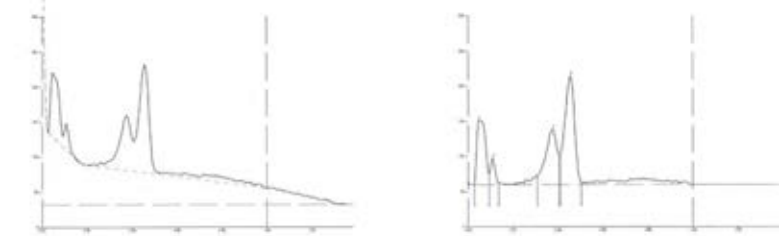
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.9	0.05	92.1	24.77	0.09	18.7	2648.7	22.79	unknown *
2	0.09	19.6	0.11	39.3	10.56	0.13	5.5	587.2	5.05	unknown *
3	0.31	11.5	0.38	81.9	22.02	0.41	42.6	3142.9	27.04	unknown *
4	0.41	42.7	0.46	158.6	42.64	0.51	4.4	5243.5	45.12	unknown *

Track 10, ID: Spike sample 25 ug/ml



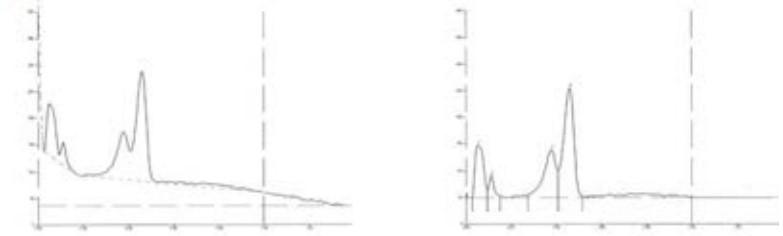
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.6	0.05	93.6	25.42	0.09	14.7	2639.1	22.75	unknown *
2	0.09	15.8	0.11	39.2	10.64	0.14	3.5	624.4	5.38	unknown *
3	0.30	9.7	0.38	79.9	21.68	0.41	41.3	3174.9	27.37	unknown *
4	0.41	42.0	0.46	155.7	42.27	0.51	4.2	5161.5	44.50	unknown *

Track 11, ID: Spike sample 25 ug/ml



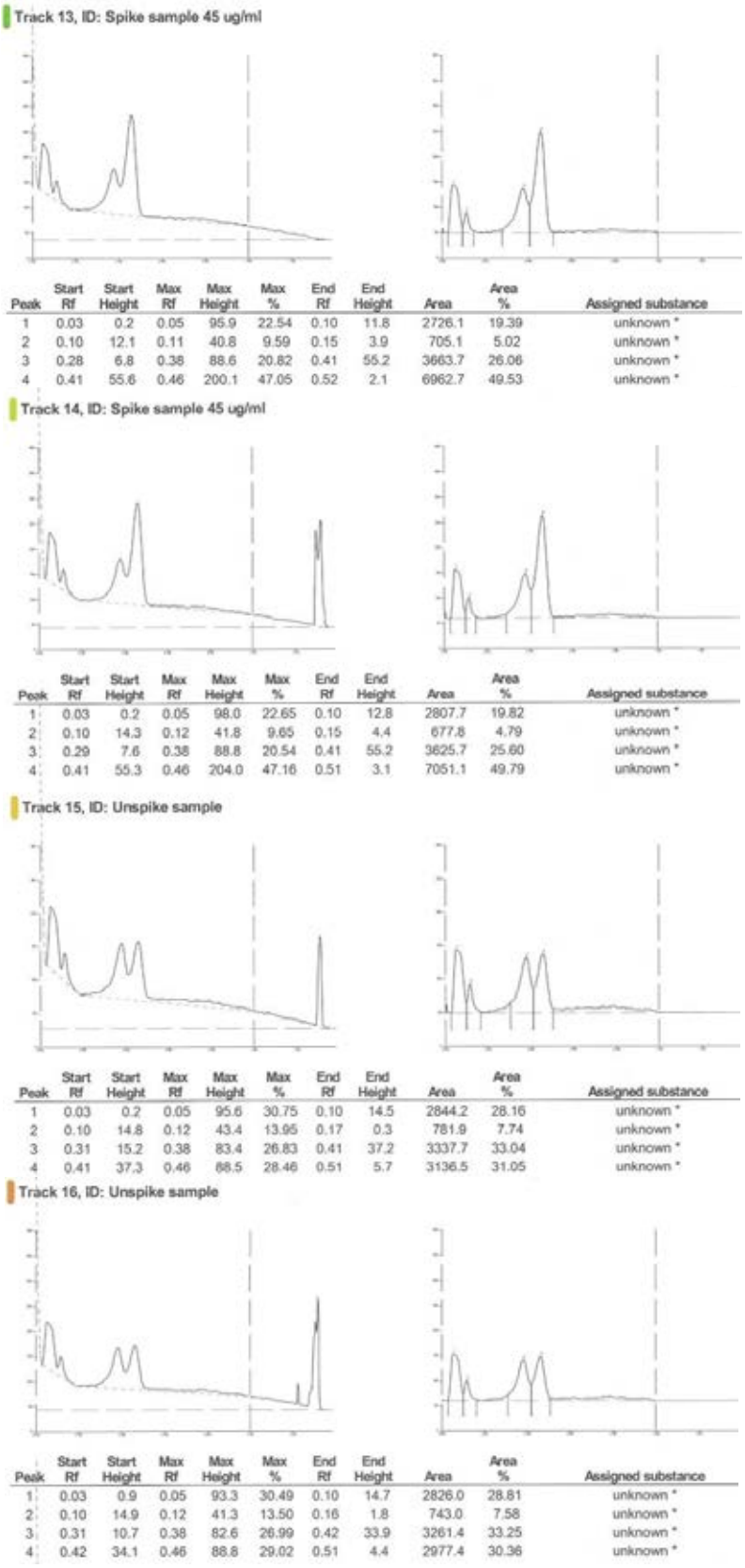
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.5	0.05	93.5	25.54	0.09	15.5	2670.0	23.18	unknown *
2	0.10	15.8	0.11	38.2	10.43	0.14	3.8	600.3	5.21	unknown *
3	0.31	13.3	0.38	79.0	21.58	0.41	42.5	3061.9	26.58	unknown *
4	0.41	42.7	0.46	155.3	42.45	0.51	4.1	5186.9	45.03	unknown *

Track 12, ID: Spike sample 45 ug/ml



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.9	0.05	97.8	22.55	0.10	13.7	2757.9	19.38	unknown *
2	0.10	14.1	0.11	42.3	9.76	0.15	2.9	723.3	5.08	unknown *
3	0.28	5.7	0.38	88.2	20.34	0.41	53.5	3700.0	26.00	unknown *
4	0.41	53.5	0.46	205.4	47.35	0.52	2.9	7048.6	49.53	unknown *

Figure 32 (cont.). TLC-densitometry chromatogram (Plate D-Accuracy)



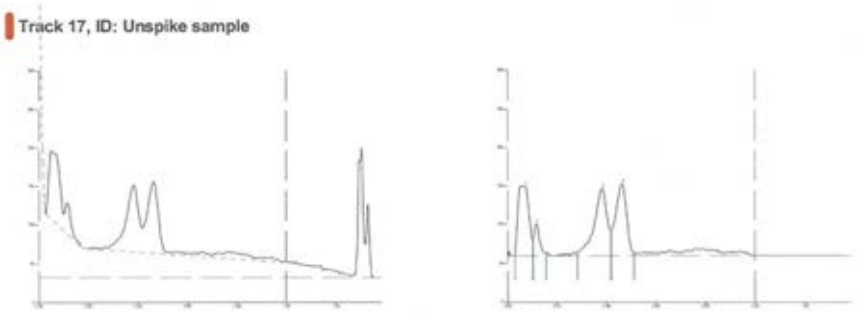
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.2	0.05	95.9	22.54	0.10	11.8	2726.1	19.39	unknown *
2	0.10	12.1	0.11	40.8	9.59	0.15	3.9	705.1	5.02	unknown *
3	0.28	6.8	0.38	88.6	20.82	0.41	55.2	3663.7	26.06	unknown *
4	0.41	55.6	0.46	200.1	47.05	0.52	2.1	6962.7	49.53	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.2	0.05	98.0	22.65	0.10	12.8	2807.7	19.82	unknown *
2	0.10	14.3	0.12	41.8	9.65	0.15	4.4	677.8	4.79	unknown *
3	0.29	7.6	0.38	88.8	20.54	0.41	55.2	3625.7	25.60	unknown *
4	0.41	55.3	0.46	204.0	47.16	0.51	3.1	7051.1	49.79	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.2	0.05	95.6	30.75	0.10	14.5	2844.2	28.16	unknown *
2	0.10	14.8	0.12	43.4	13.95	0.17	0.3	781.9	7.74	unknown *
3	0.31	15.2	0.38	83.4	26.83	0.41	37.2	3337.7	33.04	unknown *
4	0.41	37.3	0.46	88.5	28.46	0.51	5.7	3136.5	31.05	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.9	0.05	93.3	30.49	0.10	14.7	2826.0	28.81	unknown *
2	0.10	14.9	0.12	41.3	13.50	0.16	1.8	743.0	7.58	unknown *
3	0.31	10.7	0.38	82.6	26.99	0.42	33.9	3261.4	33.25	unknown *
4	0.42	34.1	0.46	88.8	29.02	0.51	4.4	2977.4	30.36	unknown *

Figure 32 (cont.). TLC-densitometry chromatogram (Plate D-Accuracy)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.7	0.07	90.8	29.19	0.10	21.2	2979.8	29.19	unknown *
2	0.10	22.3	0.12	40.6	13.04	0.16	3.0	720.3	7.06	unknown *
3	0.28	3.6	0.38	86.7	27.86	0.42	32.0	3415.3	33.46	unknown *
4	0.42	32.1	0.46	93.1	29.91	0.51	2.8	3092.4	30.29	unknown *

Figure 32 (cont.). TLC-densitometry chromatogram (Plate D-Accuracy)

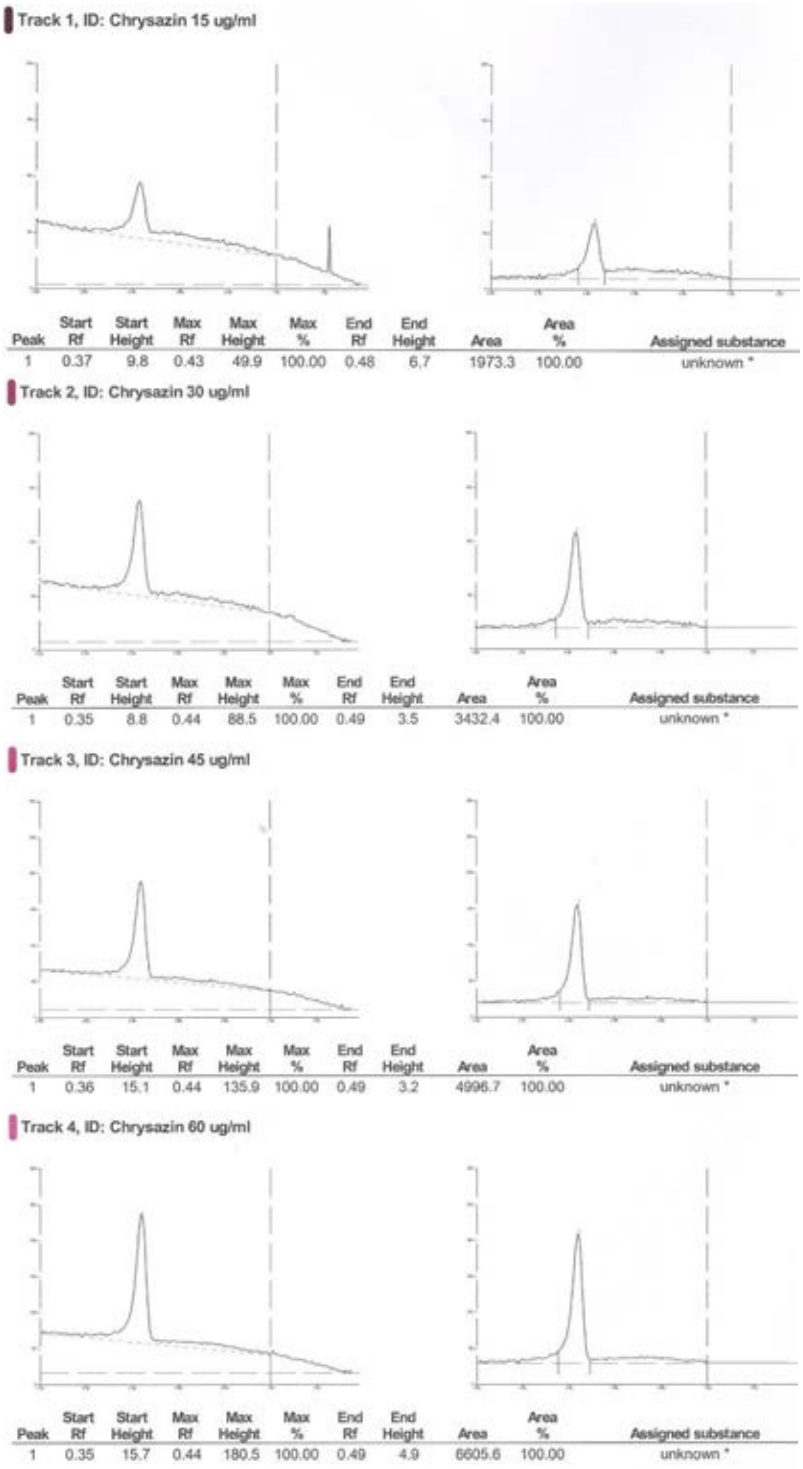
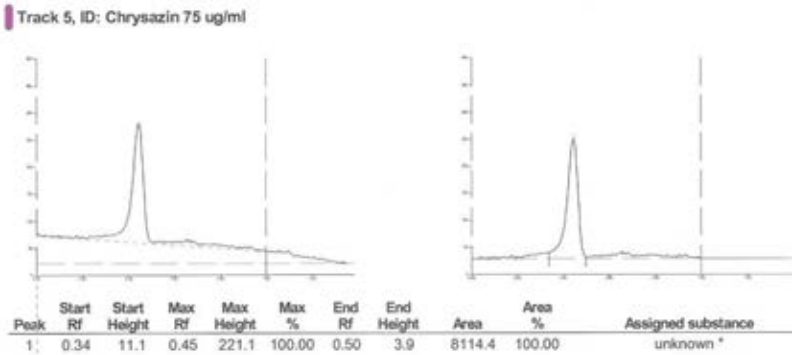
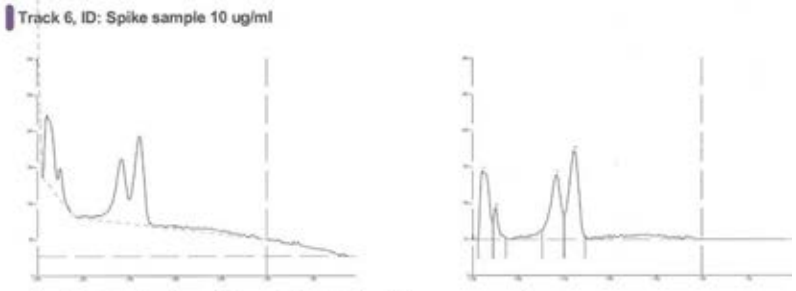


