QUANTITATIVE ANALYSIS OF CIVETONE AND NORMUSCONE IN SECRETION FROM *VIVERRICULA INDICA* AND IN AROMATIC REMEDIES BY GAS CHROMATOGRAPHY/ MASS SPECTROMETRY

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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 2011

การวิเคราะห์หาปริมาณสารซิเวทโทนและสารนอร์มัสโคนในสมุนไพรชะมดเช็ดและตำรับยาหอมโดยแกสโครมาโทกราฟี/แมสสเปกโทรเมทรี

นางสาวธิดารัตน์ ดวงยอด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์สาธารณสุข
วิทยาลัยวิทยาศาสตร์สาธารณสุข จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

Thesis Title	QUANTITATIVE ANALYSIS OF CIVETONE AND NORMUSCONE IN SECRETION FROM *VIVERRICULA INDICA* AND IN AROMATIC REMEDIES BY GAS CHROMATOGRAPHY/ MASS SPECTROMETRY
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ธิดารัตน์ ดวงยอด : การวิเคราะห์หาปริมาณสารซิเวทโทนและสารนอร์มัสโคนในสมุนไพรชะมดเช็ดและตำรับยาหอมโดยแกสโครมาโทกราฟี/แมสสเปกโทรเมทรี. (Quantitative Analysis of Civetone and Normuscone in Secretion from *Viverricula indica* and in Aromatic Remedies by Gas Chromatography/Mass Spectrometry) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ดร. ชนิดา พลานุเวช, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. นิจศิริ เรืองรังษี, 136 หน้า.

วิเคราะห์หาปริมาณสารซิเวทโทนและนอร์มัสโคนในสมุนไพรชะมดเช็ดรวมทั้งตำรับยาหอมโดยวิธีแกสโครมาโทกราฟี/แมสสเปกโทรเมสทรี สมุนไพรชะมดเช็ดประกอบด้วยสารซิเวทโทน สารไดไฮโดรซิเวทโทน และสารนอร์มัสโคน เป็นองค์ประกอบหลัก ปริมาณขององค์ประกอบทางเคมีในสมุนไพรชะมดเช็ดมีความแตกต่างกันระหว่างตัวผู้และตัวเมีย สารซิเวทโทน (23.6 ± 1.5 ไมโครกรัม/มิลลิกรัม) จัดเป็นองค์ประกอบหลักในสมุนไพรชะมดเช็ดตัวเมีย และสารนอร์มัสโคน (52.1 ± 5.9 ไมโครกรัม/มิลลิกรัม) เป็นองค์ประกอบหลักในสมุนไพรชะมดเช็ดตัวผู้ นอกจากนี้ยังพบสารซิเวทโทนและสารนอร์มัสโคนในขนของชะมดอีกด้วย แต่ไม่พบในอุจจาระ การวิเคราะห์ตำรับยาหอมที่ระบุว่ามีสมุนไพรชะมดเช็ดเป็นส่วนประกอบพบทั้งสารซิเวทโทนและนอร์มัสโคน ในปริมาณที่แตกต่างกันระหว่าง 1.073 – 8.246 และ 2.759 – 31.028 ไมโครกรัม/มิลลิกรัม ตามลำดับ วิธีวิเคราะห์สารซิเวทโทนมีช่วงความเป็นเส้นตรงระหว่าง 0 - 50 ไมโครกรัม/มิลลิกรัม และมีค่าสัมประสิทธิ์สหสัมพันธ์เท่ากับ 0.9717 วิธีวิเคราะห์สารนอร์มัสโคนมีช่วงความเป็นเส้นตรงระหว่าง 0 - 80 ไมโครกรัม/มิลลิกรัม โดยมีค่าสัมประสิทธิ์สหสัมพันธ์เท่ากับ 0.9965 ค่าเฉลี่ยการคืนกลับของสารซิเวทโทนในสมุนไพรชะมดเช็ดคือ ร้อยละ 97.3 – 98.0 และในตำรับยาหอม คือ ร้อยละ 91.4 – 105.7 สำหรับค่าเฉลี่ยการคืนกลับของสารนอร์มัสโคนในสมุนไพรชะมดเช็ดและตำรับยาหอม คือ ร้อยละ 98.5 และ 90.0 – 103.0 ตามลำดับ ระดับความเที่ยงของวิธีวิเคราะห์สารซิเวทโทนและนอร์มัสโคนประเมินจากค่าสัมประสิทธิ์ของการกระจาย มีค่าน้อยกว่าร้อยละ 8 ขีดจำกัดของการตรวจพบและขีดจำกัดของการหาปริมาณของสารซิเวทโทนมีค่า 0.0087 และ 0.0165 ไมโครกรัม/มิลลิกรัม และสารนอร์มัสโคน มีค่า 0.0596 และ 0.1154 ไมโครกรัม/มิลลิกรัม ตามลำดับ สารซิเวทโทนและนอร์มัสโคนสามารถนำไปใช้เป็นตัวบ่งชี้เชิงคุณภาพและเชิงปริมาณของสมุนไพรชะมดเช็ดในตำรับยาหอมได้ อย่างไรก็ตามปริมาณสารซิเวทโทนและนอร์มัสโคนในสมุนไพรชะมดเช็ดที่นำมาขายมีความแตกต่างกันในแต่ละครั้งของการเก็บ และปริมาณขึ้นอยู่กับสัดส่วนของชะมดเช็ดตัวผู้และตัวเมีย

ภาควิชา วิทยาลัยวิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต ธิดารัตน์ ดวงยอด
สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก ชนิดา พลานุเวช
ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม ...

5379302353 : MAJOR PUBLIC HEALTH SCIENCES

KEYWORDS : SMALL INDIAN CIVET / CIVETONE / NORMUSCONE / GAS CHROMATOGRAPHY-MASS SPECTROMERY

THIDARAT DUANGYOD : QUANTITATIVE ANALYSIS OF CIVETONE AND NORMUSCONE IN SECRETION FROM *VIVERRICULA INDICA* AND IN AROMATIC REMEDIES BY GAS CHROMATOGRAPHY/ MASS SPECTROMETRY. ADVISOR : CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR : ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 136 pp.

The quantitation of civetone and normuscone in small Indian civet (*Viverricula indica* Desmarest) secretion as well as in aromatic remedies were analyzed by gas chromatography/ mass spectrometry (GC/MS). The small Indian civet secretion consisted of civetone, dihydrocivetone and normuscone as main components. The contents of chemical constituents were different between male and female secretion. Civetone (23.6 ± 1.5 μg/mg of secretion) was identified as the major constituent in the female secretion and normuscone (52.1 ± 5.9 μg/mg of secretion) was in that of male small Indian civet. Both civetone and normuscone were also found in civet furs but not in the feces. All aromatic remedies with small Indian civet secretion ingredient in the label showed both civetone and normuscone but the quantity were different. Linearity range of civetone was 0-50 μg/ml with a correlation coefficient of 0.9717, and of normuscone was 0-80 μg/ml with a correlation coefficient of 0.9965. The average recoveries were 97.3-98.0% in secretion and 91.4-105.7% in aromatic remedy for civetone. For normuscone, average recoveries in secretion and aromatic remedy were 98.5 % and 90.0-103.0% respectively. The precision was evaluated by the intra-day and inter-day RSDs of the three concentrations which were less than 8%. LOD and LOQ for civetone were 0.0087 and 0.0165 μg/mg of secretion and for normuscone were 0.0596 and 0.1154 μg/mg of secretion respectively. Civetone and normuscone could be used as qualitative and quantitative markers for civet secretion ingredient in aromatic remedies. Civetone and normuscone contents in commercial civet secretion varied crop by crop and depended on male to female sex ratio of the small Indian civets.

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ACKNOWLEDGEMENTS

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

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

Gratitude is grateful to the thesis committee members, Professor Dr. Surasak Taneepanichskul, Dr. Tiwatt Kuljanabhagavad, and Dr. Naowarat Kanchanakhan 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.

Appreciation is also extended to College of Public Health Sciences, Chulalongkorn University and all the staff members for necessary assistance and instrumental support.

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

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LIST OF ABBREVIATIONS

amu	=	Atomic Mass Unit
AOAC	=	Association of Official Analytical Chemists
°C	=	Degree Celsius
cm	=	Centimeter
EI	=	Electron Ionization
ECG	=	Electrocardiography
g	=	Gram
GC	=	Gas chromatography
GC/MS	=	Gas chromatography/mass spectrometry
HPLC	=	High performance liquid chromatography
hr	=	Hour
IUPAC	=	International Union of Pure and Applied Chemistry
kg	=	Kilogram
KHz	=	Kilohertz
LD_{50}	=	Lethal dose 50%
LOD	=	Limit of detection
LOQ	=	Limit of quantitation
m/z	=	Mass-to-charge ratio
mg	=	Milligram
MIM	=	Multiple ions monitoring
min	=	Minute
ml	=	Microlitre
mm	=	Millimetre

mmol	=	Millimole
MS	=	Mass spectrometry
NIST	=	National Institute of Standard and Technology
nm	=	Nanometre
PTFE	=	Polytetrafluoroethylene
r^2	=	Correlation coefficients
rpm	=	Revolutions per minute
RSD	=	Relative standard deviation
SARS	=	Severe acute respiratory syndrome
SIM	=	Selected ion monitoring
US$	=	United States dollar
UV	=	Ultraviolet
α	=	Alpha
β	=	Beta

CHAPTER I
INTRODUCTION

Background and Significance of the Study

Thailand is located in tropical rain forest, so it is suitable a habitat for living things with high biodiversity. The wild animals are important species in ecology and food chain. Forest destroying causes wild animals extinction or risk on extinction including animal in the family Viverridae. A civet is small animal in a group of carnivores that found inhabiting the tropical forest area. A civet family (Viverridae) is related to the cat family (Felidae) that why most civets have catlike bodies [1]. Small Indian civet (*Viverricula indica* Desmarest) which in subfamily Viverrinae is found many countries in Southeast Asia, Pakistan, India, Nepal, Bangladesh, and South China [2]. Although it is a controlled animal that is announced in the Wildlife Protection Enactment (1992), the ministerial regulation issue 6th (1994) allows that farmer can keep the small Indian civet for the business [3]. There are several farms in central Thailand and the numbers of small Indian civets are around 800. The farmer keeps the small Indian civet for producing and exporting civet secretion to the world market. This secretion is used in medicinal and perfumeries purposes [4]. Both male and female civets produce the strong smelling secretion from the perineal gland. Civetone is the main constituent of the secretion produced from the civet of the genera *Civettictis*, *Viverra* and *Viverricula* [5, 6]. It is an animalic musk which consists of 17 carbon atoms [7].

Small Indian civet secretion is widely used in perfume industry and in traditional medicine for a long time. In Ethiopia traditional medicine, the secretion is used for treatment of headaches, skin discoloration and skin infections, by topical application. Similarly, some of the indigenous communities in India use the secretion of the small Indian civet in tribal medicine, particularly to treat respiratory ailments and skin infections, in addition to its constant use in Ayurvedic drugs which is a traditional Indian medicine [8-10]. The small Indian civet secretion is also used as an ingredient in Thai traditional medicine especially in aromatic remedy.

Thai traditional medicine uses the small Indian civet secretion for relief of faint, dizziness, nausea and vomiting; therefore it is an important ingredient in aromatic remedy. In the local markets, there are many aromatic remedies that claim to use the secretion as an ingredient in the remedies but it cannot prove that there is the secretion in the remedies. The civet secretion is often adulterated with Vaseline, butter, mashed banana, and petrolatum to increase the quantity because of the expensive secretion (US$6,600/kg) [11, 12]. In Thailand, there has been no report about the chemical constituents in secretion of small Indian civet. Hence, this present study is attempted to determine the chemical constituents of secretion from small Indian civet (*V. indica*) and investigate the civet secretion ingredient in aromatic remedies by gas chromatography/mass spectrometry (GC/MS).

Objectives of the Study

1. To determine the chemical constituents of secretion from *Viverricula indica* by GC/MS.
2. To investigate the civet secretion ingredient in aromatic remedies by GC/MS.

Expected Benefits

1. This research provides the chemical constituents of secretion from small Indian civet in Thailand.
2. This research provides the methodology to confirm small Indian civet secretion in aromatic remedy ingredient.

CHAPTER II
LITERATURE REVIEW

Family Viverridae

Animals in family Viverridae from order Canivora occupy the tropical forest area that can be found in the South-Western Europe, the Southern Asia, the Eastern Indies, Africa, and Maddascar [13]. The Viverridae family is divided into four subfamilies, Hemigalinae, Paradoxurinae, Prionodontinae, and Viverrinae, covering 35 species.

- The Hemigalinae includes four genera and four species of palm civet: Owston's civet (*Chrotogale owstoni*), otter civet (*Cynogale bennettii*), Hose's palm civet (*Diplogale hosei*) and the banded palm civet (*Hemigalus derbyanus*).

- The Paradoxurinae includes five genera and seven species: Binturong (*Arctictis binturong*), small-toothed palm civet (*Arctogalidia trivirgata*), Sulawesi palm civet (*Macrogalidia musschenbroekii*), masked palm civet (*Paguma larvata*) and three Paradoxurus species, the Asian palm civet (*P. hermaphrodites*), Jerdon's palm civet (*P. jerdoni*), and the golden palm civet (*P. zeylonensis*).

- The Prionodontinae includes two Prionodon linsang species: the banded and spotted linsangs (*P. linsang* and *P. pardicolor* respectively).

- The Viverrinae includes five genera and 22 species: African civet (*Civettictis civetta*); 14 genet species, Abyssinian genet (*Genetta abysssinica*), Angolan genet (*G. angolensis*), Bourlon's genet (*G. bourloni*), Crested Servaline genet (*G. cristata*), common or small-spotted genet (*G. genetta*), Johnston's genet (*G. johnstoni*), panther or rusty-spotted genet (*G. maculate*), Pardine genet (*G. pardina*), aquatic genet (*G. piscivora*), king genet (*G. poensis*), servaline genet (*G. servalina*), Haussa genet (*G. thierryi*), Cape or large spotted genet (*G. tigrina*), giant forest or giant genet (*G. victoriae*); two linsang species of the genus Poiana, Leighton's linsang (*P. leightoni*) and the African linsang (*P. richardsonii*); four civet species of the genus *Viverra*, the Malabar or Malabar large-spotted civet (*V. civettina*), large spotted civet (*V. megaspila*), Malayan civet (*V. tangalunga*), and large Indian civet (*V. zibetha*). Last in this subfamily is the small Indian civet (*Viverricula indica*) [14].

Viverrids (Viverrdae) are primarily carnivorous and eat small vertebrates or insects [15]. They are solitary or live in pairs or groups. Breeding may occur seasonally or throughout the year. Viverrids are small and medium-sized carnivores. Most of them have a relatively pointed muzzle and long tail (except the otter civet, *Cynogale bennetti*, which has a broad muzzle and short tail); a total of 40 teeth (the linsangs, *Prionodon* spp., have 38). Head and body length ranges from 350 to 950 mm, tail length 130 to 900 mm, and the adult weight range is 0.6 to 20 kg. There are various striped, spotted, and uniform color patterns. In some genera, the tail is banded or ringed. The body is generally long and sinewy, with short legs and typically a long, bushy tail. Footprints of most civets have five distinct toe impressions, except those of *Viverra* and *Viverricula*, which have only four toes visible and closely resemble the prints of dogs [2, 13] as shown in Figure 1.