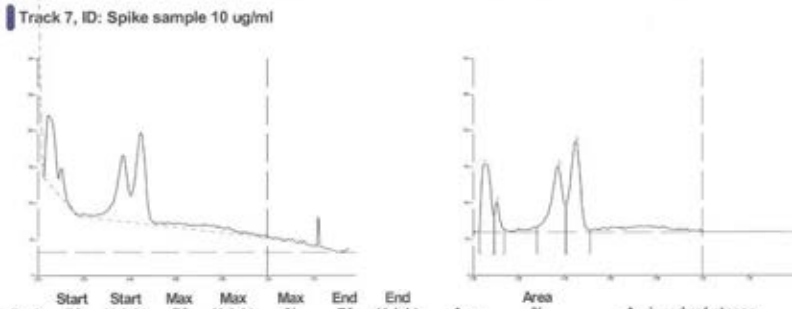
Figure 33. TLC-densitometry chromatogram (Plate E-Precision 1)



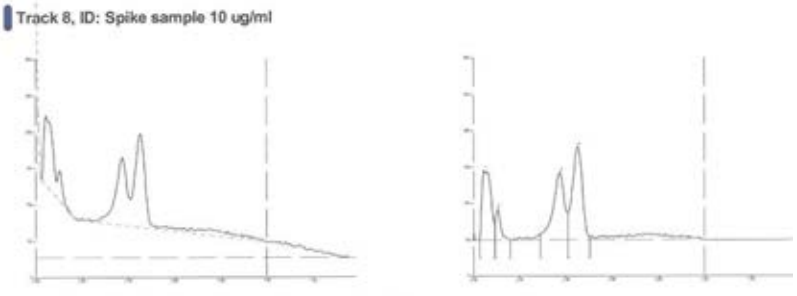
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.34	11.1	0.45	221.1	100.00	0.50	3.9	8114.4	100.00	unknown *



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	0.7	0.04	94.8	27.04	0.09	23.8	2704.0	25.79	unknown *
2	0.09	24.6	0.10	44.1	12.57	0.15	1.7	692.2	6.60	unknown *
3	0.30	12.4	0.37	88.7	25.30	0.40	34.3	3134.6	29.90	unknown *
4	0.40	34.3	0.45	123.0	35.08	0.49	3.1	3952.3	37.70	unknown *



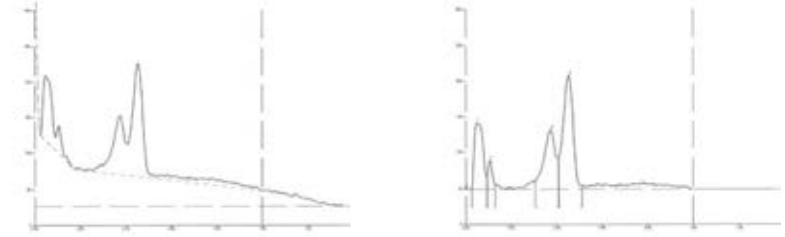
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.6	0.05	94.6	26.83	0.09	21.9	2740.0	25.07	unknown *
2	0.09	22.6	0.10	41.5	11.77	0.14	2.8	664.9	6.08	unknown *
3	0.28	6.2	0.37	91.4	25.89	0.40	37.1	3430.5	31.39	unknown *
4	0.41	37.2	0.45	125.3	35.51	0.51	1.8	4094.8	37.46	unknown *



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	2.0	0.05	96.2	26.63	0.09	22.7	2802.5	25.12	unknown *
2	0.09	23.9	0.10	42.7	11.83	0.16	0.2	721.0	6.46	unknown *
3	0.29	8.6	0.38	93.4	25.85	0.41	37.1	3484.1	31.23	unknown *
4	0.41	37.1	0.45	129.0	35.69	0.50	4.2	4150.0	37.19	unknown *

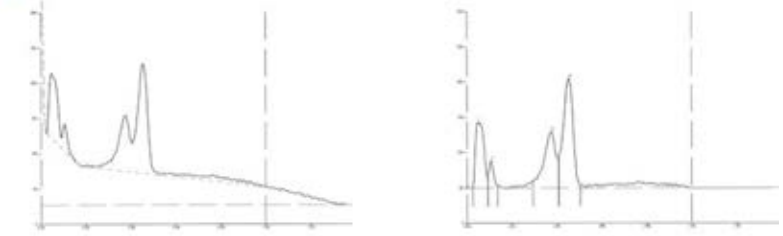
Figure 33 (cont.). TLC-densitometry chromatogram (Plate E-Precision 1)

Track 9, ID: Spike sample 25 ug/ml



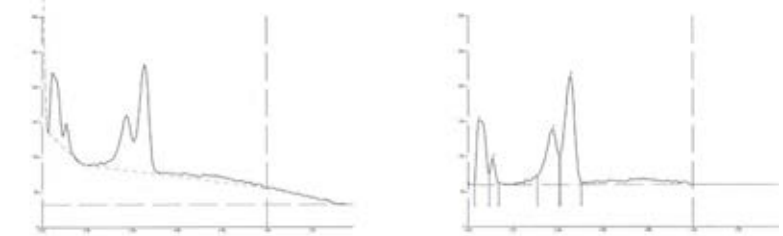
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.9	0.05	92.1	24.77	0.09	18.7	2648.7	22.79	unknown *
2	0.09	19.6	0.11	39.3	10.56	0.13	5.5	587.2	5.05	unknown *
3	0.31	11.5	0.38	81.9	22.02	0.41	42.6	3142.9	27.04	unknown *
4	0.41	42.7	0.46	158.6	42.64	0.51	4.4	5243.5	45.12	unknown *

Track 10, ID: Spike sample 25 ug/ml



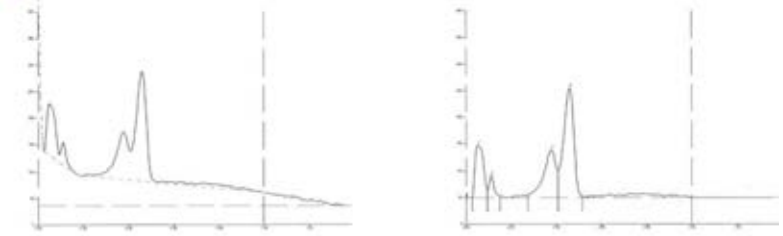
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.6	0.05	93.6	25.42	0.09	14.7	2639.1	22.75	unknown *
2	0.09	15.8	0.11	39.2	10.64	0.14	3.5	624.4	5.38	unknown *
3	0.30	9.7	0.38	79.9	21.68	0.41	41.3	3174.9	27.37	unknown *
4	0.41	42.0	0.46	155.7	42.27	0.51	4.2	5161.5	44.50	unknown *

Track 11, ID: Spike sample 25 ug/ml



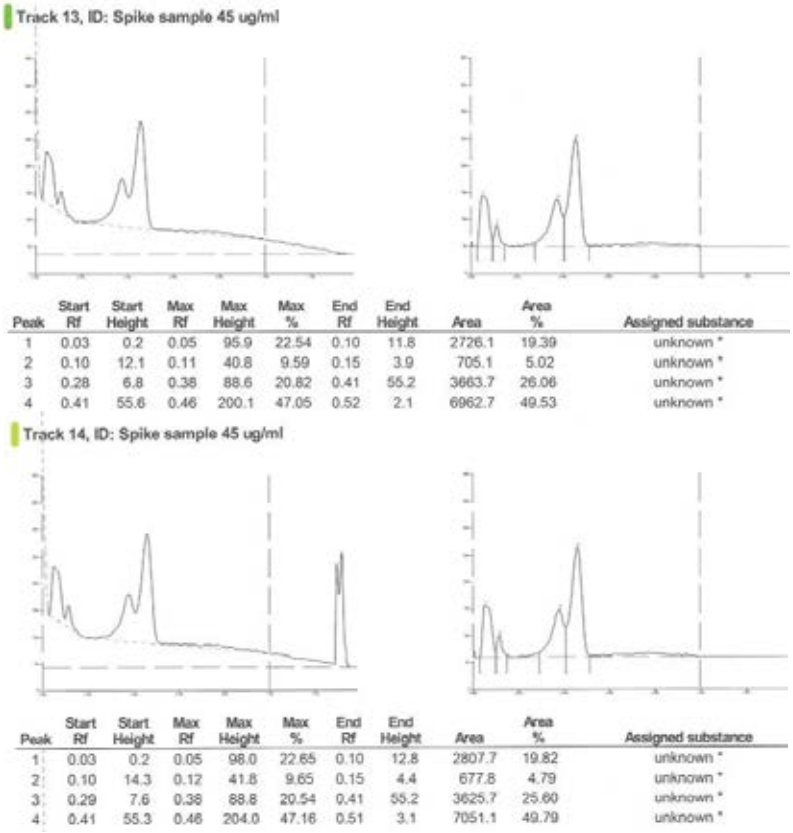
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.5	0.05	93.5	25.54	0.09	15.5	2670.0	23.18	unknown *
2	0.10	15.8	0.11	38.2	10.43	0.14	3.8	600.3	5.21	unknown *
3	0.31	13.3	0.38	79.0	21.58	0.41	42.5	3061.9	26.58	unknown *
4	0.41	42.7	0.46	155.3	42.45	0.51	4.1	5186.9	45.03	unknown *

Track 12, ID: Spike sample 45 ug/ml



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.9	0.05	97.8	22.55	0.10	13.7	2757.9	19.38	unknown *
2	0.10	14.1	0.11	42.3	9.76	0.15	2.9	723.3	5.08	unknown *
3	0.28	5.7	0.38	88.2	20.34	0.41	53.5	3700.0	26.00	unknown *
4	0.41	53.5	0.46	205.4	47.35	0.52	2.9	7048.6	49.53	unknown *

Figure 33 (cont.). TLC-densitometry chromatogram (Plate E-Precision 1)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.2	0.05	95.9	22.54	0.10	11.8	2726.1	19.39	unknown *
2	0.10	12.1	0.11	40.8	9.59	0.15	3.9	705.1	5.02	unknown *
3	0.28	6.8	0.38	88.6	20.82	0.41	55.2	3663.7	26.06	unknown *
4	0.41	55.6	0.46	200.1	47.05	0.52	2.1	6962.7	49.53	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.2	0.05	98.0	22.65	0.10	12.8	2807.7	19.82	unknown *
2	0.10	14.3	0.12	41.8	9.65	0.15	4.4	677.8	4.79	unknown *
3	0.29	7.6	0.38	88.8	20.54	0.41	55.2	3625.7	25.60	unknown *
4	0.41	55.3	0.46	204.0	47.16	0.51	3.1	7051.1	49.79	unknown *

Figure 33 (cont.). TLC-densitometry chromatogram (Plate E-Precision 1)

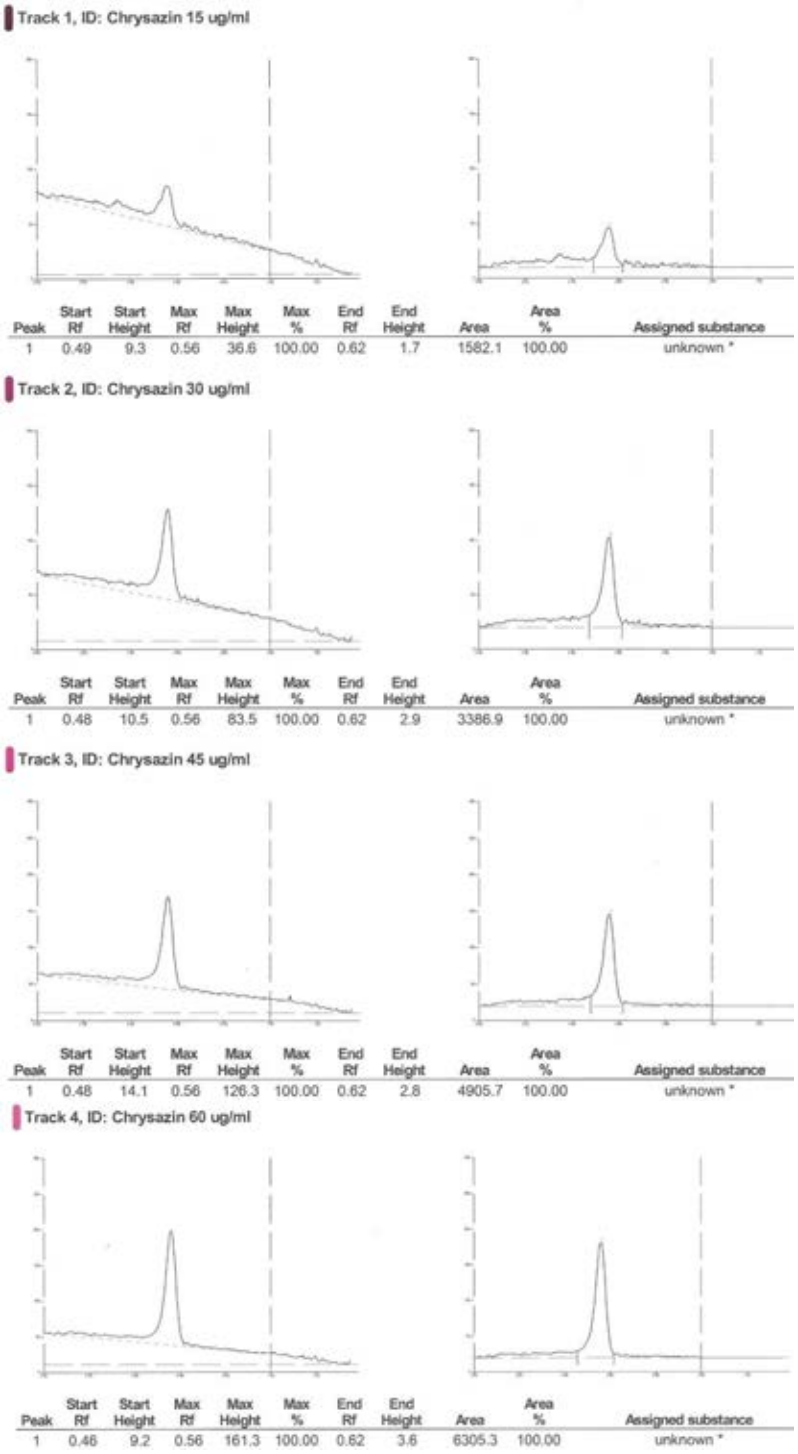
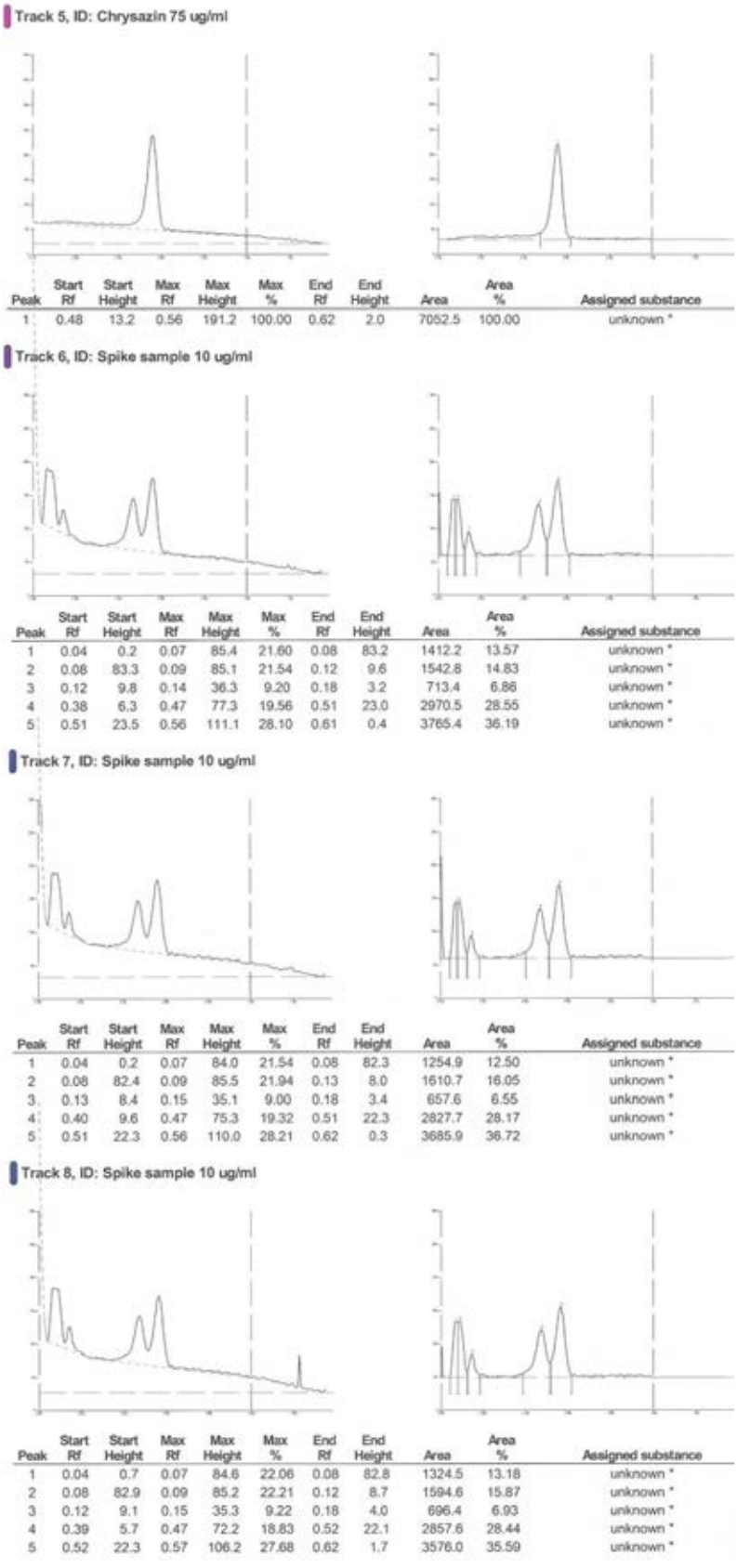


Figure 34. TLC-densitometry chromatogram (Plate F-Precision 2)



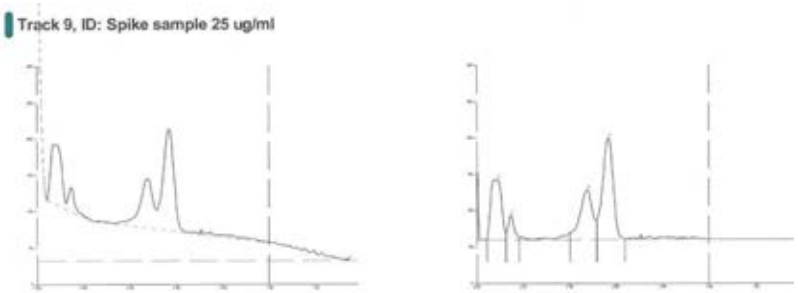
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.48	13.2	0.56	191.2	100.00	0.62	2.0	7052.5	100.00	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	0.2	0.07	85.4	21.60	0.08	83.2	1412.2	13.57	unknown *
2	0.08	83.3	0.09	85.1	21.54	0.12	9.6	1542.8	14.83	unknown *
3	0.12	9.8	0.14	36.3	9.20	0.18	3.2	713.4	6.86	unknown *
4	0.38	6.3	0.47	77.3	19.56	0.51	23.0	2970.5	28.55	unknown *
5	0.51	23.5	0.56	111.1	28.10	0.61	0.4	3765.4	36.19	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	0.2	0.07	84.0	21.54	0.08	82.3	1254.9	12.50	unknown *
2	0.08	82.4	0.09	85.5	21.94	0.13	8.0	1610.7	16.05	unknown *
3	0.13	8.4	0.15	35.1	9.00	0.18	3.4	657.6	6.55	unknown *
4	0.40	9.6	0.47	75.3	19.32	0.51	22.3	2827.7	28.17	unknown *
5	0.51	22.3	0.56	110.0	28.21	0.62	0.3	3685.9	36.72	unknown *

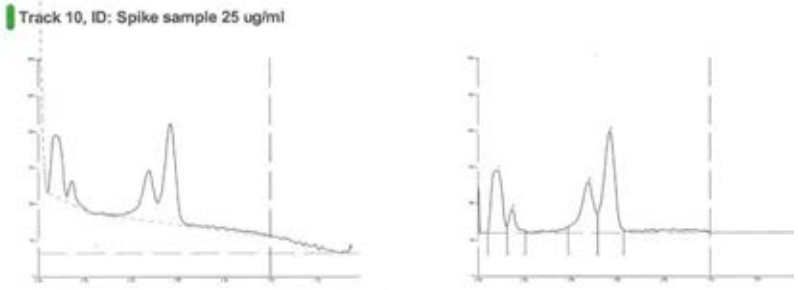
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	0.7	0.07	84.6	22.06	0.08	82.8	1324.5	13.18	unknown *
2	0.08	82.9	0.09	85.2	22.21	0.12	8.7	1594.6	15.87	unknown *
3	0.12	9.1	0.15	35.3	9.22	0.18	4.0	696.4	6.93	unknown *
4	0.39	5.7	0.47	72.2	18.83	0.52	22.1	2857.6	28.44	unknown *
5	0.52	22.3	0.57	106.2	27.68	0.62	1.7	3576.0	35.59	unknown *

Figure 34 (cont). TLC-densitometry chromatogram (Plate F-Precision 2)



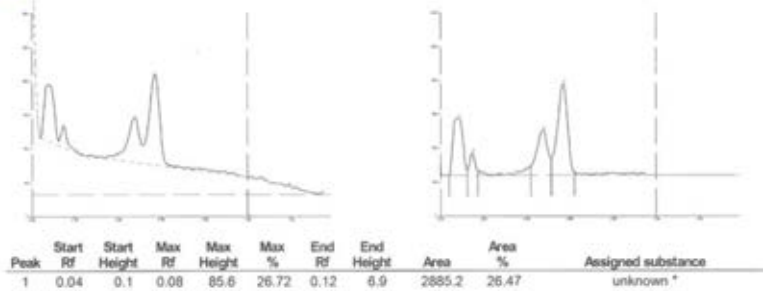
Track 9, ID: Spike sample 25 ug/ml

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	0.7	0.09	83.6	25.65	0.13	8.3	2899.0	25.81	unknown *
2	0.13	8.8	0.15	33.7	10.34	0.18	3.5	655.7	5.84	unknown *
3	0.40	9.1	0.48	68.2	20.93	0.52	26.1	2785.4	24.80	unknown *
4	0.52	26.4	0.57	140.4	43.07	0.64	0.6	4891.5	43.55	unknown *



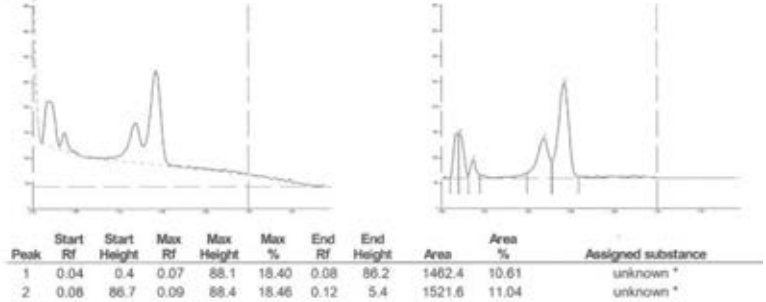
Track 10, ID: Spike sample 25 ug/ml

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	0.5	0.09	85.6	26.21	0.12	7.2	2906.5	25.43	unknown *
2	0.13	7.9	0.15	32.3	9.89	0.20	2.9	716.4	6.27	unknown *
3	0.39	6.8	0.48	69.7	21.32	0.52	26.6	2859.1	25.02	unknown *
4	0.52	26.8	0.57	139.1	42.58	0.63	4.3	4945.6	43.28	unknown *



Track 11, ID: Spike sample 25 ug/ml

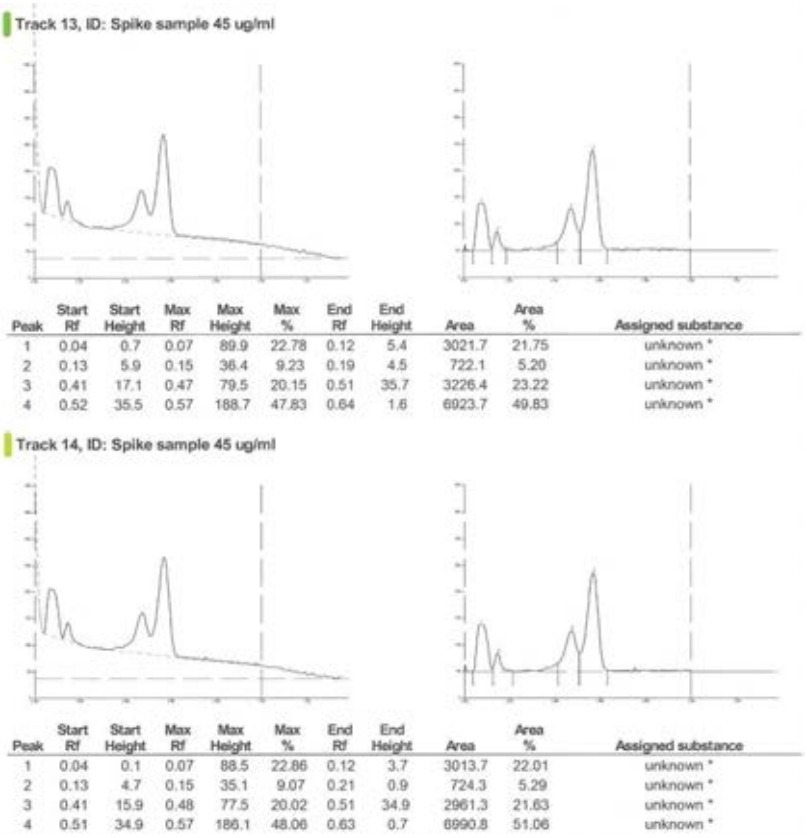
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	0.1	0.08	85.6	26.72	0.12	6.9	2885.2	26.47	unknown *
2	0.13	6.9	0.15	32.6	10.17	0.17	7.4	618.8	5.68	unknown *
3	0.42	13.4	0.48	66.9	20.87	0.51	26.7	2552.5	23.42	unknown *
4	0.52	26.8	0.57	135.4	42.24	0.62	4.8	4842.5	44.43	unknown *



Track 12, ID: Spike sample 45 ug/ml

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	0.4	0.07	88.1	18.40	0.08	86.2	1462.4	10.61	unknown *
2	0.08	86.7	0.09	88.4	18.46	0.12	5.4	1521.6	11.04	unknown *
3	0.13	5.8	0.15	36.4	7.60	0.18	6.9	706.6	5.13	unknown *
4	0.40	9.9	0.48	78.8	16.46	0.51	34.3	3192.4	23.15	unknown *
5	0.51	34.5	0.57	187.0	39.07	0.64	1.4	6905.0	50.08	unknown *

Figure 34 (cont). TLC-densitometry chromatogram (Plate F-Precision 2)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	0.7	0.07	89.9	22.78	0.12	5.4	3021.7	21.75	unknown *
2	0.13	5.9	0.15	36.4	9.23	0.19	4.5	722.1	5.20	unknown *
3	0.41	17.1	0.47	79.5	20.15	0.51	35.7	3226.4	23.22	unknown *
4	0.52	35.5	0.57	188.7	47.83	0.64	1.6	6923.7	49.83	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	0.1	0.07	88.5	22.86	0.12	3.7	3013.7	22.01	unknown *
2	0.13	4.7	0.15	35.1	9.07	0.21	0.9	724.3	5.29	unknown *
3	0.41	15.9	0.48	77.5	20.02	0.51	34.9	2961.3	21.63	unknown *
4	0.51	34.9	0.57	186.1	48.06	0.63	0.7	6990.8	51.06	unknown *

Figure 34 (cont). TLC-densitometry chromatogram (Plate F-Precision 2)