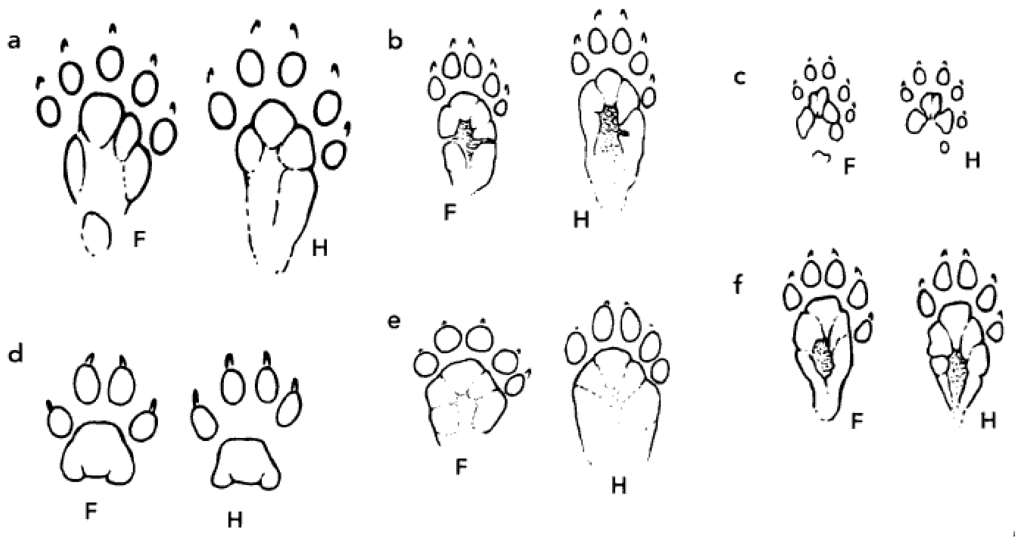

Figure 1 Left footprints of front (F) and hind (H) feet of civets: *Cynogale bennetti* (a), *Arctogalidia trivirgata* (b), *Prionodon linsang* (c), *Viverricula indica* (d), *Paradoxurus hermaphroditus* (e) and *Hemigalus derbyanus* (f) [2]

Figure 2 Scent gland of *Viverricula indica* [16]

Figure 3 Scent gland of *Viverra tangalunga* [17]

Most viverrids (except lingsangs and male small-toothed palm civet, *Arctogalidia trivirgata*) have scent gland in the anal region (Figure 2, 3) that secretes a nauseous-smelling fluid as a defensive measure. The secretion of the scent gland which obtained from several genera (*Civettictis*, *Viverra*, and *Viverricula*) is used to perfume and drug ingredients [2, 13]. In Ethiopia, it also takes in tea and coffee [18]. Some of macrocyclic musks such as civetone, dihydrocivetone and normuscone are important chemical constituents in civet secretion [19]. The secretion of African civet (*Civettictis civetta*) and small Indian civet (*Viverricula indica*) have been traded commercially for a long time. It is valued more than gold, ivory, or myrrh. Ethiopia produces 90% of the world's civet secretion [18]. Nowadays, some viverrids are tamed and kept to produce the secretion and civet coffee.

Civet coffee is one of the world's most expensive and low-production varieties of coffee. Asian palm civet (*Paradoxurus hermaphroditus*) and other related civets eat the bean of coffee berries (Figure 4), and then passed through its digestive tract. Civet cannot digest the beans; therefore, the beans can be found on the ground after the civets have passed them through their digestive tracts. After gathering, thorough washing, sun drying, light roasting and brewing these beans yield an aromatic coffee with much less bitterness, widely noted as the most expensive coffee in the world (Figure 5) [20].

Figure 4 Asian palm civet (*Paradoxurus hermaphroditus*) [21]

Figure 5 Civet coffee (Kopi Luwak) [22]

Civets are linked to SARS virus that was first recognized in China and killed nearly 800 people worldwide in 2003. A virus in civets is similar to the virus that infected in human SARS patients, or severe acute respiratory syndrome [23, 24].

Small Indian Civet (*Viverricula indica* Desmarest)

Taxonomic classification of small Indian civet

Kingdom: Animalia

Phylum: Chordata

Class: Mammalia

Order: Carnivora

Family: Viverridae

Subfamily: Viverrinae

Genus: *Viverricula*

Species: *Viverricula indica*

In Thailand, these viverrids were found 11 species, 9 genera and 3 subfamilies [19]. Three subfamilies include subfamily Paradoxurinea, subfamily Viverrinae, and subfamily Hemigalinae. Small Indian civet is in subfamily Viverrinae. It is native to Bangladesh, Bhutan, Cambodia, South and East China, India, Indonesia, Laos, Malaysia, Myanmar, Nepal, Sri Lanka, Thailand and Vietnam [25].

Small Indian civet is a small animal; weigh from 2 to 4 kg. Its body is slim, 54 to 63 cm in length. It has catlike body, long tail, and weasellike face. It has brown or tawny orange pelage ornamented with black and white rings on its neck, small spots on the body which converge into 6 to 8 dark stripes on the back toward the tail, and black and white banded tail. The tip of tail is usually white. The paws are typically dark brown or black and the breast is lighter brown or gray, with few if any marking. The small Indian civets are distinguished from closely related civets by their significantly smaller size, lack of dorsal crest of fur, smaller gap between ears, and shorter rostra (Figure 6). Male are generally larger than female [26-28]. The small Indian civet will feed on rats, small birds, lizards, insects, grubs and fruits. It likes to seek for food on the ground, although it can climb trees with agility. It digs readily and prefers to sleep in burrow [29].

Figure 6 *Viverricula indica* Desmarest [30]

Small Indian civet is usually solitary but occasionally associates in pairs. The reproduction of small Indian civet can occur throughout the year in Sri Lanka. Captive in China has two breeding seasons, the first during February to April and the second during August to September [31]. In each year it gives birth 2 to 5 young that is weaned at 2 months old. The civet gland has been shown to be of great importance to reproduction. It is likely the chemicals emitted by this gland attract mates to each other or demonstrate which animal is estrus [32, 33]. Civet secretion is produced by both sexes of civet (Viverridae). It originates from special pouched glands situated in the male civet between the testicles and the praeputium of the penis and in female between the vulva and anus [11]. Both male and female deposit the secretion from their glands on many types of objects.

The diploid chromosome numbers of small Indian civet are 2n=36, the fundamental numbers (NF) are 60 chromosomes in female and 59 in male. The autosomes consist of 6 large metacentrics, 8 large submetacentrics, 6 large acrocentrics, 2 large telocentrics, 2 medium metacentrics, 4 medium submetacentrics, 4 medium acrocentrics and 2 small metacentrics. The X chromosome is the largest submetacentric and Y chromosome is a medium tetocentric chromosome. The karyotype formula for the small Indian civet is $2n\ (36) = L^{m}_{6} + L^{sm}_{8} + L^{a}_{6} + L^{t}_{2} + M^{m}_{2} + M^{sm}_{4} + M^{a}_{4} + S^{m}_{2}$ + sex-chromosome [34].

Small Indian civet is one of the controlled animals that were designated in Wildlife Protection Enactment (1992) but according to the ministerial regulations issue 6th (1994), farmer can keep and breed the civet for commercial purposes. In Thailand, there are many civet farms and the number of small Indian civets throughout Thailand is about 800 including 500 civets of Thai civet club which consists of the several small civet farms, 100 civets of Tipprasert civet farm and 200 civets of Pa Noi' s civet farm [12, 35, 36]. All of the civets are caught from wild. They are kept in large cage made of bamboo which one cage for one civet (Figure 7). All of the civet farms are registered with the Forestry Department while officials of the department can enter and inspect the farm at any time during day time without prior notification [4]. Pa Noi's civet farm is the largest farm that produces the civet secretion to traditional medicine producers (Figure 8). At another civet farm, the small Indian civets are fed with coffee beans for civet coffee producing.

Figure 7 Small Indian civet in a cage [4]

Figure 8 Pa Noi's civet farm

Small Indian Civet Secretion

Figure 9 Small Indian civet secretion

The secretion (Figure 9) is almost strong smelling oily substance with a yellowish color. It darkens when exposed to light. The taste is bitter and the odor is less agreeable than musk. It is offensive when undiluted but agreeable when a small portion is mixed with another substance. It contains a volatile oil and free ammonia. Male civet secretion is dirty than female civet secretion because some male civet urinates during deposits the secretion on the object. The quantity and quality are not different between male and female civet secretion [3]. In extreme dilution, the civet secretion has a pleasant odor. Civetone is the main constituent of civet secretion (2.5-3.4%). The civet secretion also contains other macrocyclic ketones such as cyclohexadecanone, cycloheptadecanone, and 6-*cis*-cycloheptadecenone [37]. It has powerful holding properties for other scent and is mainly used as fixative in the manufacture of expensive perfumes. Thai traditional medicine uses the secretion for heart stimulating effect, therefore it is an important ingredient in aromatic remedy. Moreover Arabs also use civet secretion for medical purposes and in Japan; it is used as a preservative of antibiotic [11].

In Thai traditional medicine, there are 115 remedies that used the secretion as an ingredient [3]. Most of the remedies are aromatic remedies. Before used the secretion as a crude drug, the secretion had moved the impurity with the traditional process. The secretion and kaffir lime (*Citrus hystrix* D.C.) peel are placed on betel vine (*Piper betle* L.) leave, and then singed to make more cleaning and pleasing smell as shown in Figure 10 and 11.

Figure 10 Small Indian civet secretion which placed on betel vine

Figure 11 Thai traditional processing for civet secretion cleaning

Macrocyclic Ketones of Fragrance Ingredient

The common structure of macrocyclic ketone group of fragrance ingredient is a keto group, R-C (=O)-R', contained within a macrocyclic ring of C15-C17 carbon chain length. Macrocyclic ketones fragrance ingredients include 11 structurally diverse C15, C16, and C17 compounds that consist of three saturated and eight unsaturated ketones. Naturally occurring macrocyclic ketones are obtain from various animal rather than plant sources. The molecular weights of the macrocyclic ketones are range from a high of 250.43 g/mol for the C17 congener civetone to a low of 222.37 g/mol for the C15 4-cyclopentadecen-1-one. All macrocyclic ketones have similar metabolism and are detoxified in the same manner [38].

Civetone

Formula: $C_{17}H_{30}O$
IUPAC name: 9-Cycloheptadecen-1-one
Synonyms: civettone, zibeton, *cis*-civetone, *α-trans* civettone
Molecular weight: 250.43 g/mol

Civetone is a 17-membered macrocyclic ketone which is a pheromone sourced from civet (Figure 12). The structure of civetone was elucidated by Ruzicka in 1926 as a macrocyclic unsaturated ketone [39]. It contains one ethylenic double bond therefore it can exist as either the *cis* or *trans* isomer. Naturally occurring civetone has been identified as the *cis* isomer [40]. It is semi-solid, yellowish to brown, unctuous substance, bitter taste, fusible and burns without leaving much residue. It is insoluble in water but soluble in hot alcohol or in ether [41]. The previous study demonstrated the synthesis civetone from ethyl oleate which prepared from palm oil [42].

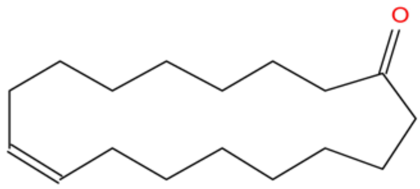

Figure 12 Structure of civetone

Civetone can be detected by thin layer chromatographic method as a yellowish green spot after spraying with mixed solution of 1 ml of 10% methanolic solution of phosphomolybdic acid and 49 ml of *p*-anisaldehyde - acetic acid - sulfuric acid - methanol (1:10:20:200) then heating for 5 min at 105 °C. In addition, civetone is easily separated from other saturated macrocyclic ketones by GC/MS method using polyethylene glycol 20 M as stationary phase [43].

Biological Data of Civetone

The acute oral LD_{50} value in rats is more than 5 g/kg body weight and the acute dermal LD_{50} value in rabbits exceeds 2 g/kg. Civetone applied to intact or abraded rabbit skin for 24 hr under occlusion is moderately irritating. Evaluation of civetone at dose of 4% in petrolatum in a 48 hr closed path test shows no irritation and no sensitization reactions. Civetone at dose of 1% and 20% in petrolatum or ethanol is negative for phototoxicity in guinea pigs. Irradiation for 30, 60, and 120 min at approximate energy level of 1.6-7.6 J/cm^2 (UV at 300-430 nm) at 15-20 cm from the skin do not produce phototoxic reactions in guinea pigs [38, 44]. *cis*-Civetone and *trans*-civetone can exhibit strong type I spectral interactions with hepatic microsomal cytochrome P-450. The *cis* isomer is a potent specific inhibitor of the in *vitro* aromatization of androgens to estrogens [45].

Usage of Civetone

Many products use civetone as a fragrance ingredient. Civetone may be found in fragrance used in cosmetic, shampoos, toilet soaps, and non-cosmetic products such as detergent and household cleaners. The worldwide volume of civetone which used as fragrance ingredient has been about 0.01-0.1 metric tons per year [38, 44].

Normuscone

Formula: $C_{15}H_{28}O$

IUPAC name: Cyclopentadecanone

Synonyms: Exaltone, normuscon

Molecular weight: 224.39

Normuscone is a macrocyclic ketone with a 15-carbon ring and colorless crystal needles (Figure 13). It is found in the scent gland of Louisiana muskrat [46]. It is insoluble in water but soluble in alcohol.

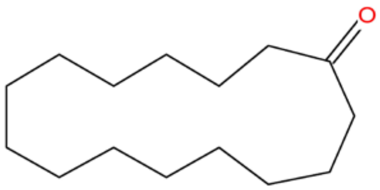

Figure 13 Structure of normuscone

Biological Data of Normuscone

The acute oral LD_{50} value in rats and acute dermal LD_{50} value in rabbits are reported as greater than 5 g/kg body weight. An intraperitoneal dose of 11.25 mmol/kg (~ 2.5 g/kg) causes no deaths. An oral dose of 45 mmol/kg (~10.0 g/kg) causes no signs of toxicity in mice. Normuscone at 10% in petrolatum produces no irritation after a 48 hr closed path test on human subjects and produces no sensitization reactions. Normuscone produces no irritation to rabbit eyes when applies an instillation of 0.1 g of 100% into the conjunctival sac of rabbits. The 30% dilution causes transient redness in the conjunctivae that cleared by 24-48 hr. Normuscone at 10% in ethanol causes no phototoxic reaction in rabbits; 1% or 20% of normuscone in petrolatum or ethanol also do not cause a phototoxic reaction in guinea pigs. Normuscone has been reported not being mutagenic in the Ames test and not clastogenic in human lymphocytes in *vitro* testing [38, 47].

Usage of Nomuscone

Normuscone is a fragrance ingredient used in many compounds such as decorative cosmetics, find fragrances, shampoos, toilet soaps, and non-cosmetic products. The worldwide volume of use for normuscone has been about 10-100 metric tons per year. This report volume is for normuscone that used as a fragrance ingredient [38, 47].

Aromatic Remedy

Aromatic remedy is one of the most Thai ancient remedies. It has been used for relief of faint, dizziness, nausea and vomiting for a long time. Aromatic remedy is usually taken by mixing 0.5-1.0 g of aromatic remedy powder with warm water and drinking the whole preparation as needed. In the local markets, there are several aromatic remedies which are different trade names and different proportion of the medicinal plants. Most aromatic remedies contain similar major ingredients such as civet secretion, clove and agarwood. Moreover, most aromatic remedy ingredients are aromatic plants. Civet secretion is also used as a fixative in aromatic remedy because of its strong holding property [11].

Figure 14 Aromatic remedies [47]

Effect of Aromatic Remedy

The water extract of aromatic remedy at concentration of 0.83-16.67 mg/ml can stimulate rat aortic ring contraction which is partially inhibited by phentolamine. The stimulatory effect of aromatic remedy is partially dependent on α receptor, similar to norepinephrine but dose not involve β receptor [49].

The major effect of aromatic remedy is to increase the blood pressure. In rat, the water extract of aromatic remedy at doses of 0.2-0.8 g/kg initially transiently decreases pressure and over time, increases blood pressure. Aromatic remedy initially decreases blood pressure for a short period and increases blood pressure over a longer period. The time to decrease blood pressure is in seconds, whereas the increasing blood pressure effect is in minutes. However, the duration of action and time to maximum response are dose dependent. Therefore, the dominant effect of aromatic remedy on increasing blood pressure supports the use of aromatic remedy for the treatment of fainting [50].

In human, aromatic remedy increases blood pressure by increasing diastolic blood pressure and decreasing pulse pressure. Aromatic remedy powder and its water extract have no effect of ECG, heart rate and peripheral blood flow. An Increasing blood pressure effect of aromatic remedy supports the use of aromatic remedy for the treatment of fainting because decreasing blood pressure is a sign of fainting [51].

Gas Chromatography/Mass Spectrometry (GC/MS)

Gas Chromatography/Mass Spectrometry (GC/MS) is one of the most popular and effective methods for separating, identifying and quantifying compound in chemical mixtures. It is made up of two parts. The gas chromatography (GC) portion separates the chemical mixtures and the mass spectrometry (MS) identifies and quantifies the chemicals. GC is a popular, powerful and easy to use analytical tool. GC is based on the repeated partition or absorption between a mobile phase and a stationary phase of the components to be separated. The chemical mixtures are injected into an inert gas stream and swept into a column with a solid or liquid support coated [52]. The chemicals in the mixture separate based on their volatility. In general, small molecules travel more quickly than larger molecules. For most GC, the identification is based on retention time. The GC method is particularly suitable for quantitative analysis of natural volatile components, and lipophilic compounds with low boiling point and good thermal stability in medicinal herbs. Although high boiling point and heat-sensitive compounds are now mostly analyzed by HPLC, some scientists still like to use GC to quantify them by making them into less polar derivatives, because GC provides better resolution due to its longer column than HPLC, and less interference by the mobile phase [53]. Mass spectrometer (MS) is the most common detector coupled with GC. The MS is used to identify chemicals based on their structures. There are four steps including ionization of the sample, acceleration of the ions by an electric field, dispersion of the ions according to their mass-to-charge ratio and detection of the ions to produce a corresponding electrical signal [54]. A mass spectrometer is an excellent tool for clear identifying the structure of a single compound but is less useful when presented with a mixture.

GC/MS is an instrument that uses to separate mixtures into individual components, identifying and then providing quantitative and qualitative information on the amounts and chemical structure of each compound [52]. It is very sensitive and has high resolution.

Full Scan Mode and Selected Ion Monitoring Mode

Full scan Mode

The continuous recording of mass spectra and the simultaneous determination of the retention time allow the identification of analyses by comparison with libraries of mass spectra [55]. The full scan mode is used to detect any molecules with a mass-to-charge ratio (*m/z*) which a certain range in the analyzed sample. Therefore, the full scan mode can be used for unambiguous confirmation of the analyst content and identifying any non-targeted analyst that may be present [56, 57].

Selected Ion Monitoring Mode (SIM)

SIM or multiple ions monitoring (MIM) mode refers to the recording of only ions current with one or multiple selected *m/z* values [56, 58]. It is used to detect the content of compounds with a specific *m/z* and used to unambiguous confirmation of the analyst from a full scan mode. It is also appropriate for quantitative analysis of low concentration of an analyzed sample [57, 58].

CHAPTER III
MATERIALS AND METHODOLOGY

Samples

1. Secretion of small Indian civets (*Viverricula indica*)
   - Male small Indian civet secretion (n=15)
   - Female small Indian civet secretion (n=15)
   - Pooled small Indian civet secretion (n = 10)
2. Feces of small Indian civets (n = 15)
3. Furs of small Indian civets (n = 3)
4. Aromatic remedies with civet secretion in ingredient
5. Aromatic remedy without civet secretion in ingredient

Materials

1. Zebron ZB 5 capillary column (30 m x 0.25 mm x 0.25 µm) (Phenomenex®, CA, USA)
2. Polytetrafluoroethylene (PTFE) syringe membrane filter (0.45 µm) (Chrom Tech, Inc., USA)

Chemicals and Reagents

1. 9-cyclohetadecon-1-one (Sigma-Aldrich Company Co., St. Louis, MO, USA)
2. Cyclopentadacanone (Sigma-Aldrich Company Co., St. Louis, MO, USA)
3. Hexane AR grade (Lab-Scan Asia Co., LTD, Bangkok, Thailand)

Instrumentations

1. Gas chromatograph (Trace GC Ultra, Thermo Finnigan, USA) equipped with MS detector (DSQ, Thermo Finnigan, USA)
2. Ultrasonic bath (Analytical Lab Science Co., LTD, Bangkok)
3. Centrifuge (Sorvall® Primo R, UK)

Methods

Sample collection

Male small Indian civet (*Viverricula indica*) secretion (n =15), female small Indian civet secretion (n =15), pooled small Indian civet secretion (n = 10), small Indian civet feces (n = 15), and small Indian civet furs (n=3) were collected from Pa Noi's civet farm in Petchaburi, Thailand. Each sample was kept in tightly capped vial and refrigerated until analysis.

Three different lot numbers of aromatic remedies with civet secretion ingredient in the description and one aromatic remedy not containing civet secretion ingredient were collected from the local markets. Each aromatic remedy stored at ambient temperature until analysis.

Small Indian Civet Secretion Analysis

One milligram of each sample was vortically mixed with 1 ml of hexane for 1 min then centrifuged at 10,000 rpm for 10 min at 25°C. One microliter of hexane supernatant was analyzed by gas chromatography/mass spectrometry (GC/MS). The analysis was performed using a Finnigan trace GC ultra gas chromatography equipped with ZB-5 capillary column (30m x 0.25mm x 0.25µm) and interfaced to a Finnigan trace DSQ MS detector. The oven temperature was ramped from 60°C to 240°C at a constant rate of 3°C/min. The injection port was held at 180°C throughout the separation. The carrier gas was helium with a flow rate of 1 ml/min and split ratio of 10:1. MS was performed by electron ionization (EI) mode at 70 electron volts. Analysis of each sample was performed in triplicates.

Small Indian Civet Furs Analysis

Fifteen milligrams of each sample was washed in aliquots of 2 ml hexane until exhaustion. Washing hexane aliquots were kept in the refrigerator for further analysis. The furs were removed, dried and cut into fine pieces. Five milligrams of the washed fine pieces of small Indian civet furs were mixed with 1 ml of hexane and sonicated at 30°C for 15 min at 53 KHz. Then, it was centrifuged at 10,000 rpm for 10 min at

25°C. Hexane extract as well as washing hexane aliquots were analyzed with the same conditions GC as used for the secretion samples. Analysis of each sample was performed in triplicates.

Small Indian Civet Feces Analysis

One hundreds milligrams of each sample was dissolved in 1 ml of hexane and vortex for 1 min. This solution was centrifuged at 10,000 rpm for 10 min at 25°C. One microliter of the hexane supernatant was analyzed by GC/MS with the same conditions as used for the secretion samples. Analysis of each sample was performed in triplicates.

Aromatic Remedies Analysis

One hundreds milligrams of each sample was mixed with 1 ml of hexane and vortex for 1 min. Then, it was filtrated though 0.45 µm PTFE membrane filters and evaporated. After this, the extract was adjusted to 250 µl of hexane and then vortex again. The solution was analyzed within the same conditions GC as used for the secretion samples. Each sample was performed in triplicates.

Qualitative Analysis of Small Indian Civet Secretion Constituents

The chromatogram of small Indian civet secretion was obtained by full scan mass spectra with a scan range of 40-650 amu at 500.0 amu/second. The chemical constituents in the secretion extract were identified by matching their mass spectra and retention time indices with Adams Essential Oils Mass Spectral library and NIST 05 Mass Spectral library. The percentages of chemical compositions were computed as the percentage of GC peak areas.

Quantitative Analysis of Civetone and Normuscone

The amount of civetone and normuscone were determined by comparing the area of the chromatogram with the calibration curve of standard solutions and were expressed in mean and standard deviation (SD) as µg/mg of samples.

Calibration Curve and Linearity

Stock solution of civetone was prepared by dissolving 1.1 µl of civetone (equivalent to 1 mg, density = 0.917 g/cm^3 at 33°C) in 1 ml of hexane. Stock solution of normuscone was prepared by dissolving 1.0 mg of normuscone in 1 ml of hexane. The stock solutions were serial diluted for linearity range and calibration curves. The standards were analyzed under the same conditions of GC as mentioned above. The calibration curves were obtained according to the linear regression analysis of the peak areas *versus* the amount of civetone and normuscone.

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

LOD and LOQ determination were based on the standard deviation of the blank [59]. The triplicates of 1 mg/ml of blank sample were prepared and analyzed with the same conditions as used for the samples. The mean and SD of concentration corresponds to analytical responses were detected and calculated the LOD and LOQ as follow [59]:

LOD = mean of blank sample + 3SD

LOQ = mean of blank sample + 10SD

Precision

The precision of the method was assessed with intra-day and inter-day analyses. For repeatability, different concentration levels (3 concentrations / triplicate) which covered the specified range were analyzed on day 1 and this were repeated on 3 consecutive days. The quantity of each component was determined by the respective calibration curve. Relative standard deviation (RSD) was used to measure precision [59].

Recovery of Small Indian Civet Secretion

The extraction efficiency method was used for recovery evaluation of civet secretion by re-extracting the residue until exhaustion [59] and determining civetone and normuscone by GC/MS. The extraction of civetone and normuscone was performed at two concentrations of the small Indian civet secretion (1 and 2 mg/ml).

The secretion was dissolved in 1 ml of hexane, vortex for 1 min and centrifuged at 10,000 rpm for 10 min at 25°C. The filtrate was removed and then analyzed by GC/MS. The marc was re-dissolved in 1 ml of hexane and performed with the aforementioned method. The extraction was repeatedly done until exhaustion. The percentage of recovery was calculated as follow:

$$\boxed{\text{Recovery (\%)} = (C_a \times 100)/C_s}$$

C_a: concentration in the first filtrate

C_s: concentration in sum of filtrates

Recovery of Aromatic Remedies

Recovery was carried out by spiking method [60] using three concentrations of standard solution. The average recoveries of every spiking concentration were calculated.

$$\boxed{\text{Recovery (\%)} = [(A_s\text{-}A)/A_a] \times 100}$$

A_s: the amount of civetone or normuscone that found after spiking of the standard solution

A: the amount of those found that before spiking

A_a: the amount of reference standards actually added to the sample

Selected Ion Monitoring (SIM) Analysis of Civetone and Normuscone

For analysis of trace amount of civetone and normuscone, SIM mode was performed simultaneously with full scan mass spectra monitoring. A selected ion mornitoring chromatogram at *m/z* 250 and 224 were measured for representing civeone and normuscone respectively.

CHAPTER IV
RESULTS

Small Indian Civet Farm

Pa Noi's civet farm is the largest civet farm in Thailand that keeps civets for over 80 years. The farm is run by 60 years old couple. It is a family business and they do it for almost a century. There are about 200 small Indian civets in this farm and can get almost a kilogram of civet secretion monthly. Most of the civets were caught from wild and some civets were bought from the neighbor who stopped to keep the civets. Male small Indian civets are more than female civets. The farmers try to breed the civets but they have never been able to raise civet babies to adult size. Most of them died before reach the age of 6 weeks. All of the small Indian civets are kept in individual cages made of bamboo (Figure 15, 16). The size of the cage is 0.90 X 1.0 meter and 0.50 meter high. The cages are under roof to protect the small Indian civets from direct sun and rain. A timber wood stick about 1.0 inches square and about 0.60 meter long is put in the middle of the cage that used to deposit the civet secretion. The farmers take the stick out every morning at around 6 a.m. and take the secretion out with a stainless steel tea spoon. Then, they pool the secretion gathering from all small Indian civets together. Therefore, the commercial civet secretion from the civet farm does not separate male and female secretion. The amount of secretion collected from one civet depends on the health of the civets and the temperature. If the weather is cold, there is more civet secretion onto the wooden stick. The secretion of each small Indian civet was of 3-4 g/month. When the civet is too old, farmer will release it back into the wild or keep it until die. This farm produces the civet secretion to most traditional medicine producers in Thailand [12].

Figure 15 A small Indian civet in the bamboo cage

Figure 16 Small Indian civet cages

Small Indian Civet Secretion

Small Indian civet secretion was an oily substance with yellowish in color. It had an offensive animal odor. Male small Indian civet secretion was stronger smelling and darker than female secretion (Figure 17).

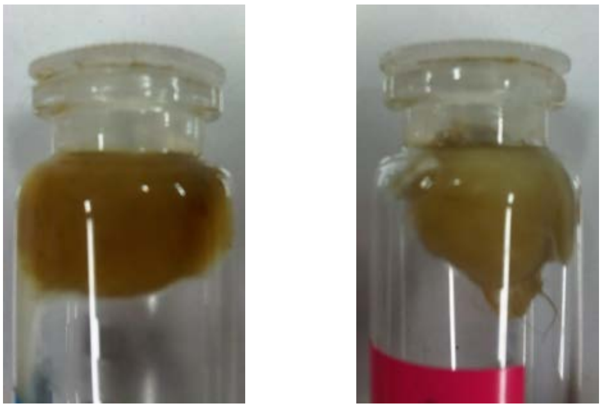

Figure 17 Male (left) and female (right) small Indian civet secretion

Small Indian Civet Furs

Small Indian civet furs were dark brown and white (Figure 18). It has the same smell as the secretion.

Figure 18 small Indian civet furs

Small Indian Civet Feces

The small Indian civet feces was a soft and paste substance with foul smell. It was yellowish in color (Figure 19). There was broken-milled rice in the feces. The male small Indian civet feces was darker than female feces.

Figure 19 Male (left) and female (right) small Indian civet feces

Small Indian Civet Secretion Constituents

Three main chemical constituents of male small Indian civet secretion (Figure 20) were normuscone, dihydrocivetone and civetone with the percent area of 73.4 ± 7.0, 5.1 ± 1.5, and 3.2 ± 1.9 % respectively. The female small Indian civet secretion exhibited four main chemical constituents (Figure 21) including civetone, dihydrocivetone, normuscone, and cyclohexadecanone with the percent area of 56.1 ± 5.2, 16.8 ± 2.9, 11.8 ± 2.1, and 3.2 ± 0.6 % respectively.