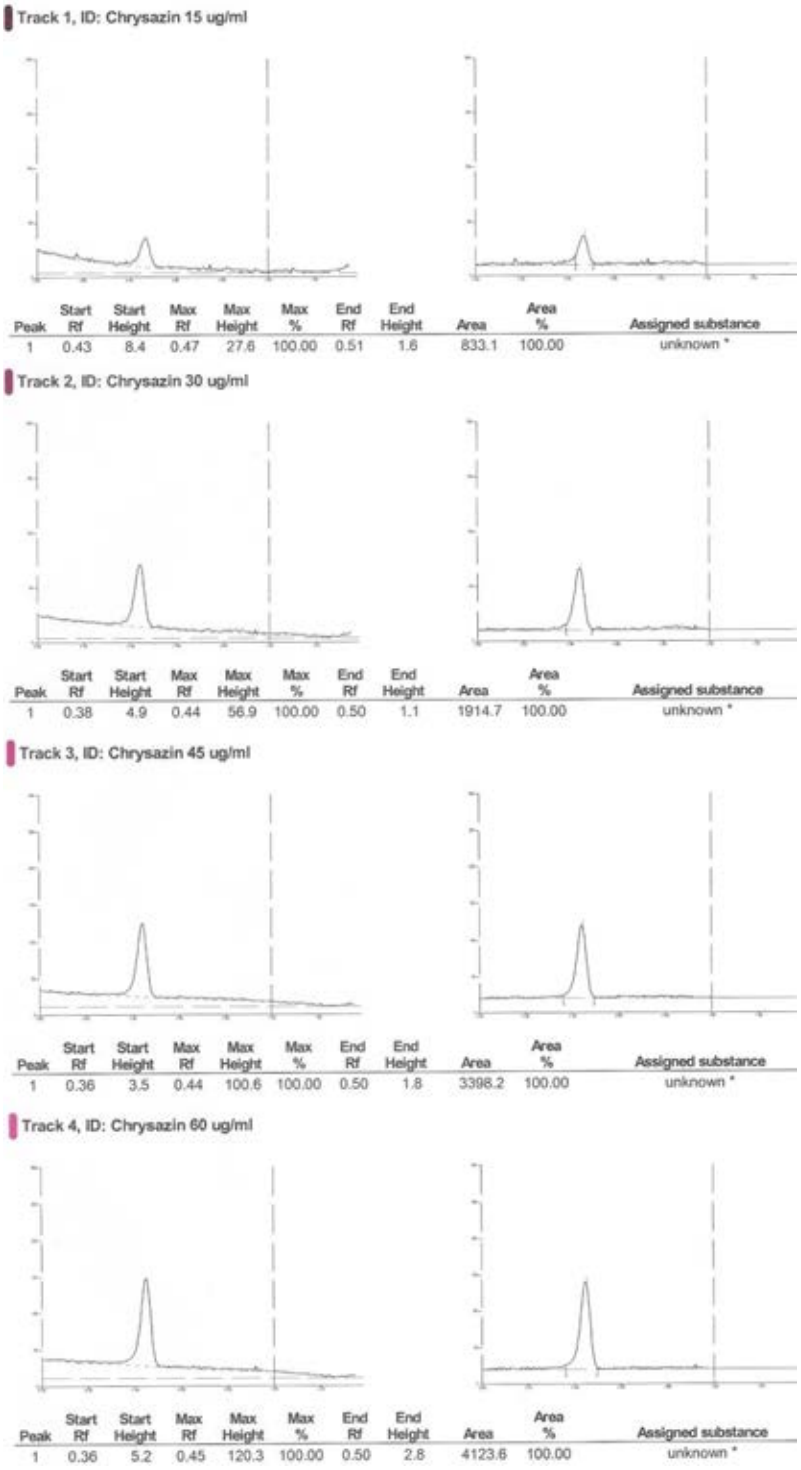
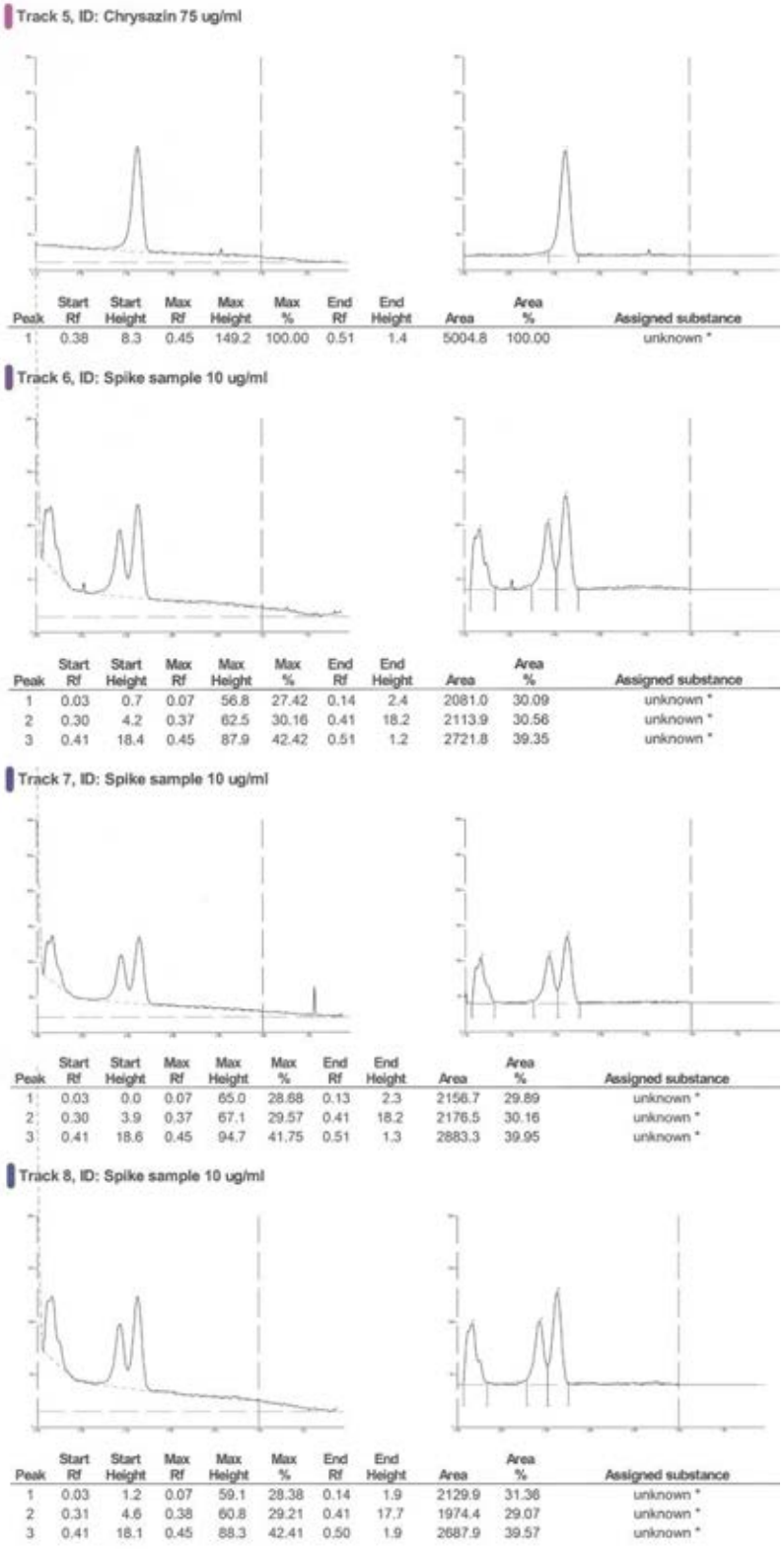


Figure 35. TLC-densitometry chromatogram (Plate G-Precision 3)



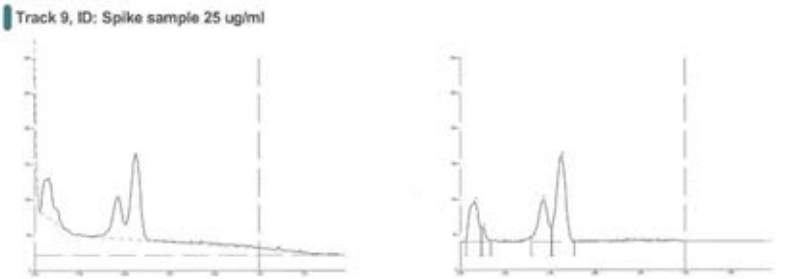
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.38	8.3	0.45	149.2	100.00	0.51	1.4	5004.8	100.00	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.7	0.07	56.8	27.42	0.14	2.4	2081.0	30.09	unknown *
2	0.30	4.2	0.37	62.5	30.16	0.41	18.2	2113.9	30.56	unknown *
3	0.41	18.4	0.45	87.9	42.42	0.51	1.2	2721.8	39.35	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.0	0.07	65.0	28.68	0.13	2.3	2156.7	29.89	unknown *
2	0.30	3.9	0.37	67.1	29.57	0.41	18.2	2176.5	30.16	unknown *
3	0.41	18.6	0.45	94.7	41.75	0.51	1.3	2883.3	39.95	unknown *

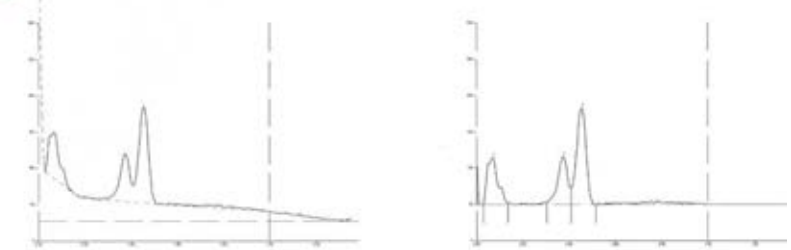
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.2	0.07	59.1	28.38	0.14	1.9	2129.9	31.36	unknown *
2	0.31	4.6	0.38	60.8	29.21	0.41	17.7	1974.4	29.07	unknown *
3	0.41	18.1	0.45	88.3	42.41	0.50	1.9	2687.9	39.57	unknown *

Figure 35 (cont). TLC-densitometry chromatogram (Plate G-Precision 3)



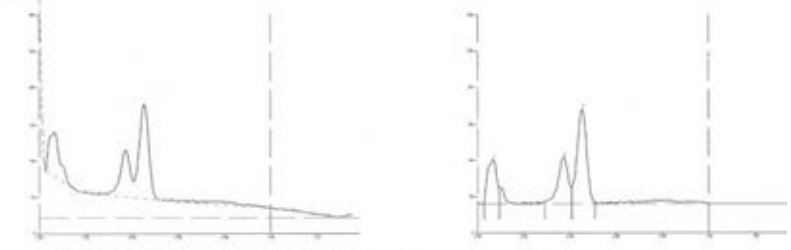
Track 9, ID: Spike sample 25 ug/ml

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	0.7	0.06	57.3	22.07	0.09	20.1	1710.9	22.25	unknown *
2	0.09	20.2	0.10	21.1	8.11	0.14	1.4	303.2	3.94	unknown *
3	0.32	7.2	0.37	59.7	22.99	0.41	20.8	1925.7	25.04	unknown *
4	0.41	20.9	0.45	121.6	46.82	0.51	0.7	3750.3	48.77	unknown *



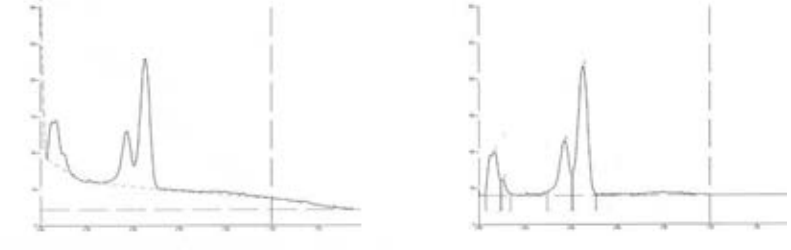
Track 10, ID: Spike sample 25 ug/ml

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.2	0.07	65.2	24.56	0.14	1.4	2269.1	26.29	unknown *
2	0.30	3.1	0.38	66.4	25.02	0.41	23.2	2172.0	25.16	unknown *
3	0.41	23.1	0.46	133.8	50.42	0.52	0.1	4191.0	48.55	unknown *



Track 11, ID: Spike sample 25 ug/ml

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.1	0.07	62.3	24.17	0.09	21.6	1841.7	22.68	unknown *
2	0.29	3.2	0.38	65.6	25.44	0.41	23.1	2182.6	26.88	unknown *
3	0.41	22.8	0.46	129.9	50.39	0.51	1.9	4095.8	50.44	unknown *

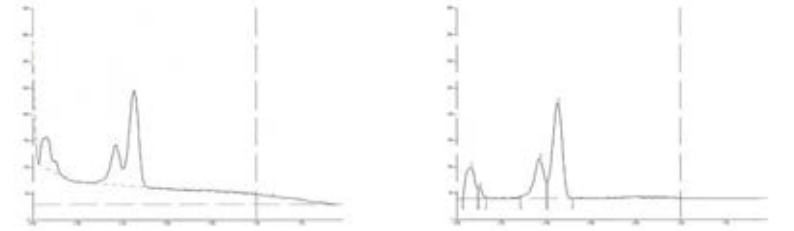


Track 12, ID: Spike sample 45 ug/ml

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.2	0.07	61.4	18.13	0.10	21.1	1846.4	17.74	unknown *
2	0.10	21.6	0.11	22.6	6.67	0.14	2.1	358.1	3.44	unknown *
3	0.30	4.1	0.37	75.9	22.43	0.40	29.1	2475.9	23.79	unknown *
4	0.41	29.2	0.46	178.6	52.77	0.51	1.1	5728.6	55.04	unknown *

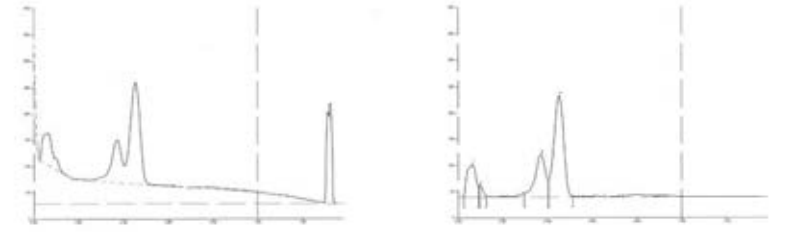
Figure 35 (cont). TLC-densitometry chromatogram (Plate G-Precision 3)

Track 13, ID: Spike sample 45 ug/ml



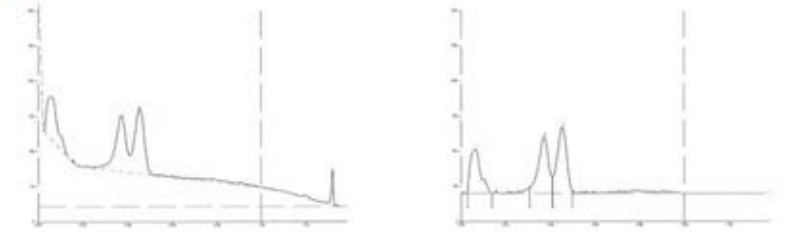
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.3	0.06	60.0	17.55	0.10	20.7	1824.0	17.04	unknown *
2	0.10	20.8	0.10	22.8	6.68	0.13	2.3	303.2	2.83	unknown *
3	0.29	4.0	0.37	76.7	22.43	0.40	32.3	2617.7	24.45	unknown *
4	0.40	32.6	0.45	182.4	53.33	0.52	0.5	5960.5	55.68	unknown *

Track 14, ID: Spike sample 45 ug/ml



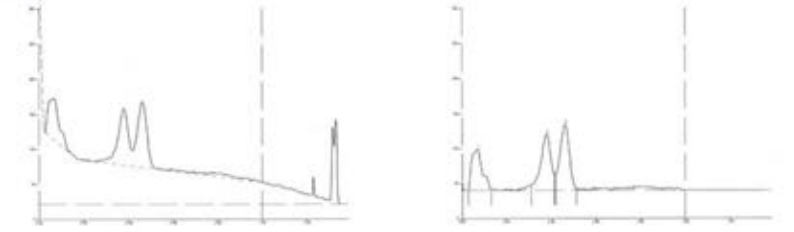
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.7	0.07	63.0	17.46	0.09	20.2	1882.1	16.56	unknown *
2	0.10	22.7	0.10	23.0	6.38	0.13	1.1	277.5	2.44	unknown *
3	0.30	7.7	0.37	81.2	22.53	0.40	33.3	2838.9	24.99	unknown *
4	0.41	33.6	0.45	193.4	53.64	0.51	0.8	6363.5	56.01	unknown *

Track 15, ID: Unspike sample



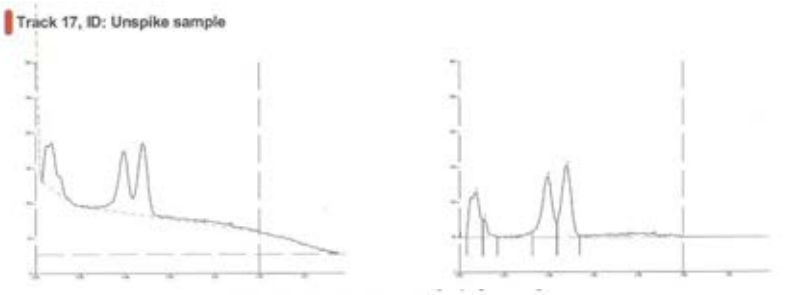
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	1.1	0.07	62.7	26.33	0.14	0.3	2188.3	27.40	unknown *
2	0.31	8.6	0.37	80.8	33.94	0.41	23.5	2781.1	34.82	unknown *
3	0.41	23.9	0.46	94.6	39.73	0.50	4.7	3017.5	37.78	unknown *

Track 16, ID: Unspike sample



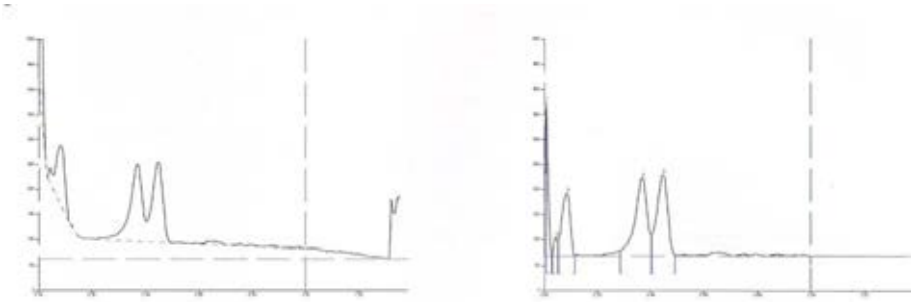
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.9	0.07	59.9	25.34	0.13	1.6	2170.7	26.99	unknown *
2	0.31	7.7	0.38	81.6	34.51	0.42	24.1	2801.3	34.83	unknown *
3	0.42	22.9	0.46	95.0	40.16	0.51	2.7	3070.4	38.18	unknown *

Figure 35 (cont). TLC-densitometry chromatogram (Plate G-Precision 3)



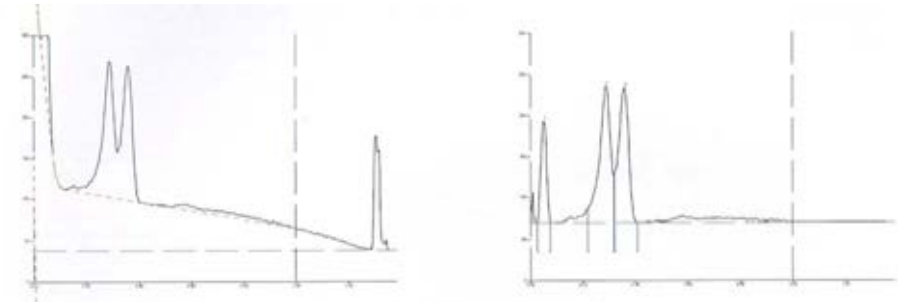
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.8	0.07	65.0	23.09	0.10	24.5	2136.8	23.76	unknown *
2	0.10	24.6	0.11	26.6	9.44	0.17	0.2	436.7	4.86	unknown *
3	0.33	7.9	0.40	87.5	31.06	0.44	22.2	3058.2	34.01	unknown *
4	0.44	22.3	0.48	102.6	36.41	0.54	2.5	3360.1	37.37	unknown *

Figure 35 (cont). TLC-densitometry chromatogram (Plate G-Precision 3)



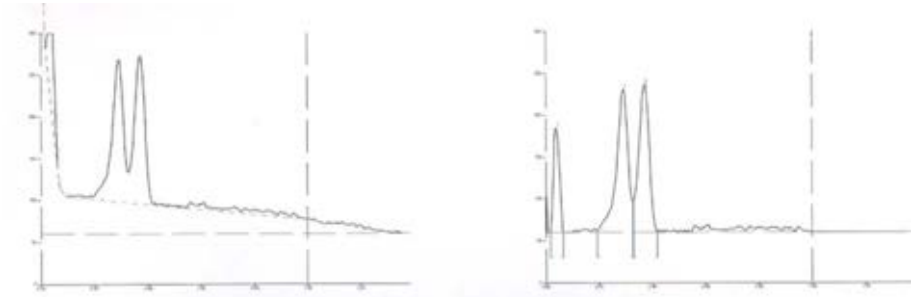
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	306.7	0.01	306.7	39.34	0.03	1.6	1878.9	12.38	unknown *
2	0.03	1.6	0.04	35.2	4.51	0.05	30.8	349.3	2.30	unknown *
3	0.05	31.3	0.09	122.8	15.75	0.12	0.0	2883.8	18.99	unknown *
4	0.29	10.4	0.37	154.7	19.84	0.40	39.8	5252.4	34.59	unknown *
5	0.40	39.9	0.45	160.2	20.55	0.49	1.6	4818.4	31.74	unknown *

Figure 36. TLC-densitometry chromatogram (Plate H-Robustness, 8:1 v/v)



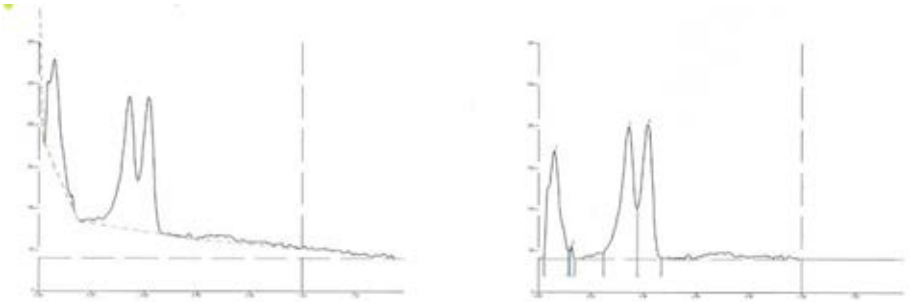
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	0.4	0.05	124.0	27.25	0.08	1.2	2062.4	16.76	unknown *
2	0.22	15.4	0.29	166.3	36.56	0.32	57.4	5427.8	44.11	unknown *
3	0.32	58.4	0.36	164.7	36.20	0.41	0.1	4813.9	39.12	unknown *

Figure 37. TLC-densitometry chromatogram (Plate I-Robustness, 8.1:0.9 v/v)



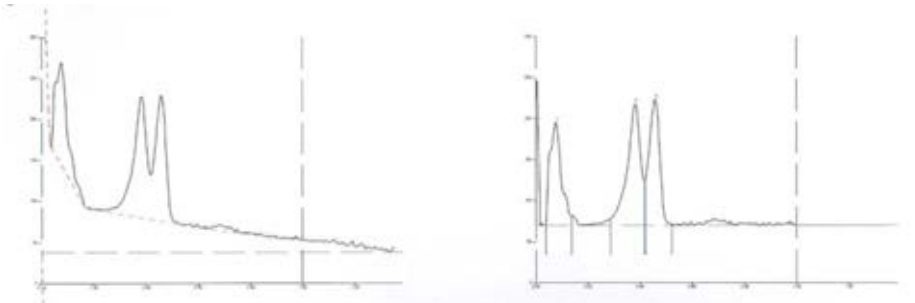
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	1.3	0.04	125.2	26.47	0.07	0.5	2142.5	16.32	unknown *
2	0.19	2.8	0.29	170.5	36.04	0.33	36.4	5668.2	43.17	unknown *
3	0.33	36.4	0.37	177.3	37.49	0.42	0.7	5319.4	40.51	unknown *

Figure 38. TLC-densitometry chromatogram (Plate J-Robustness, 8.2:0.8 v/v)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.02	0.5	0.06	130.3	27.85	0.12	10.3	4248.9	27.63	unknown *
2	0.12	11.1	0.13	15.5	3.31	0.14	0.1	113.6	0.74	unknown *
3	0.25	7.3	0.34	159.8	34.16	0.38	60.3	5999.8	39.02	unknown *
4	0.38	60.6	0.42	162.2	34.67	0.47	1.5	5014.9	32.61	unknown *

Figure 39. TLC-densitometry chromatogram (Plate K-Robustness, 7.9:1.1 v/v)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.04	1.3	0.08	126.1	29.45	0.14	11.4	4212.6	28.51	unknown *
2	0.29	7.9	0.38	148.2	34.62	0.42	54.6	5745.9	38.88	unknown *
3	0.42	55.1	0.46	153.9	35.93	0.52	0.7	4819.0	32.61	unknown *

Figure 40. TLC-densitometry chromatogram (Plate L-Robustness, 7.8:1.2 v/v)

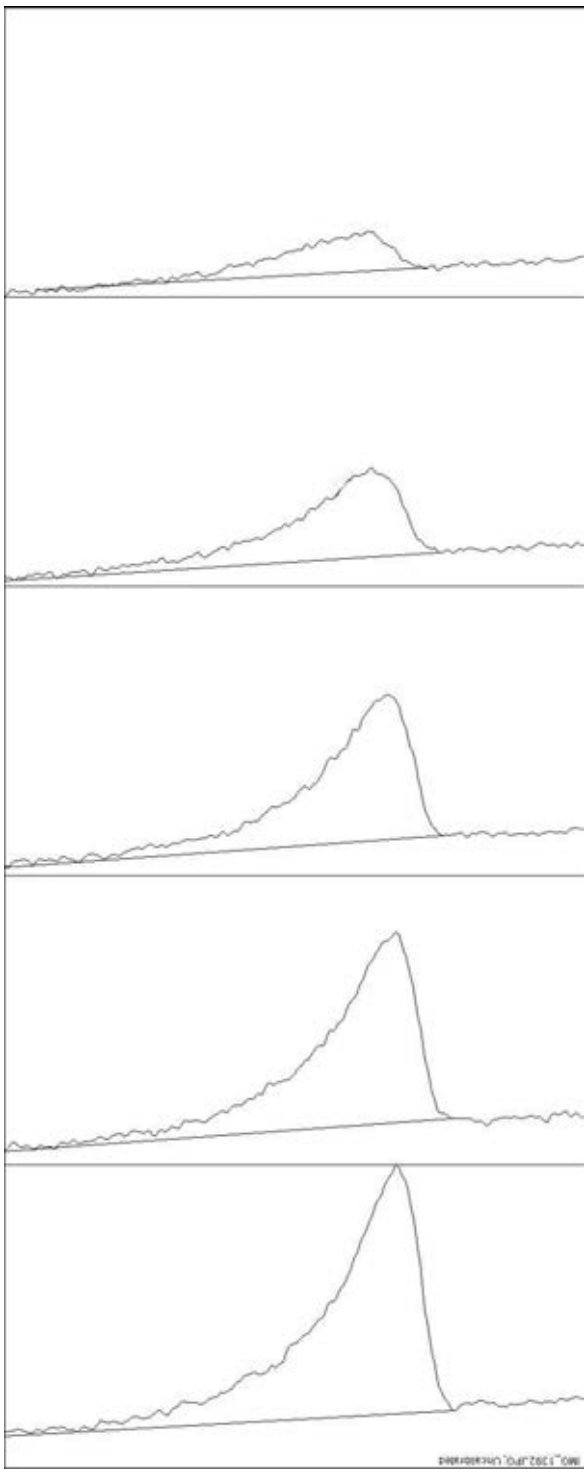


Figure 41. TLC image analysis chromatogram by ImageJ software (Plate A-Calibration curve)

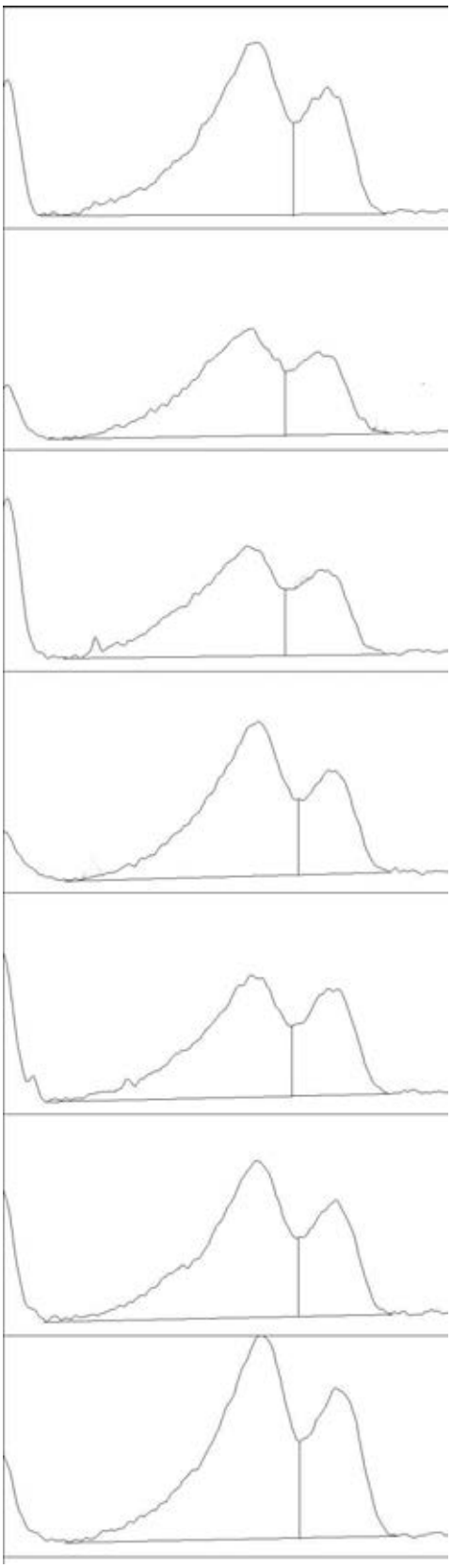


Figure 42. TLC image analysis chromatogram by ImageJ software (Plate A-Sample No. 1-7)