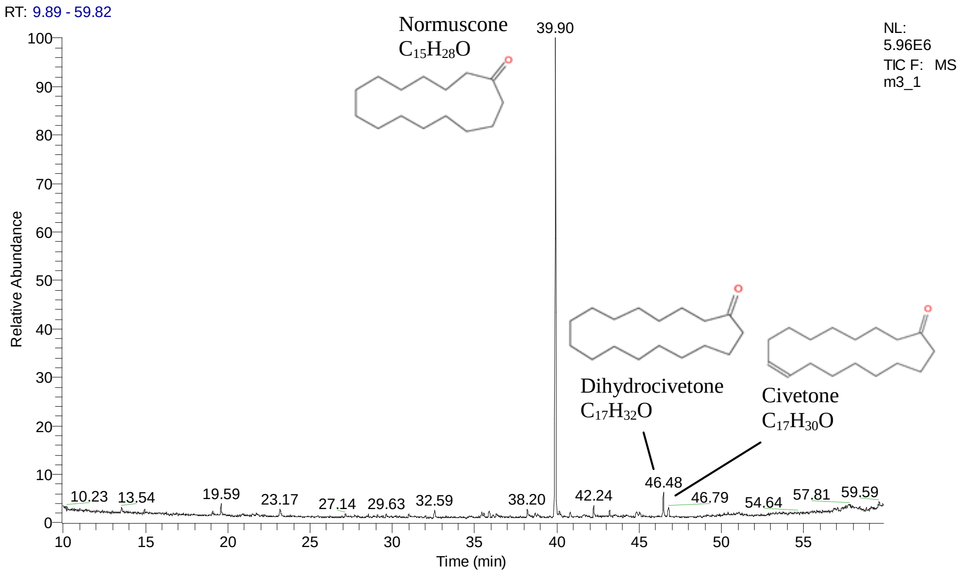

Figure 20 GC chromatogram of male small Indian civet secretion

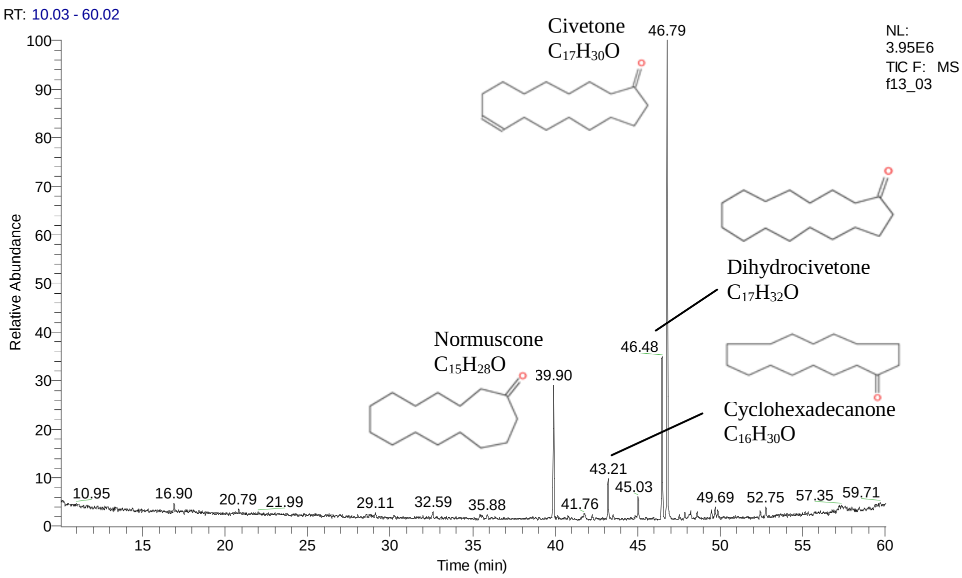

Figure 21 GC chromatogram of female small Indian civet secretion

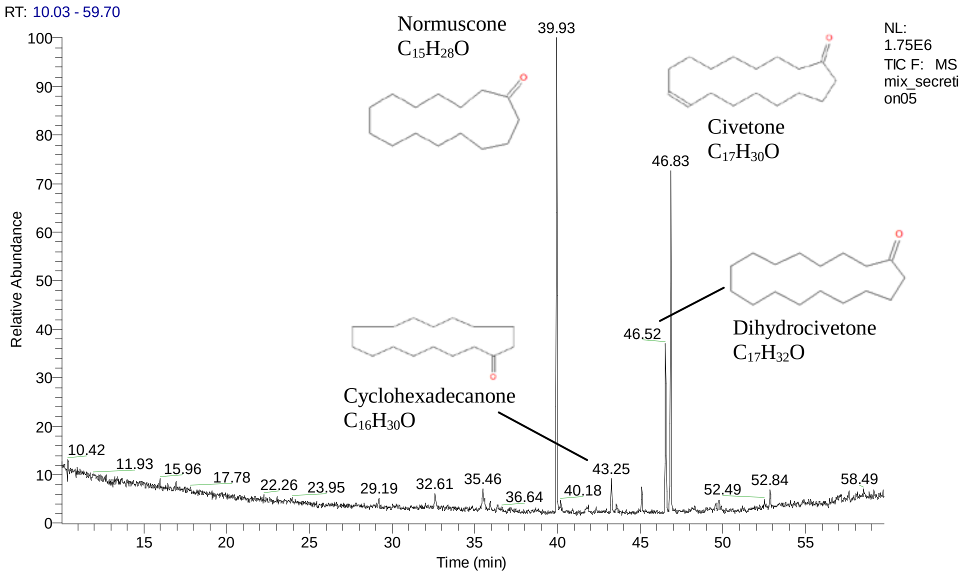

Figure 22 GC chromatogram of pooled small Indian civet secretion

Commercial civet secretion from civet farms did not separate male and female secretion. The secretion was gathered from all small Indian civets every morning and pooled together. Ten crops of secretion were analyzed and found that the secretion consisted of normuscone, civetone, and dihydrocivetone as main components with the percent area of 45.8 ± 1.3, 34.9 ± 0.7, and 15.7 ± 0.7 % respectively. The chemical cyclohexadecanone which found only in the secretion of female civet could be expressed in minor component with the percent area of 3.6 ± 0.3 % (Figure 22).

The mass spectrum of civetone (Figure 23) showed a parent peak at *m/z* 250 corresponding to the molecular formula $C_{17}H_{30}O$. The spectrum contained peaks corresponding to major fragments at *m/z* value of 250, 96, 95, 82, 81, 80, 69, 55, 54, and 41. Similarly, the mass spectrum of normuscone (Figure 24) showed a parent peak at *m/z* 224 corresponding to the molecular formula $C_{15}H_{28}O$. The spectrum contained peaks corresponding to major fragments at *m/z* value of 96, 83, 82, 71, 69, 58, 55, 43, 42, and 41.

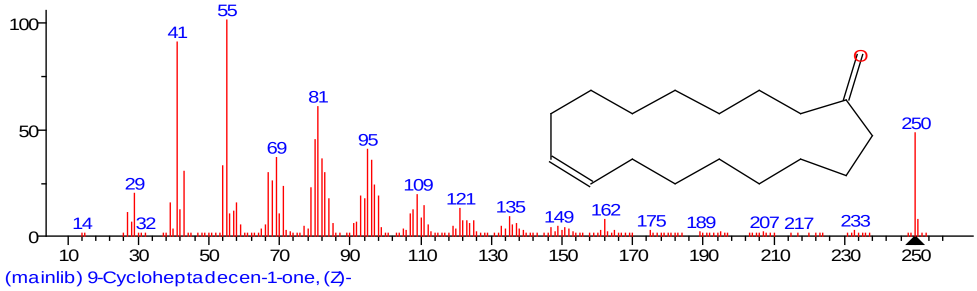

Figure 23 Mass spectrum of civetone

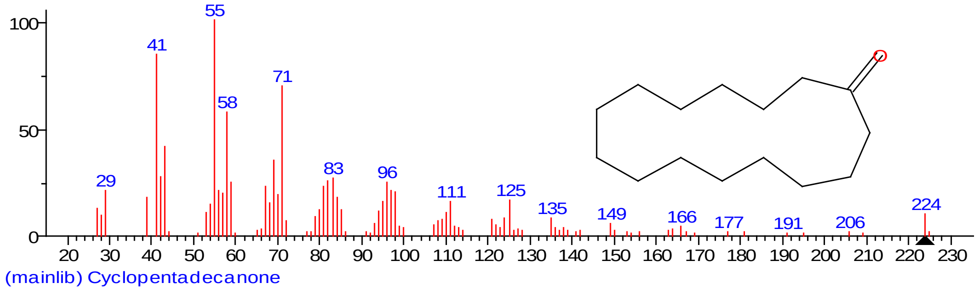

Figure 24 Mass spectrum of normuscone

Linearity

The calibration curves were constructed by plotting the peak area of the standards against their concentration. The regression equations for the linear portion of the calibration curves of civetone and normuscone were $y = 910946x$ and $y = -735543 + 354744x$ respectively (y referred to the peak area; x referred to the concentration of the unknown).

The correlation coefficients (r^2) were 0.9717 and 0.9965 for civetone and normuscone respectively. Linearity range of civetone was 0-50 µg/mg and of normuscone was 0-80 µg/mg (Figure 25, 26).

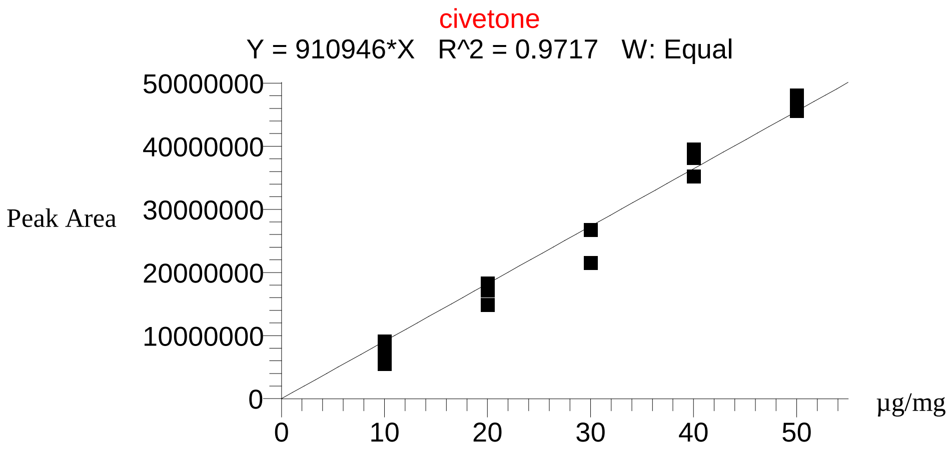

Figure 25 Calibration curve of civetone

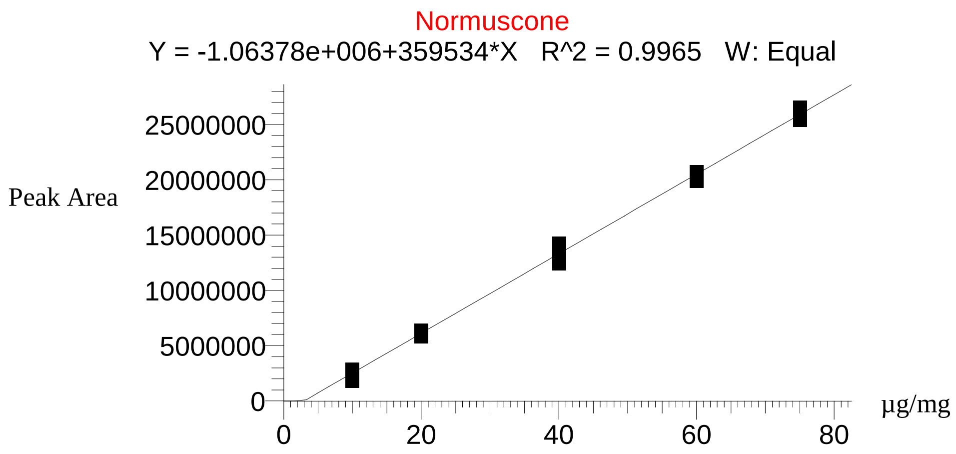

Figure 26 Calibration curve of normuscone

LOD and LOQ

In the present study, LOD and LOQ determination were based on the standard deviation of the sample blank. LOD and LOQ for civetone were 0.0087 and 0.0165 µg/mg of sample, 0.0596 and 0.1154 µg/mg of sample for normuscone respectively.

Precision

The triplicates of each sample within 3 consecutive days for intra-day and inter-day precision were determined. The results were presented in Table 1. The intra-day and inter-day RSDs were less than 8%.

Table 1 Percentage of relative standard deviation of intra-day and inter-day analysis

	Concentration (mg/ml)	Intra-day RSD%			Inter-day RSD%
		Day 1	Day 2	Day 3	
Civetone	0.025	3.212	0.821	1.276	0.472
	0.5	1.008	3.530	5.507	0.139
	1	1.660	4.693	3.185	0.180
normuscone	0.025	3.446	4.739	1.889	0.531
	0.5	1.117	4.856	7.649	0.929
	1	1.114	3.920	0.872	0.464

Recovery

The triplicates of each concentration group were analyzed for the recovery. This recovery was shown as the percentage amount of civetone and normuscone which extracted from the sample to validate the method. The average recoveries were 97.3-98.0% in secretion and 91.4-105.7% in aromatic remedy for civetone. For normuscone, average recoveries in secretion and aromatic remedy were 98.5% and 90.0-103.0% respectively. The percentage recoveries were presented in Table 2-4.

Table 2 The percentage recoveries of civetone and normuscone in civet secretion

% recovery of civetone		% recovery of normuscone	
Secretion 1 mg/ml	Secretion 2 mg/ml	Secretion 1 mg/ml	Secretion 2 mg/ml
95.7 %	98.7 %	99.4 %	98.3 %
97.9 %	97.3 %	97.6 %	98.9 %
98.3 %	97.9 %	98.5 %	98.3 %

Table 3 The percentage recoveries of civetone in aromatic remedy

No. of sample	% recovery of civetone		
	Civetone 10 µg/mg of sample	Civetone 20 µg/mg of sample	Civetone 30 µg/mg of sample
1	112.6%	96.4%	99.9%
2	66.1%	90.3%	110.4%
3	95.3%	102.2%	105.7%

Table 4 The percentage recoveries of normuscone in aromatic remedy

No. of sample	% recovery of normusone		
	Normuscone 10 µg/mg of sample	Normuscone 20 µg/mg of sample	Normuscone 30 µg/mg of sample
1	116.4%	91.9%	103.1%
2	102.8%	85.5%	90.0%
3	95.3%	95.2%	92.6%

Civetone and Normuscone Contents in Small Indian Civet Secretion

The civetone and normuscone were main chemical constituents in the secretion of small Indian civet. Table 5 demonstrated that the concentration of chemical constituents were different between secretion of male and female small Indian civet. Civetone was dominated in female whereas normuscone was dominated in male small Indian civet secretion. The analysis of secretion crops which containing both male and female secretion showed higher concentration of normuscone than civetone.

Table 5 The concentration of civetone and normuscone in small Indian civet secretion (µg/mg of secretion)

	Male	Female	Crop
civetone (µg/mg)	0.788 ± 0.138	23.614 ± 1.469	5.931 ± 1.728
normuscone (µg/mg)	52.121 ± 5.931	19.218 ± 1.584	22.304 ± 5.162

Civetone and Normuscone Contents in Small Indian Civet Furs

The collected civet secretion was frequently mixed up with civet furs. The furs sticked with civet secretion were one of commercial products form civet farms. Besides full scan analysis of mass spectrum, selected ion monitoring (SIM) spectra were analyzed for trace amount of civetone and normuscone. Figure 27 showed neither normuscone nor civetone in the last aliquot of hexane from civet furs washing thus represented exhaustively washing. However after successively washing (as shown in Figure 27), it was still found that the small Indian civet furs presented four chemical constituents (Figure 28) including civetone, dihydrocivetone, normuscone, and cyclohexadecanone that related to the small Indian civet secretion. The concentration of civetone and normuscone in small Indian civet furs were 0.23 ± 0.09 and 1.27 ± 0.17 µg/mg of washed furs respectively.

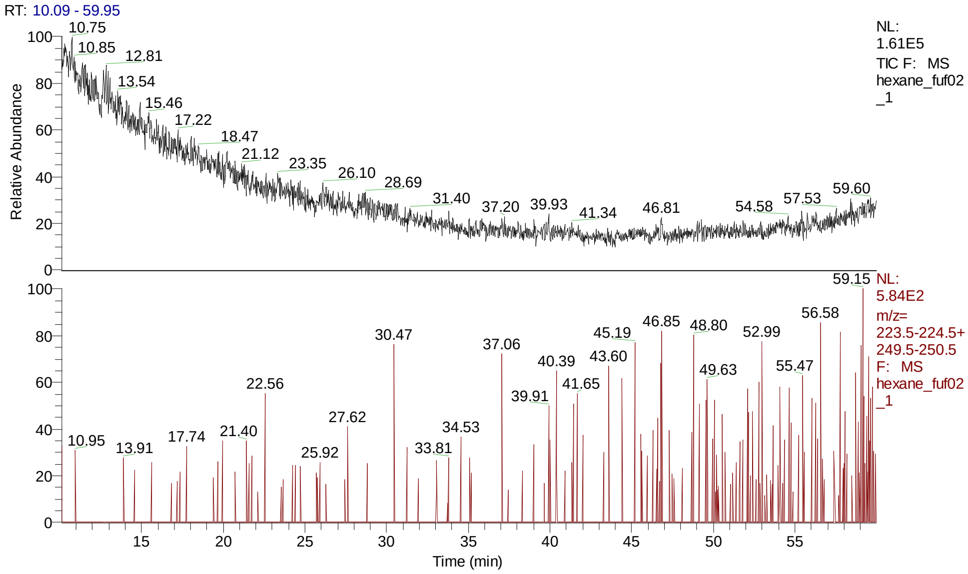

Figure 27 Full scan (upper) and SIM (lower) chromatogram of the last aliquot of hexane from civet furs washing

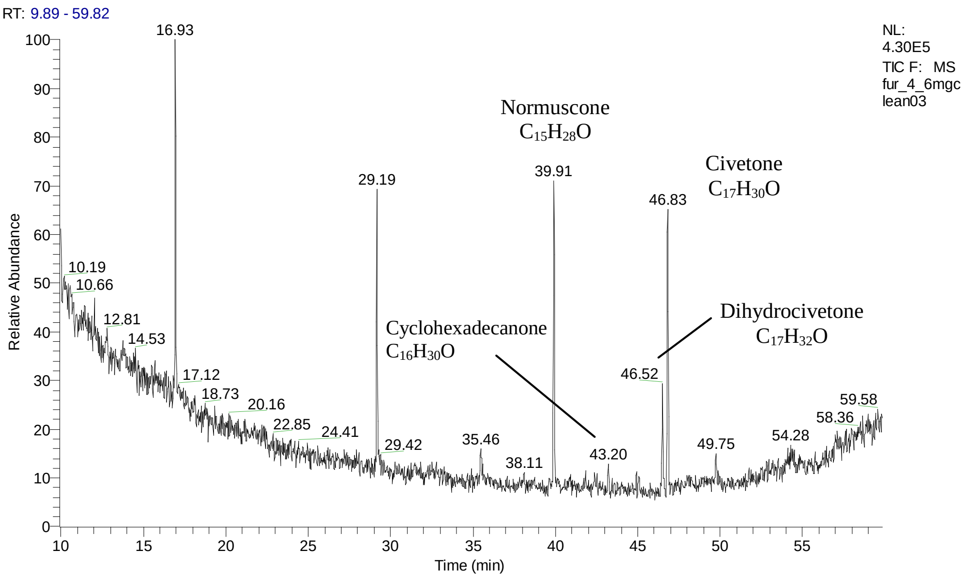

Figure 28 GC chromatogram of small Indian civet furs

Civetone and Normuscone Contents in Small Indian Civet Feces

There was no civetone and normuscone in the chemical constituents of both male and female small Indian civet feces as shown in figure 29 and figure 30.

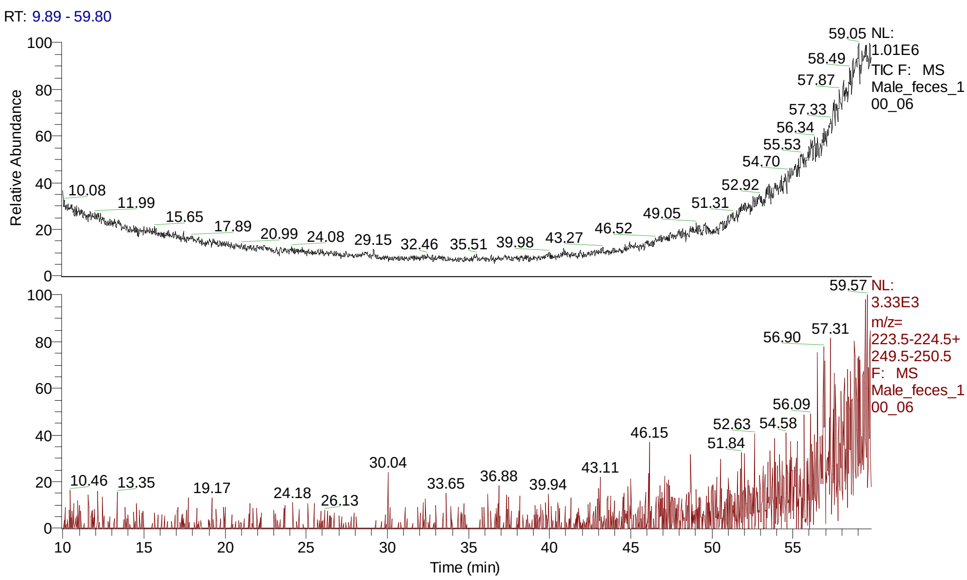

Figure 29 GC chromatogram of male small Indian civet feces

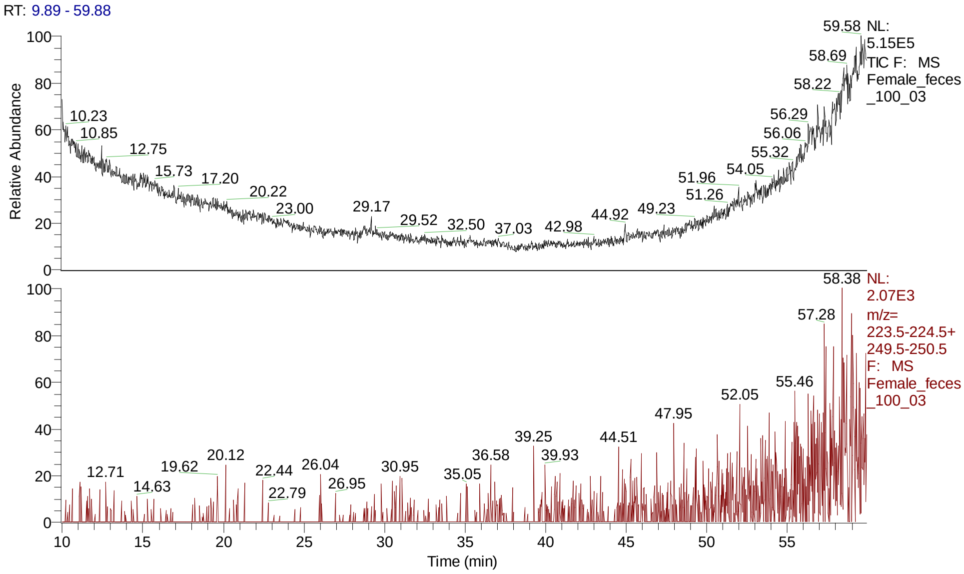

Figure 30 GC chromatogram of female small Indian civet feces

Civetone and Normuscone Contents in aromatic remedies

Civetone and normuscone were found in all aromatic remedies which claimed to use the small Indian civet secretion as an ingredient. The contents of civetone and normuscone were different in each lot number of each remedy (Table 6, 7). In addition, the concentration of normuscone was higher than civetone in all remedies.

The average concentration of civetone in aromatic remedies A, B, C, D, E, F, and G were 3.397 ± 0.531, 4.520 ± 2.085, 4.774 ± 0.663, 8.247 ± 2.778, 12.413 ± 2.869, 1.073 ± 0.213, and 3.029 ± 2.990 µg/mg of aromatic remedies respectively. Similarly, the average concentration of normuscone in aromatic remedies A, B, C, D, E, F, and G were 7.753 ± 1.151, 10.238 ± 4.087, 11.687 ± 1.877, 15.865 ± 6.880, 31.028 ± 8.849, 2.759 ± 0.620, and 6.268 ± 6.088 µg/mg of aromatic remedies respectively.

Table 6 The concentration of civetone (µg/mg) in aromatic remedies obtained by GC/MS

Aromatic remedies	**Lot number**			**Mean**	**SD**
	1	**2**	**3**		
A	3.679 ± 0.32	2.785 ± 0.169	3.727 ± 0.773	3.397	0.531
B	2.913 ± 0.263	3.772 ± 0.341	6.876 ± 0.355	4.520	2.085
C	4.259 ± 0.323	4.540 ± 0.141	5.523 ± 0.521	4.774	0.663
D	10.011 ± 1.471	5.045 ± 0.459	9.686 ± 1.502	8.247	2.778
E	15.696 ± 1.601	10.386 ± 0.713	11.158 ± 1.152	12.413	2.869
F	1.233 ± 0.084	0.830 ± 0.520	1.155 ± 0.250	1.073	0.213
G	< LOQ	3.108 ± 0.213	5.978 ± 0.321	3.029	2.990

Table 7 The concentration of normuscone (µg/mg) in aromatic remedies obtained by GC/MS

Aromatic remedies	**Lot number**			**Mean**	**SD**
	1	**2**	**3**		
A	8.225 ± 0.429	6.441 ± 0.552	8.593 ± 0.782	7.753	1.151
B	6.754 ± 0.727	9.219 ± 1.54	14.739 ± 0.799	10.238	4.087
C	10.447 ± 0.979	10.767 ± 1.257	13.846 ± 1.069	11.687	1.877
D	23.696 ± 2.701	10.792 ± 0.167	13.106 ± 2.467	15.865	6.880
E	41.040 ± 0.585	27.792 ± 2.162	24.251 ± 2.140	31.028	8.849
F	2.874 ± 0.884	2.089 ± 0.474	3.313 ± 0.186	2.759	0.620
G	< LOQ	6.644 ± 0.520	12.159 ± 0.675	6.268	6.088

CHAPTER V
DISCUSSIONS

In Thailand and some countries in Southeast Asia, small Indian civet and another civet are kept as captive animal for producing and exporting the secretion or civet coffee to the world market. Pa Noi's civet farm has been the largest civet farm in Thailand where produce the secretion to the world market. The civet secretion from this farm was around US$6,600 per kilogram [12]. It is so expensive secretion that the aromatic remedies which use the secretion as an ingredient are also high priced. The farm had an offensive smell as same as the secretion. All of the small Indian civets were kept in individual cages. The air flowed freely under the cage's legs. It had a good atmospheric environment due to healthy small Indian civets. In India, captive small Indian civets were also kept in individual wooden cages. A reed pole about 65 to 75 cm long was placed vertically in the center of the cage to facilitate secretion by perineal gland rubbing. The marked secretion from the pole was scraped out by a piece of coconut leaf. The secretory output was of 2-6 g/month in each civet [9]. From the results, maintenance and quantity of small Indian civet secretion were not different between Thai farm and Indian farm but the harvest of secretion was different. Thai civet farm used a stainless steel tea spoon to scrap the secretion from a pole while in India used a piece of coconut leaf. The small Indian civets in this farm were fed rice mixed with milk daily. Chicken, fish and dessert were supplied 2-3 times per week. Puddle frogs and house gecko were also supplied when available. The regular diet for captive small Indian civets including milk, cooked rice, and banana are in accordance with the captive small Indian civet in India [61].

Civet secretion is also called civet or civet musk. It is produced by both sexes of civet (Viverrdae). Two species are well known for civet secretion producing. They are African civet (*Civettictis civetta*), which inhabits Ethiopia, and large Indian civet (*Viverra zibetha*), found in India and Southeast Asia [11, 62]. The secretion that obtained from these two species was a semisolid, yellowish to brown, foul smelling substance and had a consistency of thick grease [11, 40, and 63]. The powerful and disagreeable of secretion smell remained detectable for at least four months [64]. The

African civet yielded more quantity of the secretion than other species of civet. It produced about 3-4 g of secretion per week [63, 65, 66]. In the present study, the properties of small Indian civet secretion were related with the secretion that produced from different species of civet. The characteristic odor of the civet secretion came from civetone [63, 65]. Both civetone and normuscone were macrocyclic ketones carrying musky odor [42, 46]. The standard civetone and normuscone which used in this study had unpleasant odor as same as the secretion. The standard civetone was more pleasant odor than normuscone. From the results, male small Indian civet secretion was stronger smell than female secretion might due to normuscone which was the main component in male secretion. In Ethiopia, African civet secretion from the male was preferred to the female secretion. The male yielded more the secretion than female. The female African civet secretion was less productive because the secretion was washed out or diluted with urine before the civet deposited the secretion on the wooden pole [11, 67, 68]. In contrast, Thai female small Indian civet secretion was preferred to the male secretion; the male secretion was darker than the female secretion because male small Indian civets urinate during deposit the secretion on the object [3]. The quantity of the small Indian civet secretion was not different between male and female secretion [12]. The small Indian civet furs sticked with the secretion were one of commercial products from Pa Noi’s civet farm but there was no report about its usage. The chemical constituents in small Indian civet furs were related with the secretion. Therefore, the small Indian civet furs may have holding properties as same as the secretion. However, the concentration of civetone and normuscone in the civet furs were considerably low. In Africa, the African civet furs were also used in some African folk medicine [69]. In *Genetta genetta* and *Genetta tigrina*, the feces have the same smell as their anal gland secretion [70]. Both smell and chemical constituents of small Indian civet feces was not related with the secretion. The small Indian civet might not digest rice thus there was broken-milled rice in the feces.