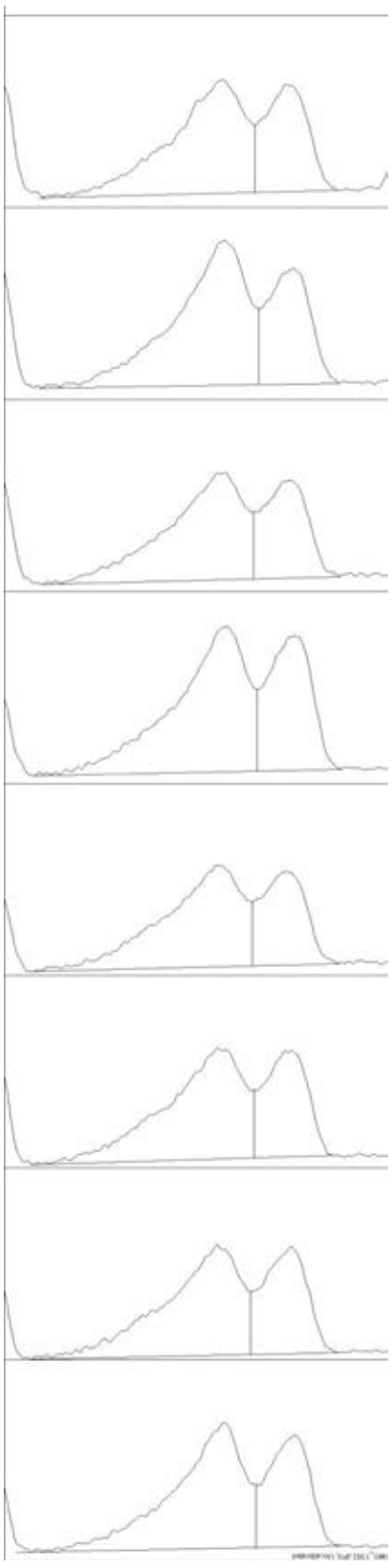


Figure 43. TLC image analysis chromatogram by ImageJ software (Plate A-Sample No. 8-15)

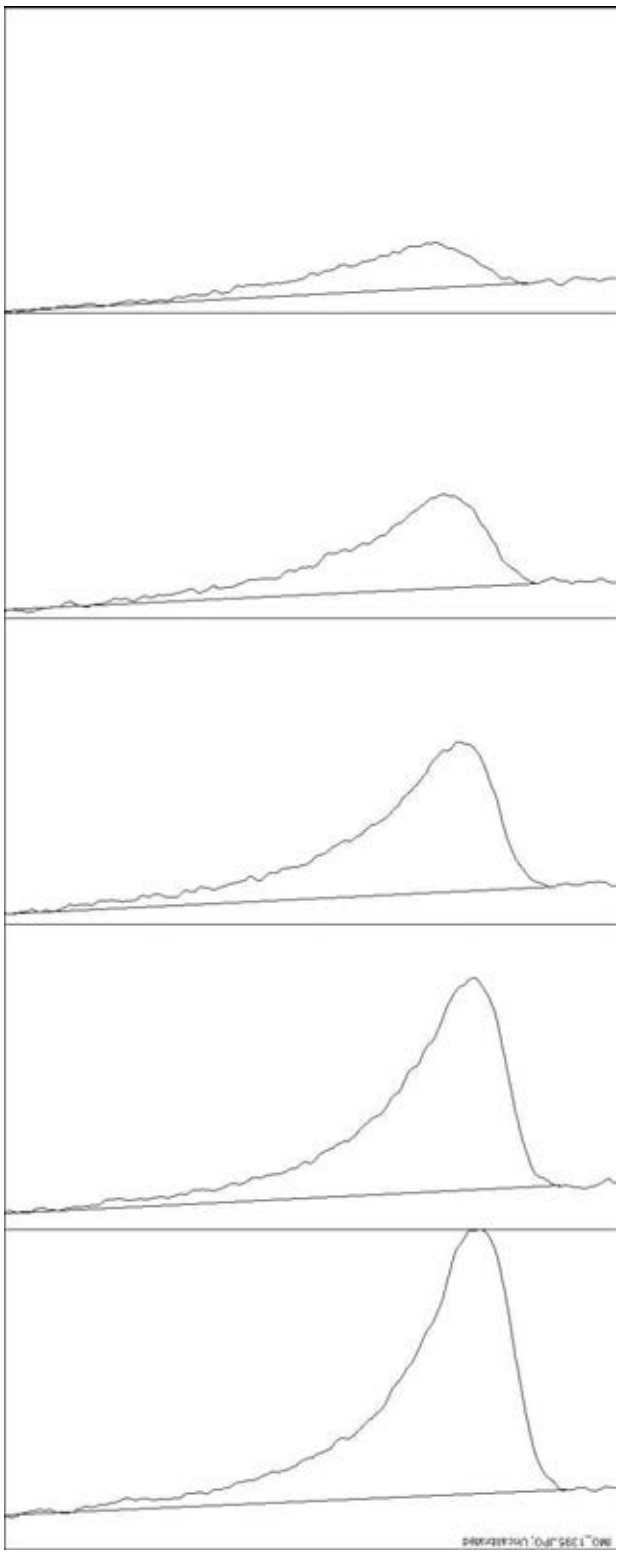


Figure 44. TLC image analysis chromatogram by ImageJ software (Plate B-Calibration curve)

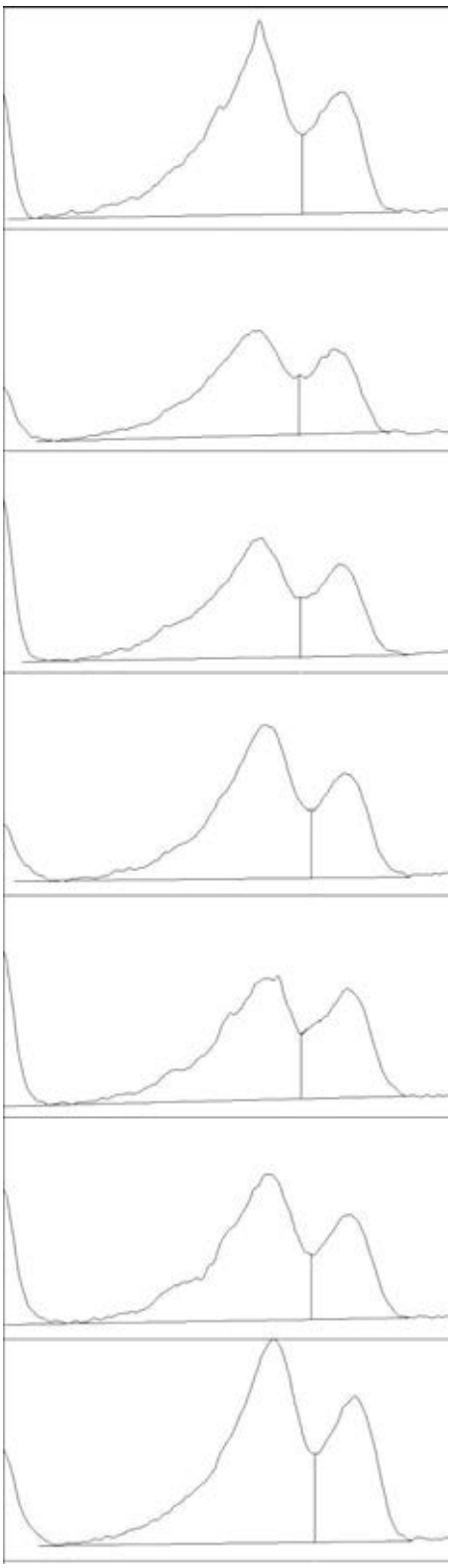


Figure 45. TLC image analysis chromatogram by ImageJ software (Plate B-Sample No. 1-7)

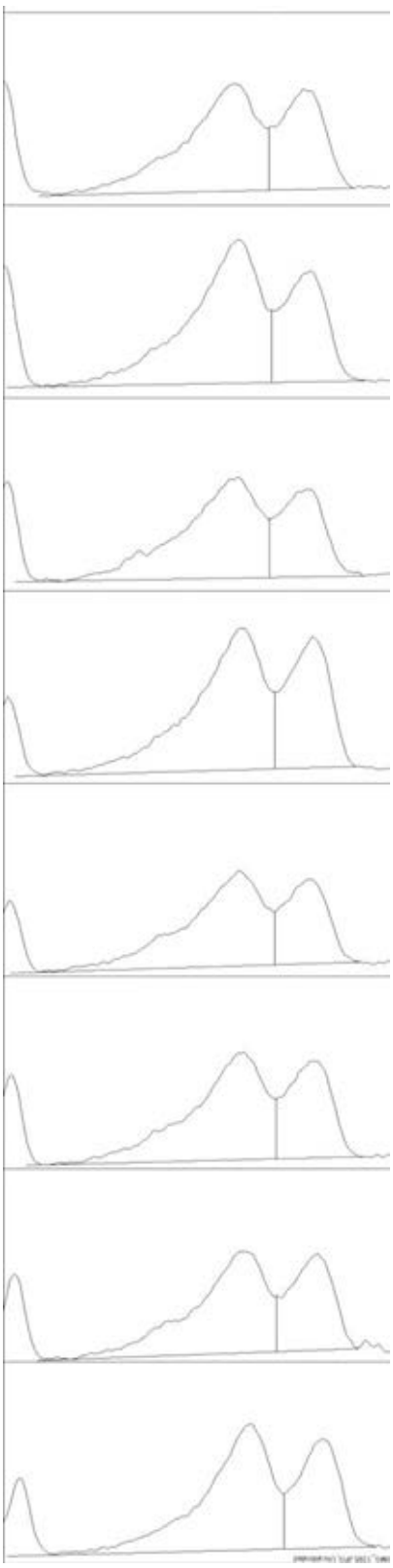


Figure 46. TLC image analysis chromatogram by ImageJ software (Plate B-Sample No. 8-15)

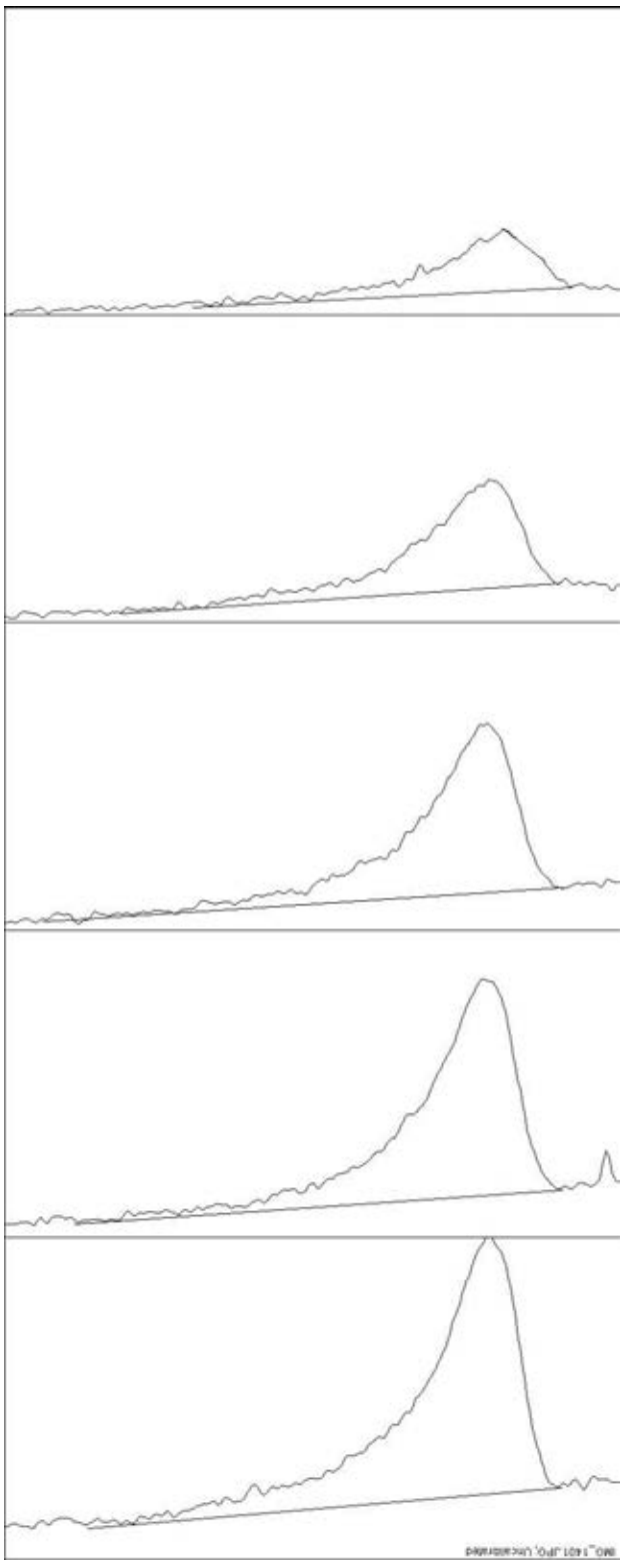


Figure 47. TLC image analysis chromatogram by ImageJ software (Plate C-Calibration curve)

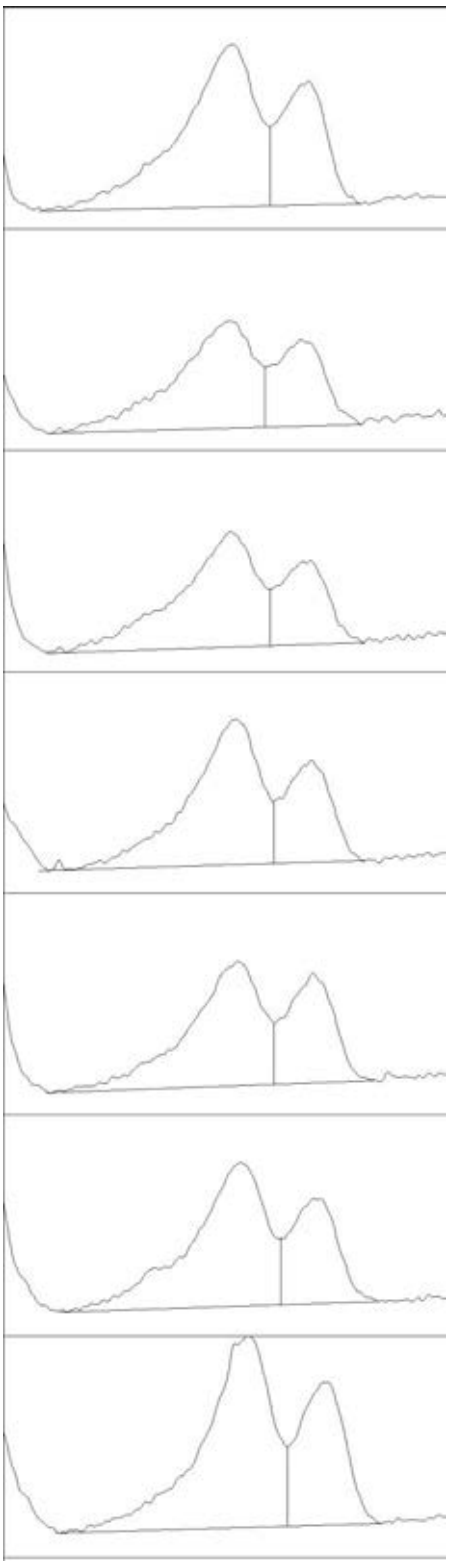


Figure 48. TLC image analysis chromatogram by ImageJ software (Plate C-Sample No. 1-7)