In the present study, hexane was used as solvent because of low noise and absence of interfering peaks on the chromatogram at the retention time of civetone and normuscone. External standard method was used for quantifying civetone and normuscone in the secretion of small Indian civet and in aromatic remedies because the external procedure was simple (single extraction) and extraneous peaks was in concern [71]. For the external standard method, the peak areas of the analytes in the samples are compared to the peak area of the standard solution. The calibration curves are used to determine the concentrations of the analyte in the sample. The calibration range of the standards should be around the concentration of analytes expected in the sample [72, 73]. According to AOAC guidelines, the extraction efficiency method was used for recovery evaluation of small Indian civet secretion by re-extracting the residue until exhaustion since the matrix was not homogeneous and hardly miscible with added standard solution. The precision determinations in range of expected concentrations level should not exceed 15% of RSD [74]. From this study, linear calibration curves were obtained with good correlation (r^2 = 0.9717 and 0.9965) for civetone and normuscone respectively. The average recoveries were 97.3-98.0% in secretion and 91.4-105.7% in aromatic remedy for civetone. For normuscone, average recoveries in secretion and aromatic remedy were 98.5 % and 90.0-103.0% respectively. The intra-day and inter-day RSDs of the three concentrations were less than 8%. The results demonstrated that the method was sufficiently accurate and precise for determination of civetone and normuscone in small Indian civet secretion and in aromatic remedies. Hence, GC/MS method was beneficially implemented for qualitative and quantitative analysis of civetone and normuscone in secretion from small Indian civet and in aromatic remedies.

The qualitative result showed that the secretion of small Indian civet consisted of civetone, dihydrocivetone and normuscone as main components which were related to the previous study [43]. Cyclohexadecanone was found only in the female small Indian civet secretion therefore, the results suggested that the content of chemical constituents was different between male and female small Indian civet secretion. African civet secretion contained scatone, indole, ethylamine, propylamine, some free acid, and civetone [65]. The results indicated that the chemical constituents of the secretion were different between the African civet and small Indian civet secretion. The GC analysis of macrocyclic ketones in the odoriferous gland of small Indian civet in China demonstrated that civetone was identified as the major constituent in the odoriferous gland of female civet and normuscone in that of male civet [75]. The quantitative results found that civetone was dominated in female (23.6 ± 1.5 μg/mg of secretion) whereas normuscone was dominated in male small Indian civet secretion (51.1 ± 5.9 μg/mg of secretion). This finding was in accordance with the civet in China [75]. In Pa Not's civet farm, male small Indian civets are more than female civets therefore; the secretion which obtained from this farm consisted of male secretion more than female secretion. This farm has produced the civet secretion to most traditional medicine producers throughout Thailand [12]. The results indicated that concentration of normuscone was higher than civetone in all remedies. The quantity of civetone and normuscone in aromatic remedy sample G, lot number 1 were less than LOQ might be owing to the older manufacture date. The difference in content of civetone and normuscone among each aromatic remedy might be due to specific formulary. The difference content of civetone and normuscone among each lot of same remedy might be due to the variety of each crop of civet secretion.

The SIM mode is a mode in which only of a few *m/z* values are monitored rather than monitoring all the *m/z* values in a specified interval. This mode is usually used when performing target compound analyses [76, 77]. The chromatographic signal was obtained by the sum of the MS signals due to all ions detected in the selected mass or due to selected ions when resolution in the *m/z* domain was utilized to improve inadequate resolution in the time domain. It should be emphasized that molecules of different structures exhibited different ionization yield and consequently the number of ions and the chromatographic peak areas could be different for different compounds present in equal molar ratio [78]. In present study, the peak at the retention time around 46.80 min corresponded to civetone, whose EI mass spectrum was characterized by the presence of high abundant ions at *m/z* 250. The abundant ions of civetone was higher than normuscone (*m/z* = 224) that presented in the peak at the retention time around 39.90 min. In the SIM mode, the selection of the ions at *m/z* 250 and 224 led a chromatogram with only the peaks of civetone and normuscone. The civetone peak was higher than peak of normuscone in the SIM mode because of its abundant ions. From this study, the full scan chromatogram of most aromatic remedies showed the small peaks of civetone and normuscone because the concentration was too low. The SIM mode was sensitive than full scan mode because all scan time was used to focus on a specific *m/z* rather than a broad mass range in full scan mode. Therefore the SIM mode was useful for confirmation of civetone and normuscone contents in aromatic remedies.

In Ethiopia, African civet secretion intended for the export would be controlled for the percentage of civetone contents. If civetone contents were less than 40%, export would not be allowed [11]. However, there have been a few studies about the analysis of the chemical constituents in civet secretion. It is important to point that this present study represents the first report on the quantitative analysis of civetone and normuscone in small Indian civet secretion and in aromatic remedies in Thailand by GC/MS.

CONCLUSION AND RECOMMEMDATION

The small Indian civet secretion has been well known as the fragrance ingredient and the traditional medicine ingredient for a long time. Aromatic remedies are the Thai ancient remedies that commonly used for relief of faint. Both small Indian civet secretion and aromatic remedies with civet secretion ingredient were investigated for quantitation of civetone and normuscone by GC/MS method. There were three main of macrocyclic ketone constituents in the secretion and furs of Thai small Indian civets including civetone, normuscone and dihydrocivetone. Sex dependent difference in chemical constituents of Thai small Indian civet secretion was demonstrated and should be concerned for their application. The contents of civetone and normuscone in commercial civet secretion depended on male and female sex ratio of the small Indian civet. GC/MS method was found to be precise and accurate for civetone and normuscone determination in small Indian civet secretion as well as in aromatic remedies. Civetone and normuscone could be used as quantitatively marker for civet secretion ingredient in aromatic remedies. Further researches are needed to establish the bioactivity and toxicity of the small Indian civet secretion.

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APPENDICES

APPENDIX A

Data of Civetone and Normuscone Determination

Formulas

- LOD = mean of blank sample + 3SD
- LOQ = mean of blank sample + 10SD
- RSD % = (SD/mean) x 100
- Recovery % of small Indian civet secretion = (C_a x 100)/C_s

    C_a: concentration in the first filtrated

    C_s: concentration in sum of filtrated
- Recovery % of aromatic remedies = [(A_s-A)/A_a] x 100

    A_s: amount of civetone or normuscone that found after spiking of the standard solution

    A: amount of those found that before spiking

    A_a: amount of reference standards actually added to the sample

Table 8 Percent Areas of Male Small Indian Civet Secretion Constituents

No. of secretion	**Area %**		
	Normucone	**Dihydrocivetone**	**civetone**
Male1	78.20	3.19	2.71
Male2	80.87	4.75	3.44
Male3	83.48	3.24	1.35
Male4	81.19	5.19	1.39
Male5	77.33	5.08	2.17
Male6	69.04	4.44	2.87
Male7	74.70	3.80	2.36
Male8	67.77	4.56	3.14
Male9	77.96	4.92	2.16
Male10	71.28	5.08	1.07
Male11	79.25	3.17	2.49
Male12	58.94	6.92	4.69
Male13	67.52	8.08	4.46
Male14	66.77	7.49	7.62
Male15	67.28	6.27	6.81
Mean	**73.44**	**5.08**	**3.25**
SD	**7.0**	**1.5**	**1.9**

Table 9 Percent Areas of Female Small Indian Civet Secretion Constituents

No. of secretion	**Area %**			
	Normuscone	**Cyclohexadecanone**	**Dihydrocivetone**	**civetone**
Female1	12.07	3.43	12.29	61.90
Female2	10.78	3.23	15.23	58.57
Female3	8.69	2.30	14.12	64.47
Female4	10.11	2.23	18.67	60.45
Female5	12.34	2.62	20.02	58.41
Female6	14.15	4.48	20.59	46.30
Female7	10.30	3.43	14.19	59.66
Female8	12.63	3.14	13.63	57.52
Female9	11.32	3.34	16.53	52.43
Female10	9.40	3.10	16.62	56.02
Female11	15.52	3.09	20.86	49.82
Female12	14.83	4.29	17.16	52.25
Female13	9.49	3.43	17.11	53.46
Female14	11.73	2.64	13.71	60.48
Female15	13.67	3.88	21.16	50.15
Mean	**11.80**	**3.24**	**16.79**	**56.13**
SD	**2.1**	**0.6**	**2.9**	**5.2**

Table 10 Percent Areas of Pooled Small Indian Civet Secretion Constituents

No. of secretion	**Area %**			
	Normuscone	**Cyclohexadecanone**	**Dihydrocivetone**	**civetone**
Pooled 1	46.71	3.37	15.31	34.61
Pooled 2	48.10	3.60	14.70	33.60
Pooled 3	44.26	3.93	15.82	35.99
Pooled 4	44.83	3.52	16.32	35.33
Pooled 5	46.28	3.35	16.20	34.17
Pooled 6	45.31	3.26	16.55	34.88
Pooled 7	44.83	3.41	16.08	35.69
Pooled 8	47.43	3.47	14.49	34.62
Pooled 9	45.54	4.08	15.34	35.04
Pooled 10	44.82	3.88	15.77	35.53
Mean	**45.81**	**3.59**	**15.66**	**34.95**
SD	**1.3**	**0.3**	**0.7**	**0.7**

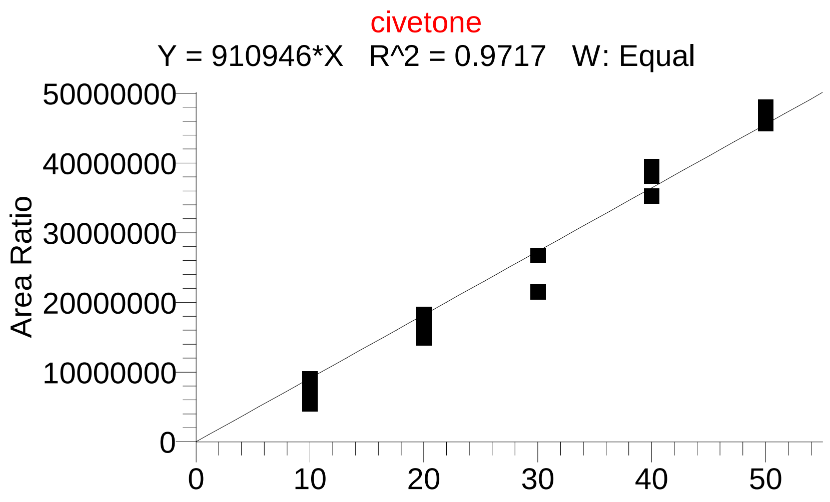

Figure 31 Calibration curve for civetone determination by GC/MS

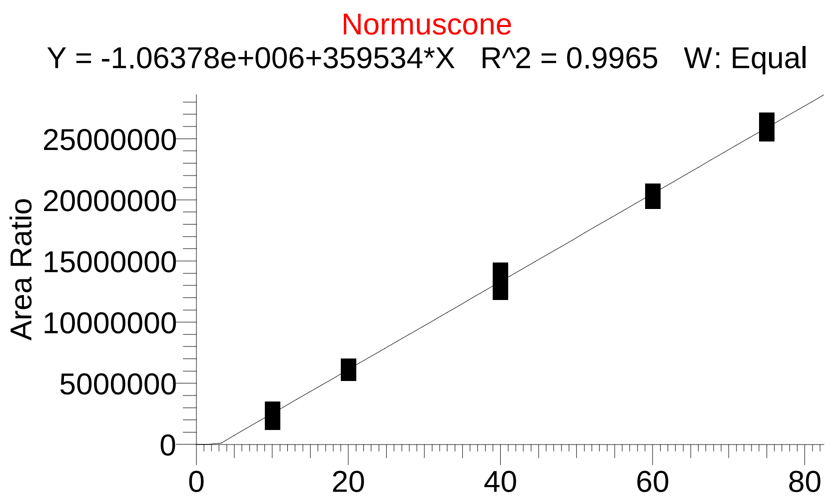

Figure 32 Calibration curve for normuscone determination by GC/MS

Table 11 Civetone and Normuscone Contents (µg/mg of secretion) in Male Small Indian Civet (n = 3)

No. of male secretion	Cvetone	Normuscone
1	0.589 ± 0.042	55.718 ± 9.393
2	1.344 ± 0.110	70.301 ± 6.064
3	0.771 ± 0.157	73.673 ± 3.129
4	0.500 ± 0.063	41.550 ± 1.863
5	0.809 ± 0.062	65.956 ± 5.200
6	0.928 ± 0.346	56.586 ± 3.338
7	0.636 ± 0.122	72.583 ± 11.657
8	1.350 ± 0.249	64.993 ± 6.787
9	0.430 ± 0.071	48.526 ± 9.286
10	0.303 ± 0.057	33.246 ± 2.677
11	0.489 ± 0.062	44.277 ± 4.856
12	0.941 ± 0.064	36.674 ± 4.139
13	1.031 ± 0.094	68.207 ± 2.185
14	1.194 ± 0.094	31.627 ± 4.729
15	0.504 ± 0.101	17.893 ± 2.839
Grand mean	**0.788 ± 0.138**	**52.121 ± 5.931**

Table 12 Civetone and Normuscone Contents (µg/mg of secretion) in Female Small Indian Civet (n = 3)

No. of male secretion	Cvetone	Normuscone
1	28.584 ± 2.761	21.661 ± 2.102
2	21.364 ± 1.100	16.116 ± 1.297
3	25.781 ± 1.054	13.787 ± 0.891
4	28.817 ± 1.762	20.466 ± 1.880
5	23.744 ± 2.268	20.236 ± 2.461
6	18.262 ± 0.945	20.494 ± 2.179
7	26.246 ± 1.162	18.640 ± 0.483
8	25.090 ± 1.023	20.353 ± 0.875
9	22.788 ± 1.435	18.413 ± 1.306
10	24.858 ± 1.237	16.520 ± 1.458
11	24.659 ± 0.290	27.246 ± 0.958
12	18.999 ± 0.567	19.734 ± 0.219
13	18.101 ± 1.050	13.652 ± 0.667
14	27.850 ± 0.794	22.821 ± 2.052
15	19.067 ± 2.273	18.135 ± 2.444
Grand mean	**23.614 ± 1.469**	**19.218 ±1.584**

Table 13 Civetone and Normuscone Contents (µg/mg of aromatic remedy) in Aromatic Remedy A

Normuscone	**Lot 1**	**Lot 2**	**Lot 3**
1	8.251	6.092	8.004
2	8.640	6.154	8.294
3	7.784	7.077	9.481
Mean	**8.225**	**6.441**	**8.593**
SD	**0.429**	**0.552**	**0.782**

Civetone	**Lot 1**	**Lot 2**	**Lot 3**
1	3.652	2.701	3.305
2	3.714	2.674	3.258
3	3.671	2.979	4.619
Mean	**3.679**	**2.785**	**3.727**
SD	**0.032**	**0.169**	**0.773**

Table 14 Civetone and Normuscone Contents (µg/mg of aromatic remedy) in Aromatic Remedy B

Normuscone	**Lot 1**	**Lot 2**	**Lot 3**
1	6.218	7.645	15.545
2	6.464	9.349	13.948
3	7.581	10.664	14.725
Mean	**6.754**	**9.219**	**14.739**
SD	**0.727**	**1.514**	**0.799**

Civetone	**Lot 1**	**Lot 2**	**Lot 3**
1	2.811	4.132	6.482
2	2.717	3.453	6.975
3	3.212	3.732	7.171
Mean	**2.913**	**3.772**	**6.876**
SD	**0.263**	**0.341**	**0.355**

Table 15 Civetone and Normuscone Contents (µg/mg of aromatic remedy) in Aromatic Remedy C

Normuscone/4	Lot 1	Lot 2	Lot 3
1	10.163	9.482	12.873
2	11.537	10.827	13.674
3	9.641	11.994	14.991
Mean	**10.447**	**10.767**	**13.846**
SD	**0.979**	**1.257**	**1.069**

Civetone	Lot 1	Lot 2	Lot 3
1	4.336	4.448	4.957
2	4.538	4.702	5.632
3	3.905	4.469	5.981
Mean	**4.259**	**4.540**	**5.523**
SD	**0.323**	**0.141**	**0.521**

Table 16 Civetone and Normuscone Contents (µg/mg of aromatic remedy) in Aromatic Remedy D

Normuscone	**Lot 1**	**Lot 2**	**Lot 3**
1	20.599	10.628	12.983
2	24.929	10.961	15.632
3	25.561	10.788	10.704
Mean	**23.696**	**10.792**	**13.106**
SD	**2.701**	**0.167**	**2.467**

Civetone	**Lot 1**	**Lot 2**	**Lot 3**
1	8.313	4.772	10.241
2	10.821	5.575	10.831
3	10.901	4.789	7.985
Mean	**10.011**	**5.045**	**9.686**
SD	**1.471**	**0.459**	**1.502**

Table 17 Civetone and Normuscone Contents (μg/mg of aromatic remedy) in Aromatic Remedy E

Normuscone	Lot 1	Lot 2	Lot 3
1	40.675	25.978	21.963
2	41.714	30.184	24.589
3	40.730	27.215	26.202
Mean	**41.040**	**27.792**	**24.251**
SD	**0.585**	**2.162**	**2.140**

Civetone	Lot 1	Lot 2	Lot 3
1	16.443	9.971	9.930
2	13.858	11.210	11.332
3	16.787	9.979	12.214
Mean	**15.696**	**10.386**	**11.158**
SD	**1.601**	**0.713**	**1.152**

Table 18 Civetone and Normuscone Contents (µg/mg of aromatic remedy) in Aromatic Remedy F

Normuscone	**Lot 1**	**Lot 2**	**Lot 3**
1	3.040	1.542	3.134
2	3.663	2.385	3.300
3	1.919	2.341	3.506
Mean	**2.874**	**2.089**	**3.313**
SD	**0.884**	**0.474**	**0.186**

Civetone	**Lot 1**	**Lot 2**	**Lot 3**
1	1.328	0.386	0.866
2	1.169	0.702	1.316
3	1.202	1.403	1.281
Mean	**1.233**	**0.830**	**1.155**
SD	**0.084**	**0.520**	**0.250**

Table 19 Civetone and Normuscone Contents (µg/mg of aromatic remedy) in Aromatic Remedy G

Normuscone	**Lot 1**	**Lot 2**	**Lot 3**
1	< LOQ	6.161	12.431
2	< LOQ	6.579	11.391
3	< LOQ	7.194	12.657
Mean	**-**	**6.644**	**12.159**
SD	**-**	**0.520**	**0.675**

Civetone	**Lot 1**	**Lot 2**	**Lot 3**
1	< LOQ	2.893	5.906
2	< LOQ	3.114	5.699
3	< LOQ	3.318	6.329
Mean	**-**	**3.108**	**5.978**
SD	**-**	**0.213**	**0.321**

APPENDIX B

GC Chromatogram and Mass Spectrum

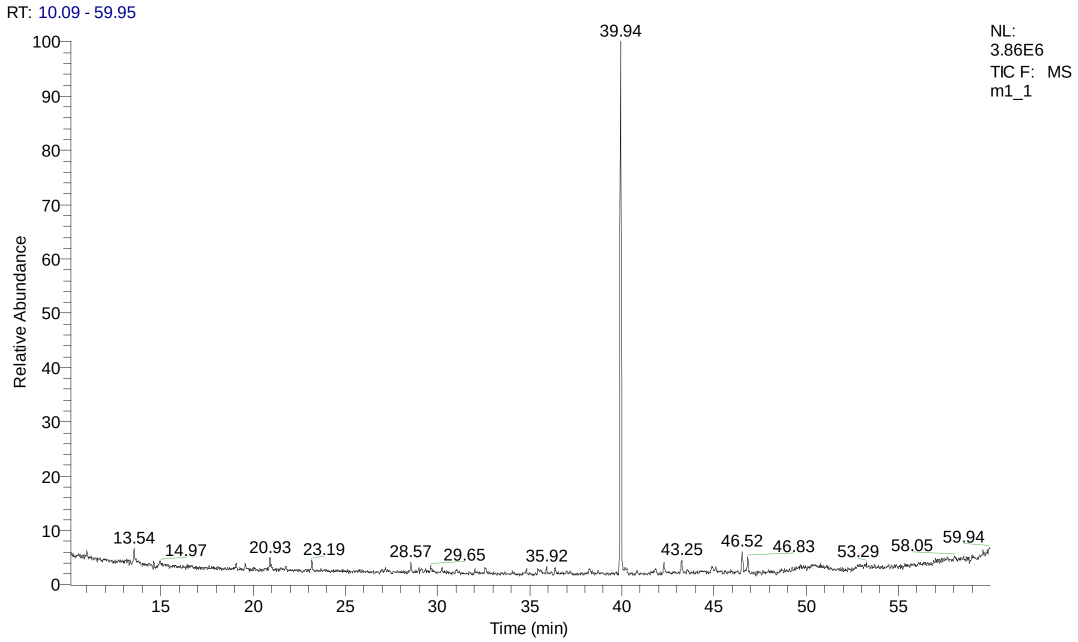

Figure 33 GC chromatogram of male small Indian civet secretion (1)

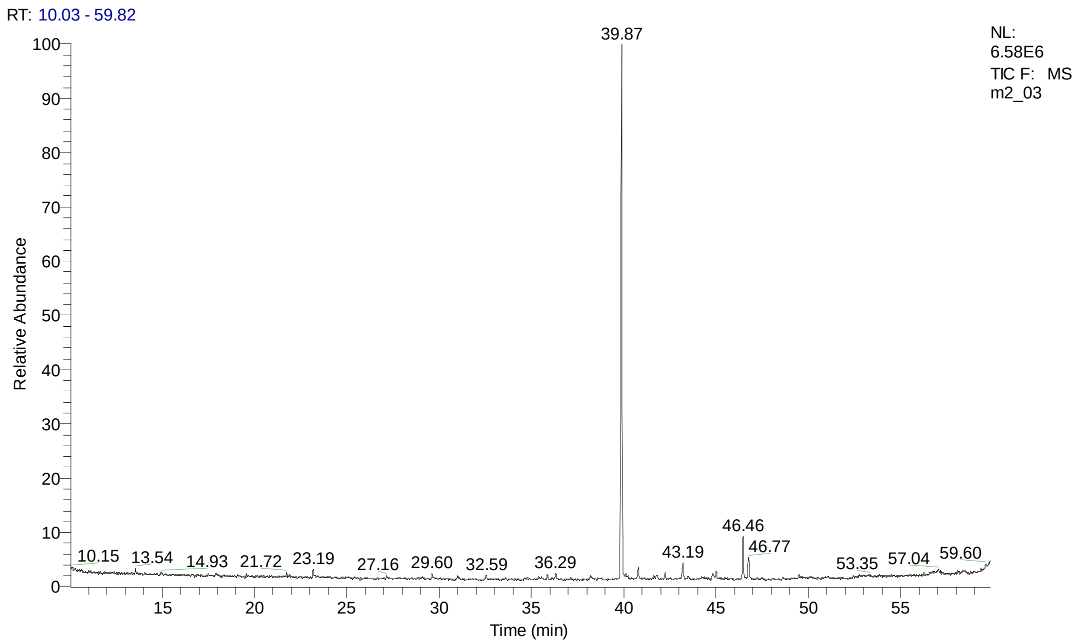

Figure 34 GC chromatogram of male small Indian civet secretion (2)

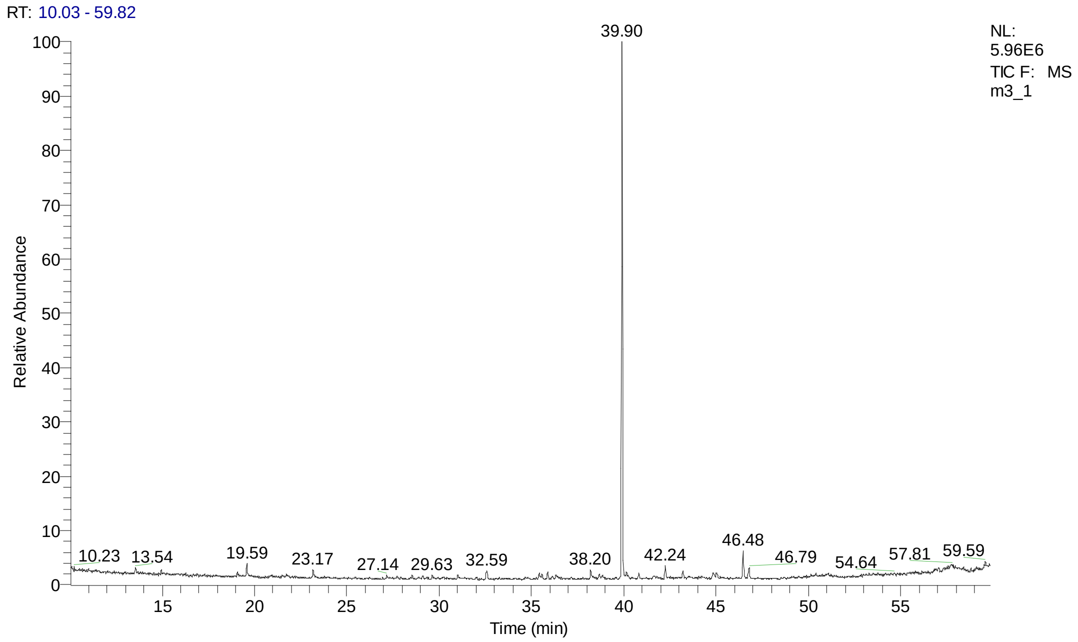

Figure 35 GC chromatogram of male small Indian civet secretion (3)

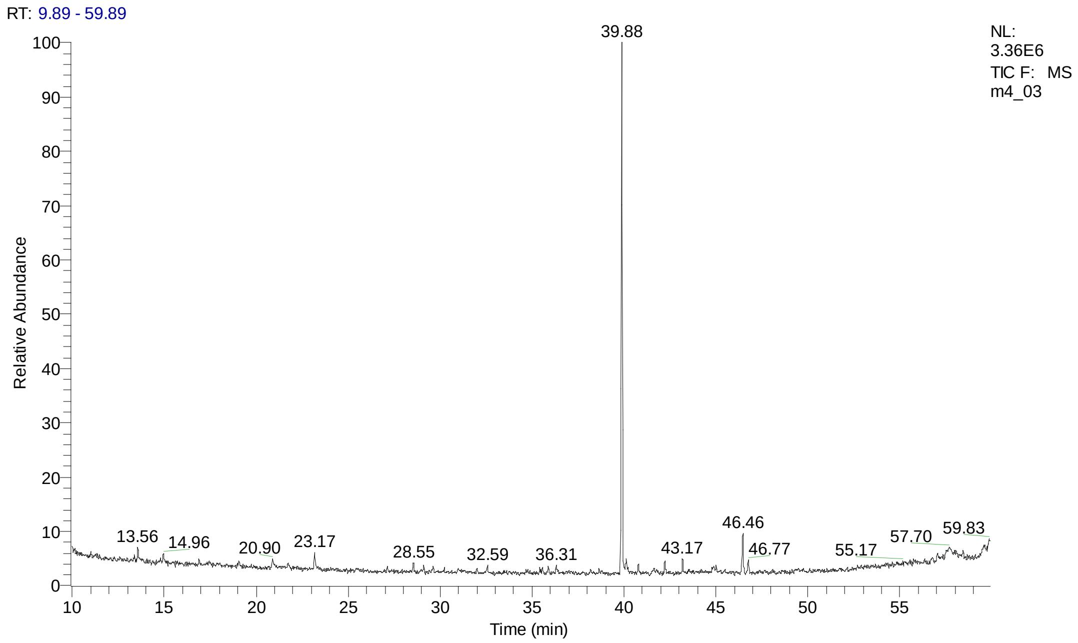

Figure 36 GC chromatogram of male small Indian civet secretion (4)

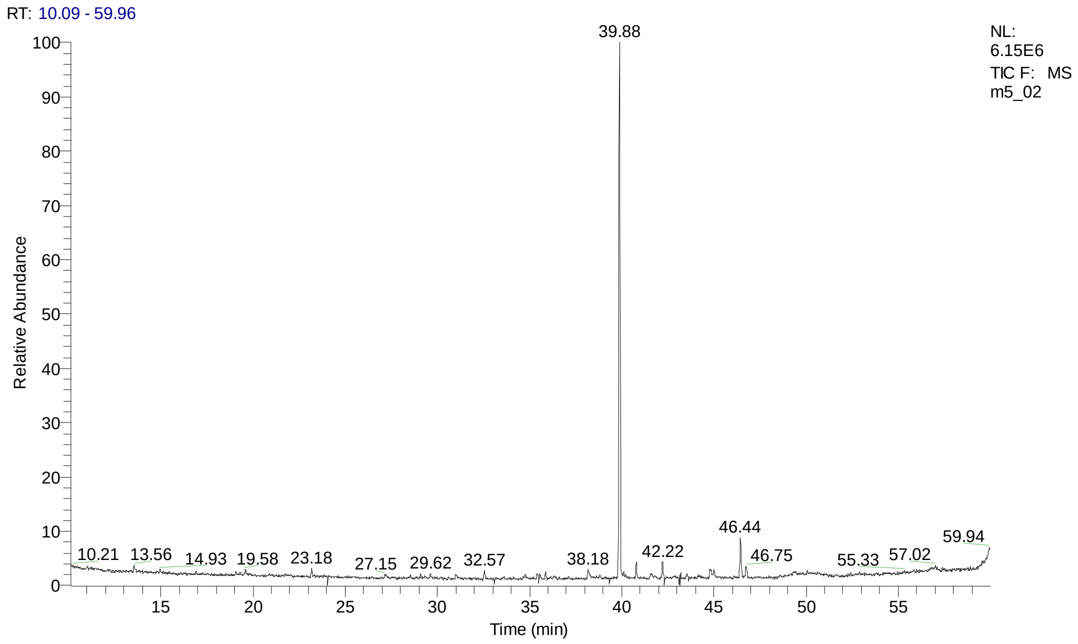

Figure 37 GC chromatogram of male small Indian civet secretion (5)