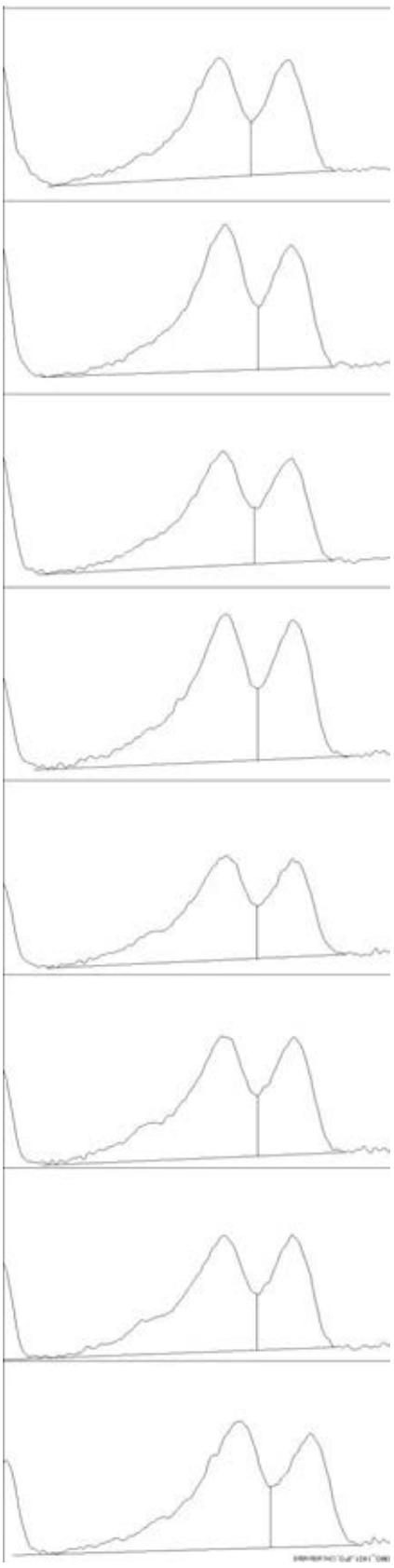


Figure 49. TLC image analysis chromatogram by ImageJ software (Plate C-Sample No. 8-15)

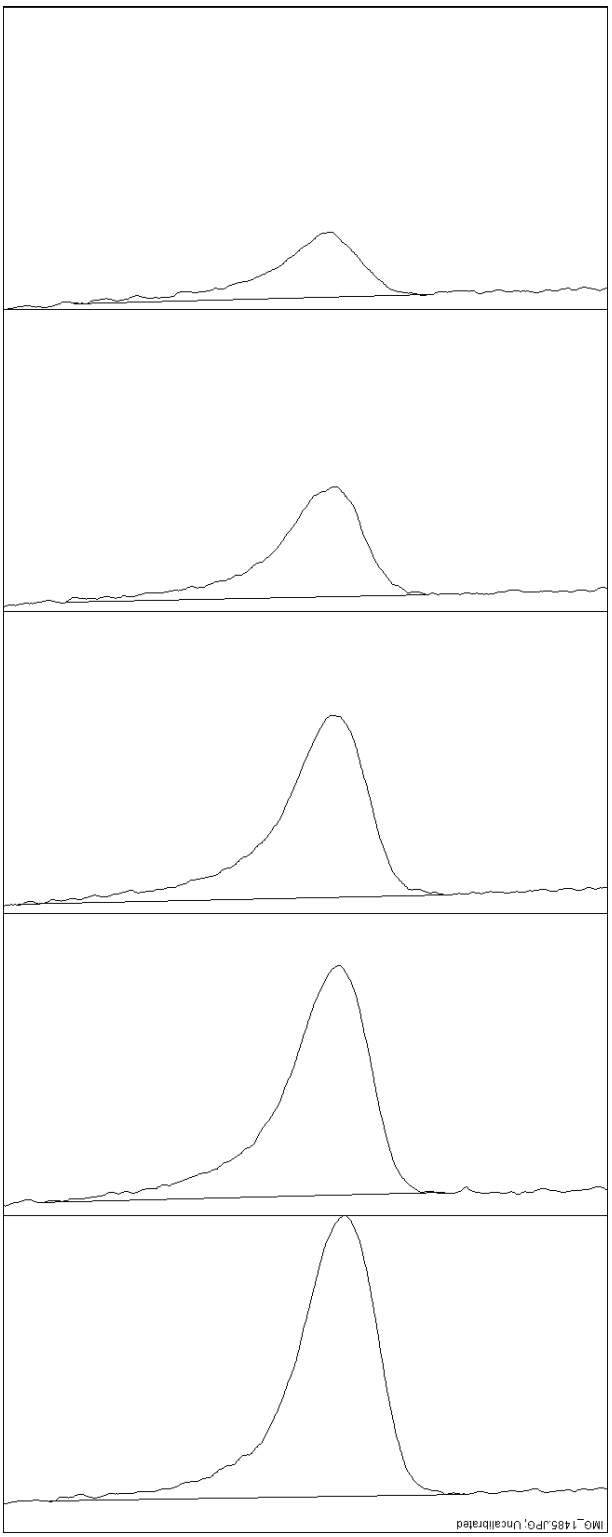


Figure 50. TLC image analysis chromatogram by ImageJ software (Plate D-Accuracy, calibration curve)

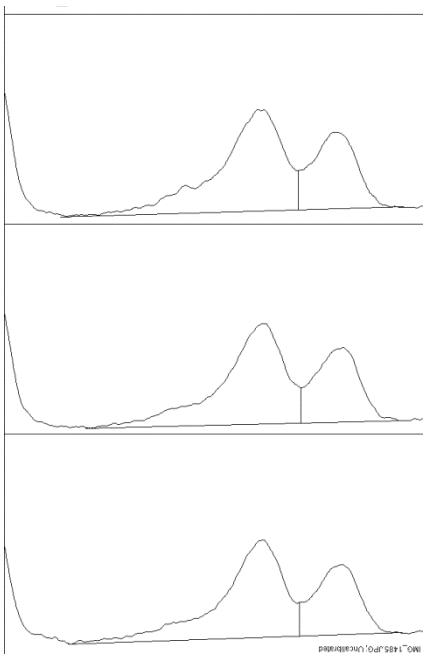


Figure 51. TLC image analysis chromatogram by ImageJ software (Plate D-Accuracy, un-spiked sample)

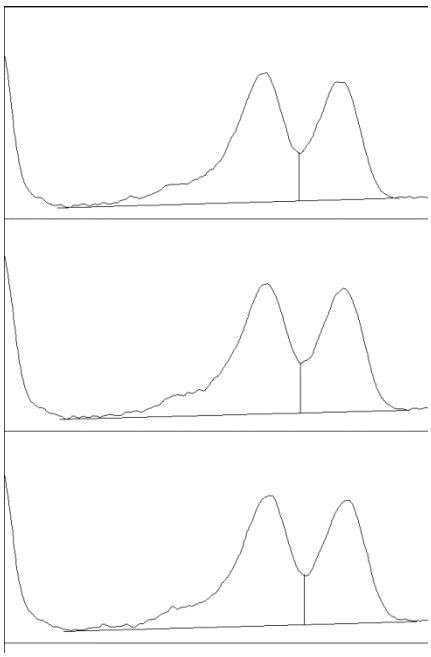


Figure 52. TLC image analysis chromatogram by ImageJ software (Plate D-Accuracy, spiked 10 µg/ml in sample)

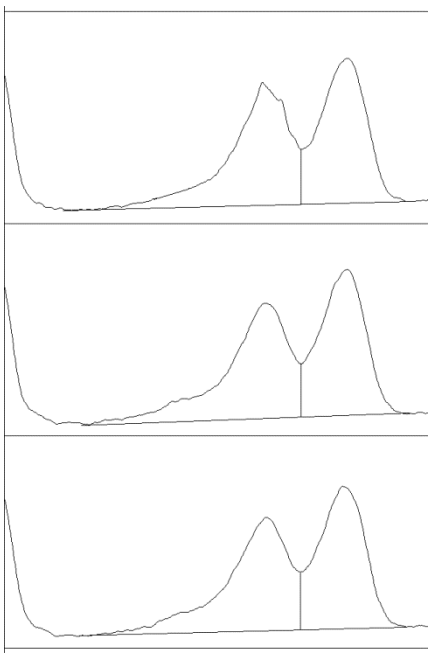


Figure 53. TLC image analysis chromatogram by ImageJ software (Plate D-Accuracy, spiked 25 µg/ml in sample)

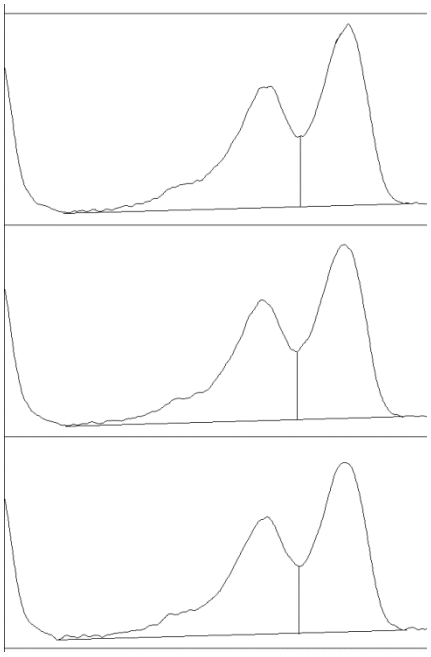


Figure 54. TLC image analysis chromatogram by ImageJ software (Plate D-Accuracy, spiked 45 µg/ml in sample)

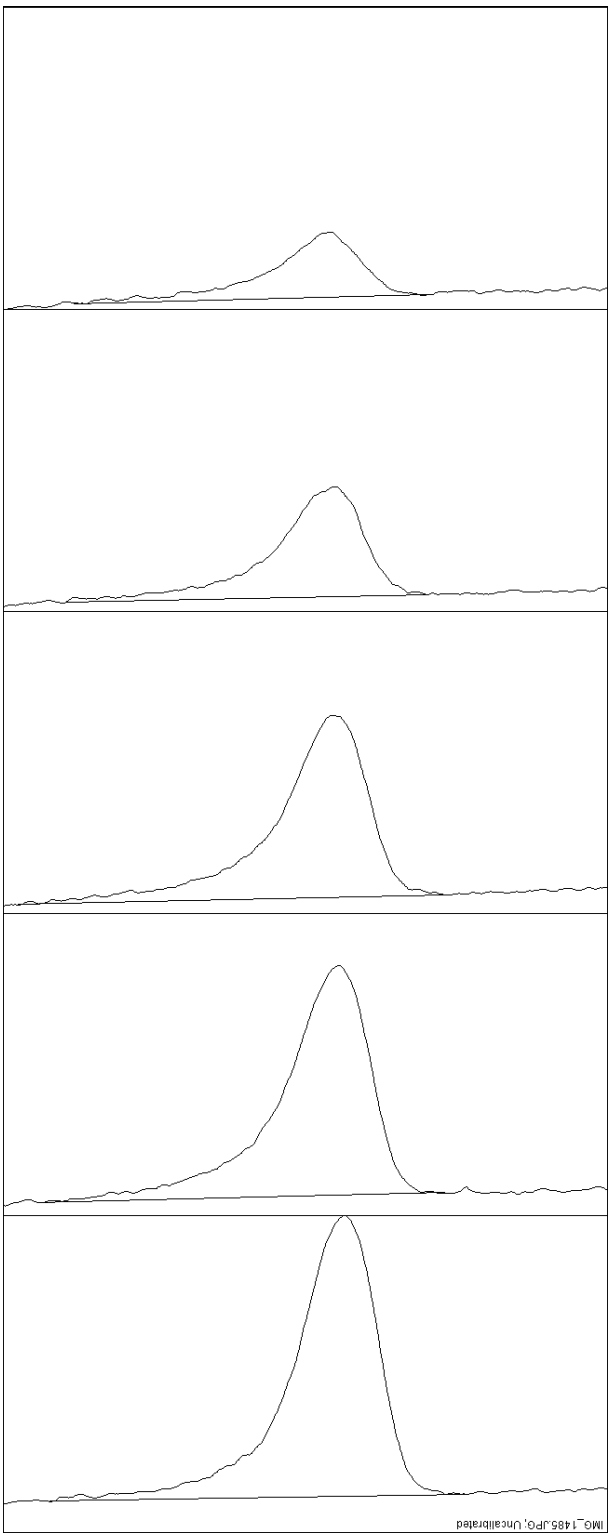


Figure 55. TLC image analysis chromatogram by ImageJ software (Plate E-Precision 1, calibration curve)

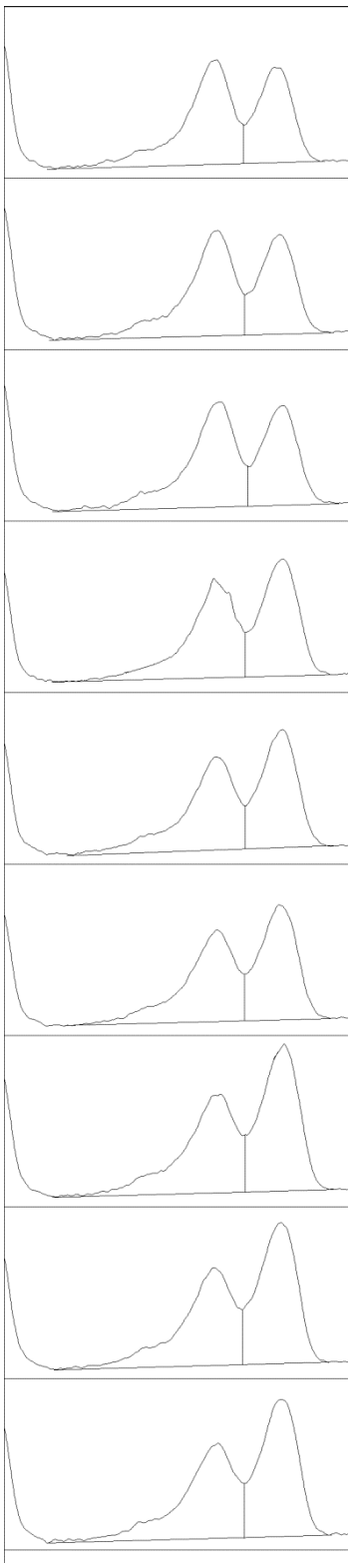


Figure 56. TLC image analysis chromatogram by ImageJ software (Plate E-Precision 1)

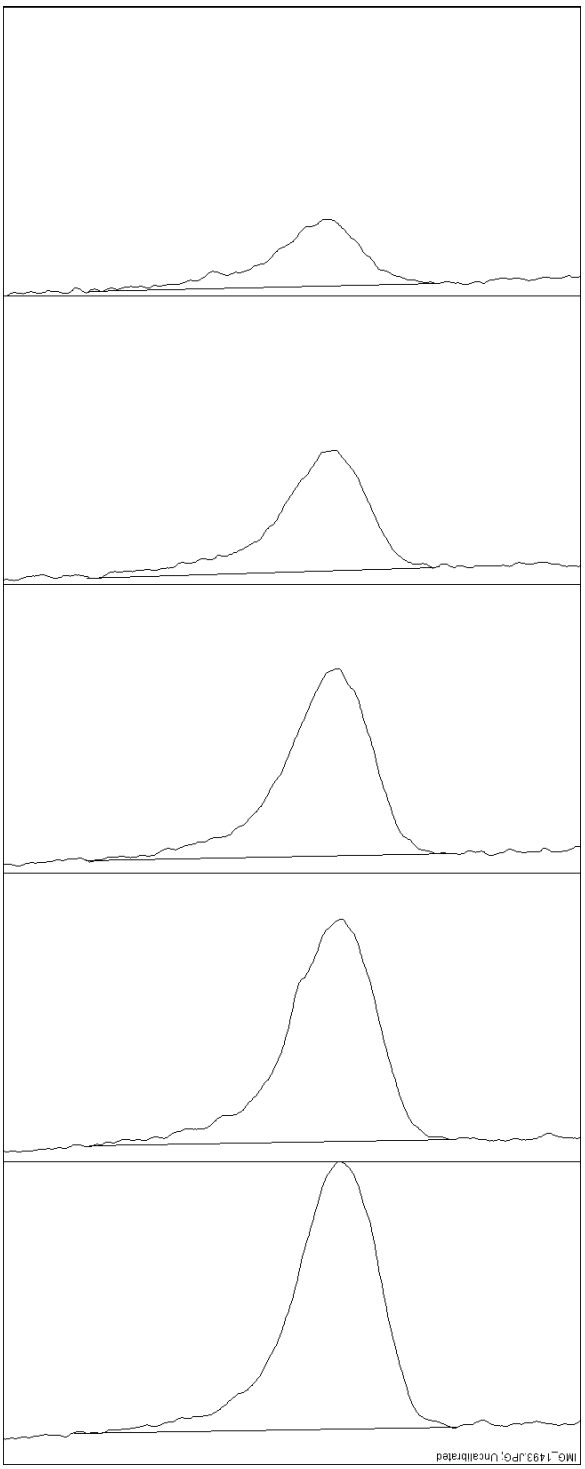


Figure 57. TLC image analysis chromatogram by ImageJ software (Plate F-Precision 2, calibration curve)

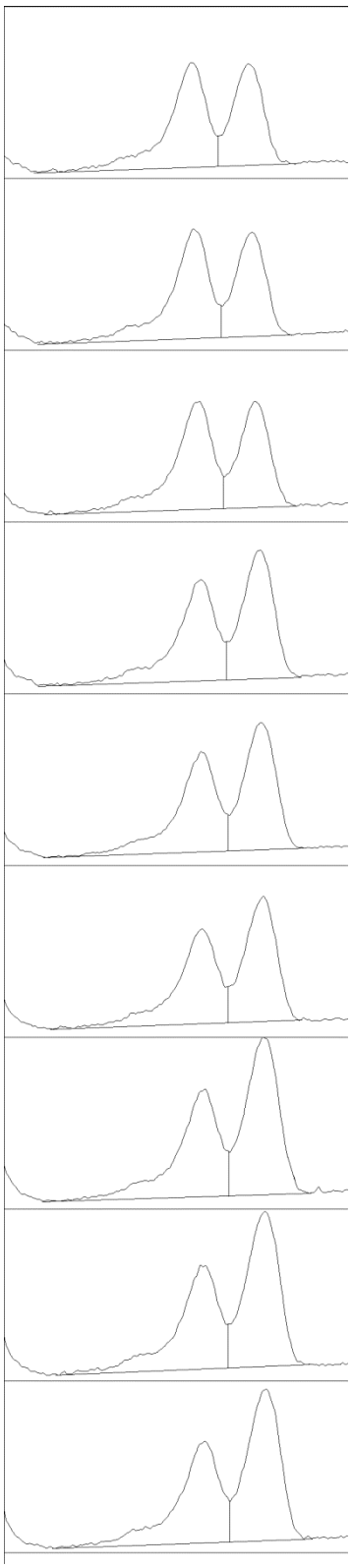


Figure 58. TLC image analysis chromatogram by ImageJ software (Plate F-Precision 2)

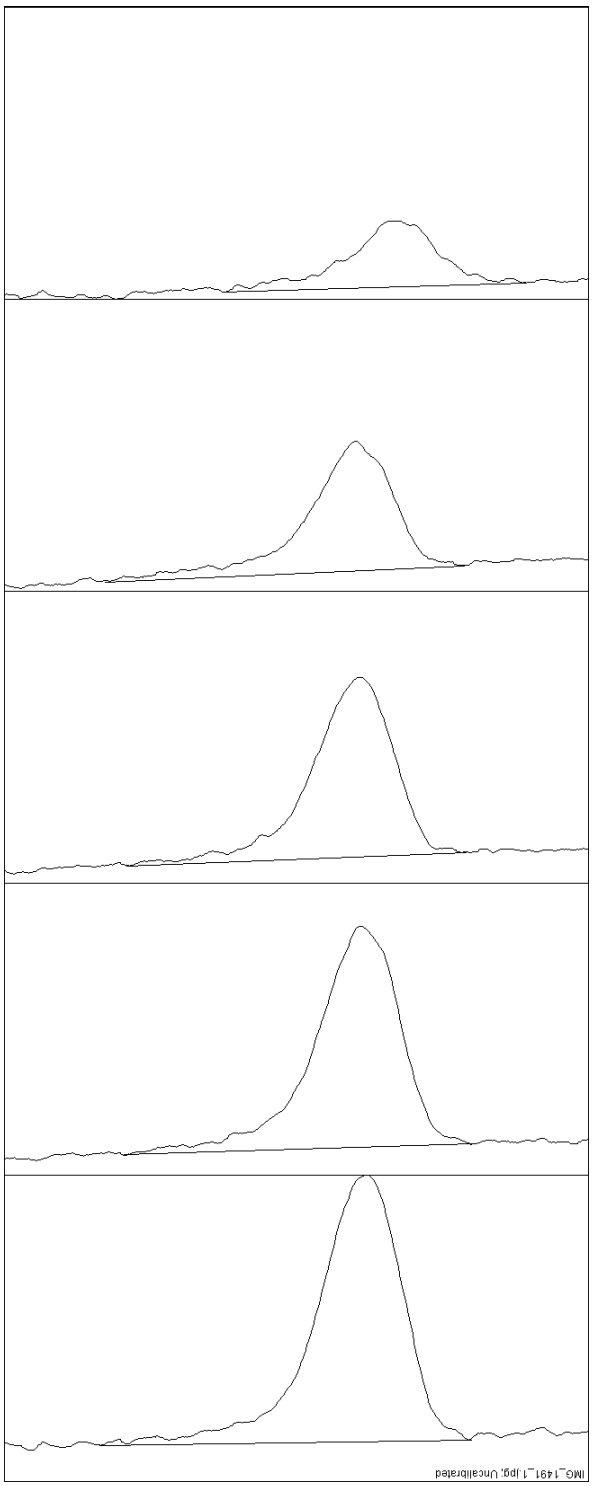


Figure 59. TLC image analysis chromatogram by ImageJ software (Plate G-Precision 3, calibration curve)

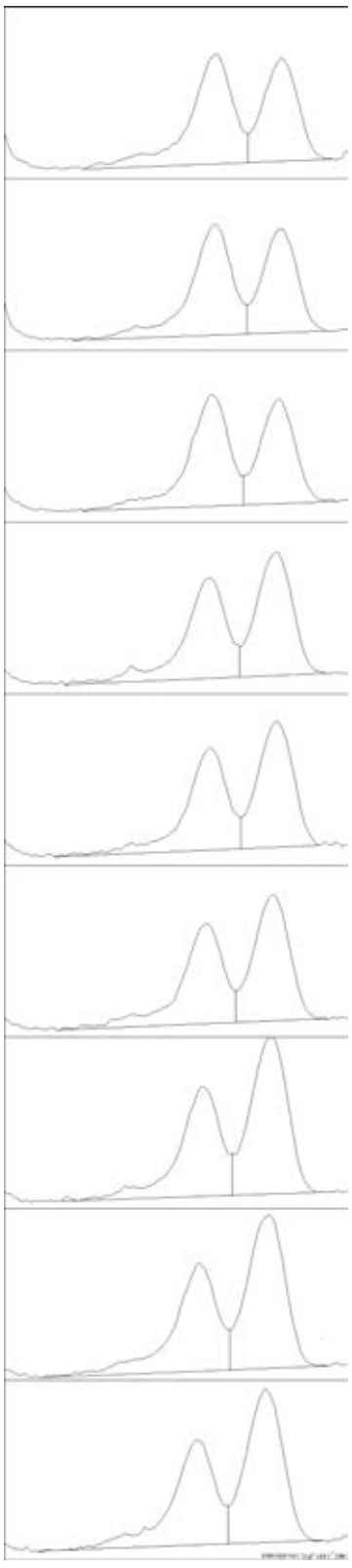


Figure 60. TLC image analysis chromatogram by ImageJ software (Plate G-Precision3)

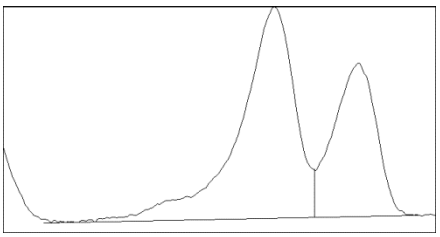


Figure 61. TLC image analysis chromatogram by ImageJ software (Plate H-Robustness, 8:1 v/v)

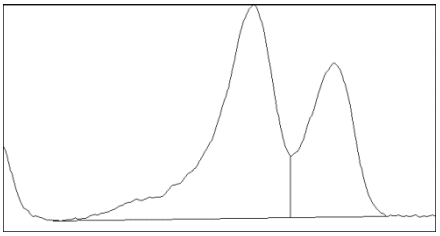


Figure 62. TLC image analysis chromatogram by ImageJ software (Plate I-Robustness, 8.1:0.9 v/v)

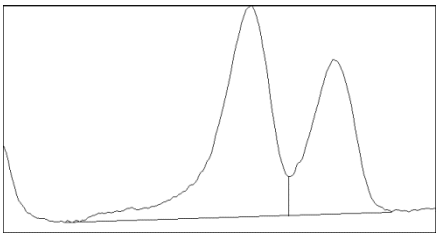


Figure 63. TLC image analysis chromatogram by ImageJ software (Plate J-Robustness, 8.2:0.8 v/v)

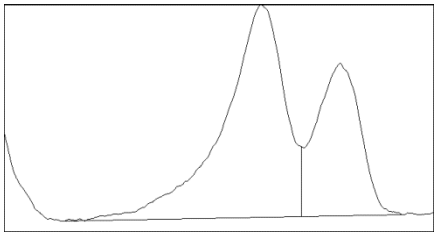


Figure 64. TLC image analysis chromatogram by ImageJ software (Plate K-Robustness, 7.9:1.1 v/v)

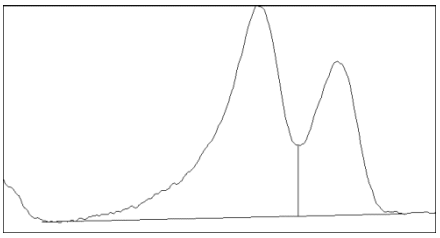


Figure 65. TLC image analysis chromatogram by ImageJ software (Plate L-Robustness, 7.8:1.2 v/v)

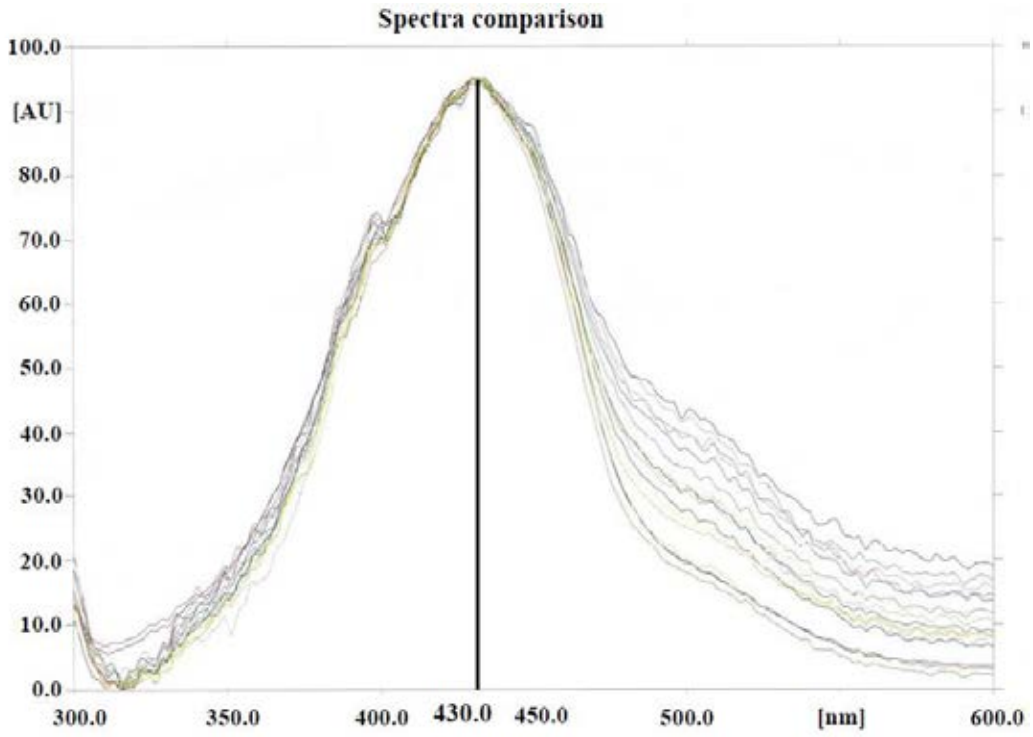


Figure 66. The UV spectrum of chrysazin separated from sample and standard chrysazin

VITAE

Miss Chuanchom Khuniad was born on July 11, 1988 in Phatthalung, Thailand. She receives her Bachelor's degree of Science (Thai Traditional Medicine) with second class honor from faculty of Traditional Thai Medicine, Prince of Songkla University, Thailand in 2010.

Publication

Khuniad, C., Chansukh, K., Palanuvej, C. and Ruangrungsi, N. Pharmacognostic Evaluation and Chrysazin Quantitation of *Xyris indica* flowering heads. Proceedings of 39th Congress on Science and Technology of Thailand, Bangkok, 2013