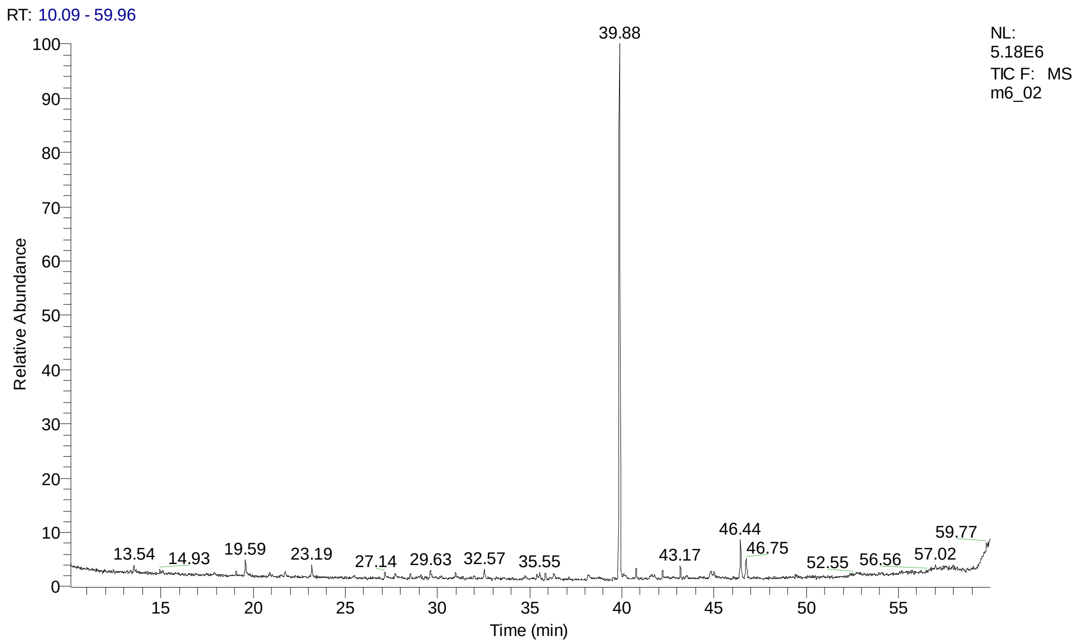

Figure 38 GC chromatogram of male small Indian civet secretion (6)

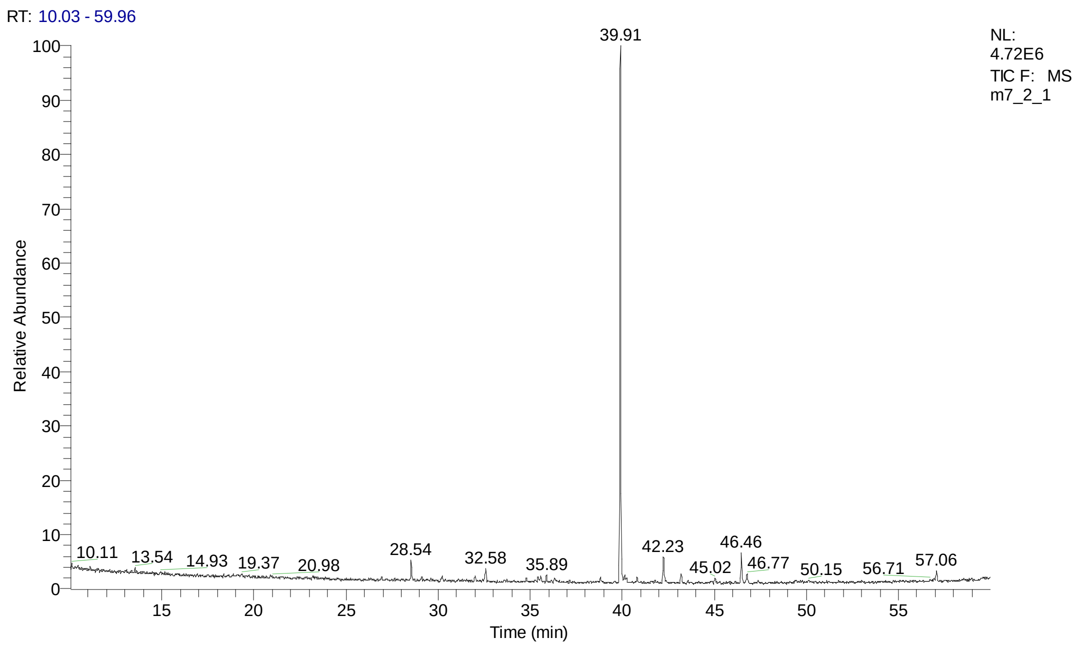

Figure 39 GC chromatogram of male small Indian civet secretion (7)

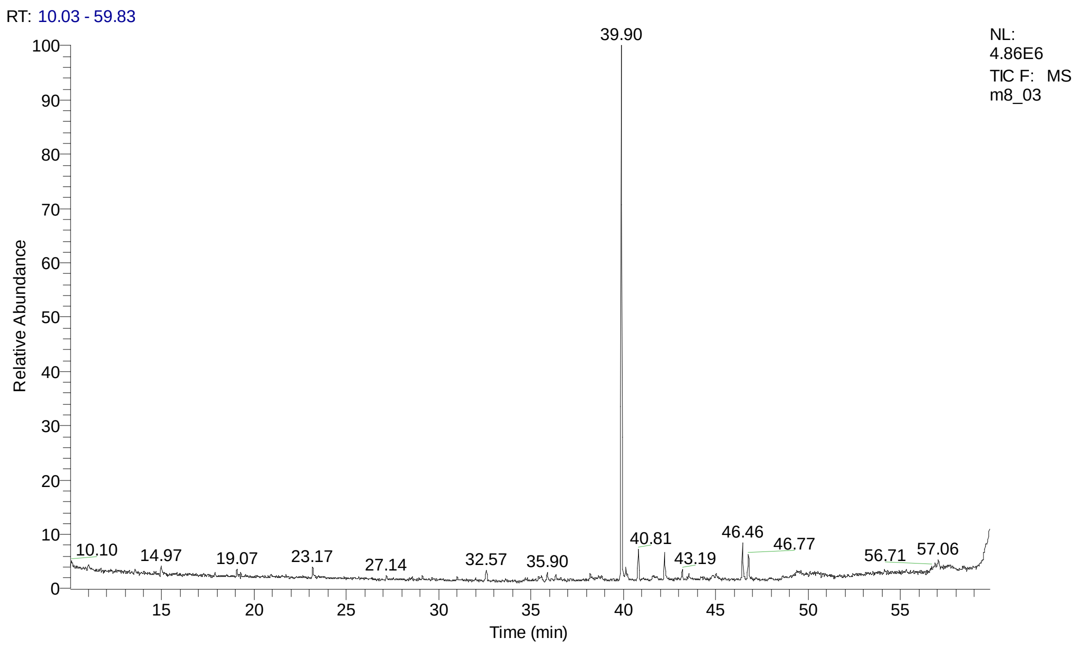

Figure 40 GC chromatogram of male small Indian civet secretion (8)

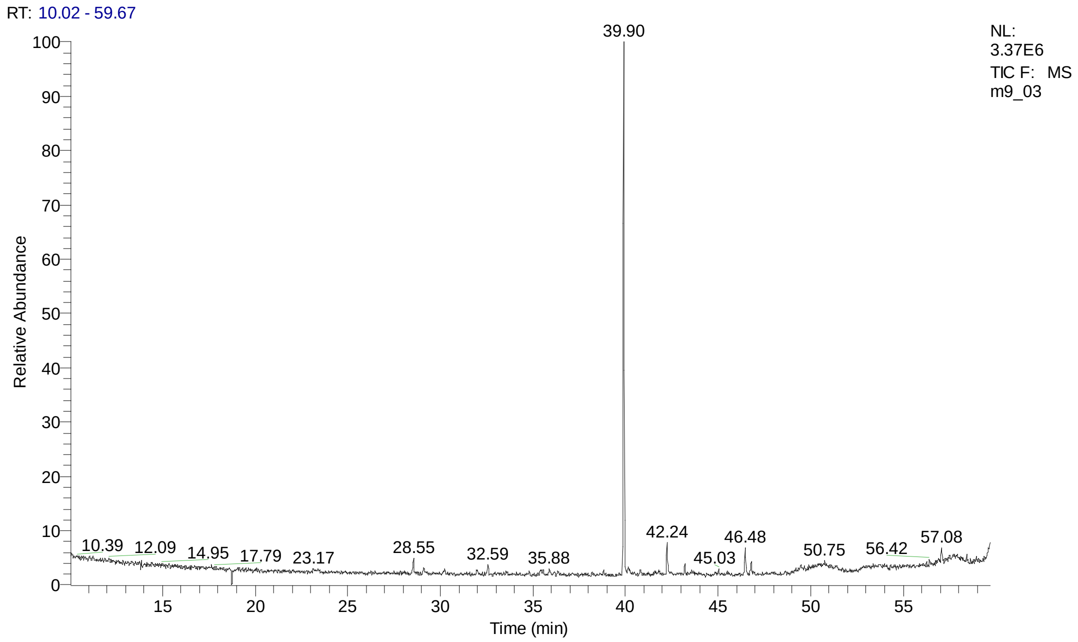

Figure 41 GC chromatogram of male small Indian civet secretion (9)

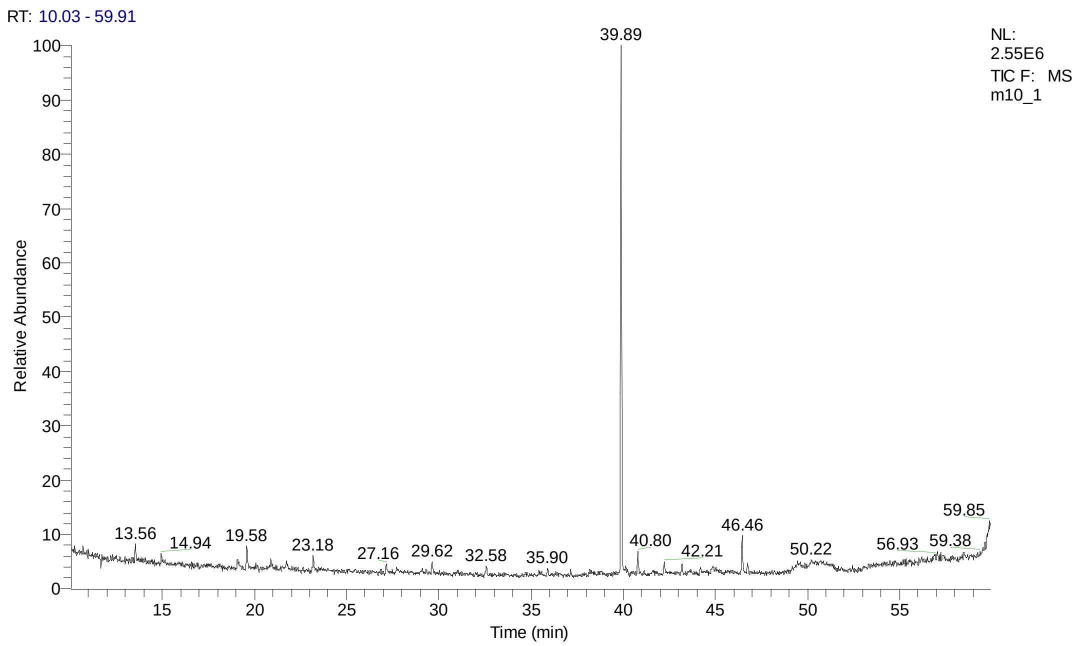

Figure 42 GC chromatogram of male small Indian civet secretion (10)

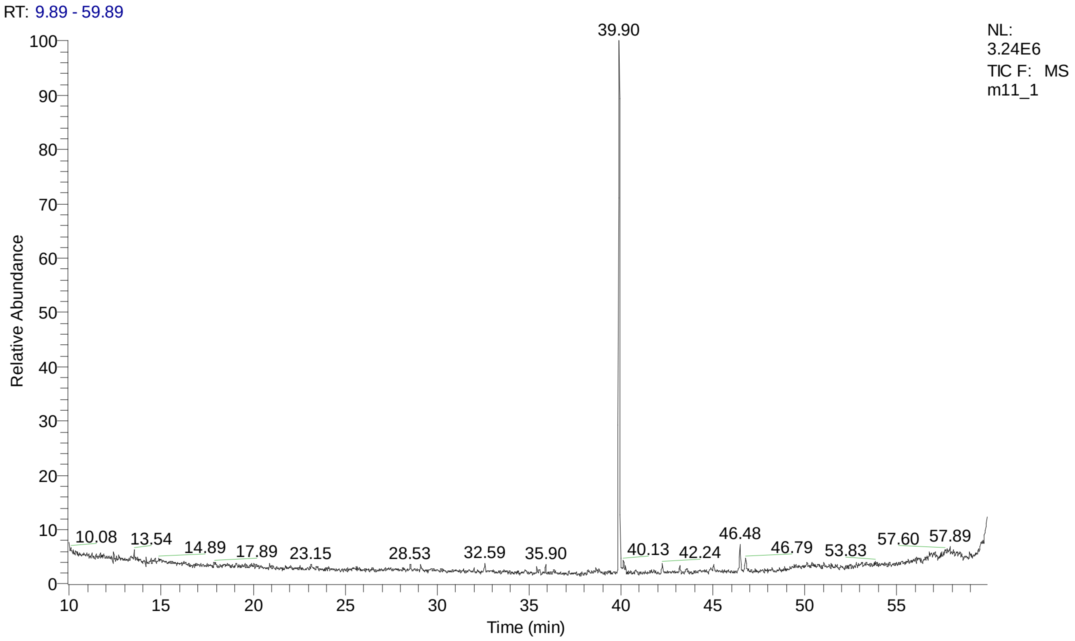

Figure 43 GC chromatogram of male small Indian civet secretion (11)

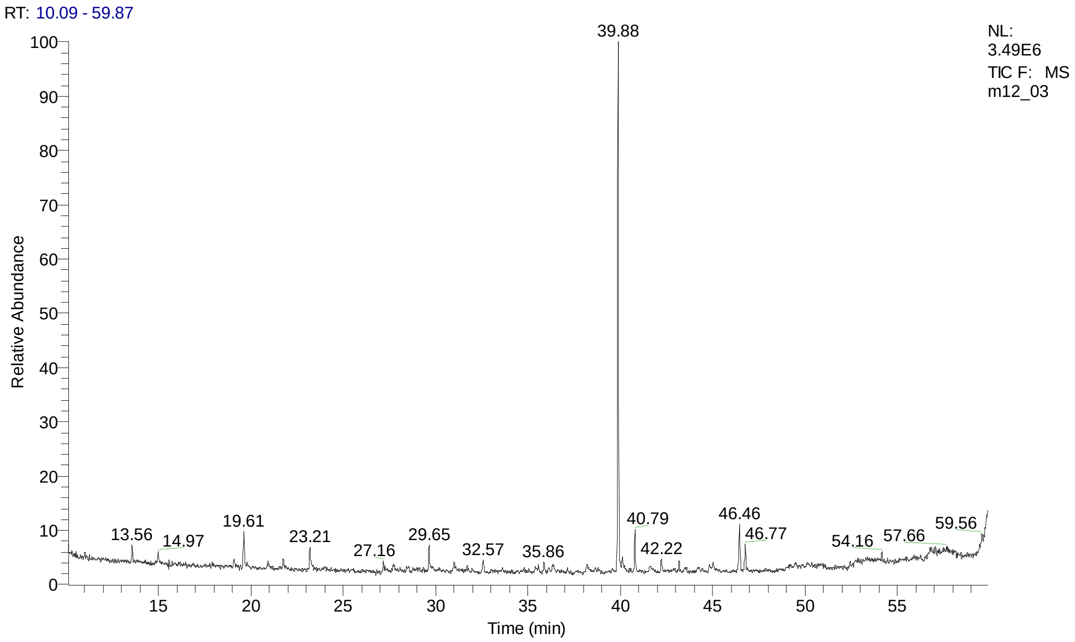

Figure 44 GC chromatogram of male small Indian civet secretion (12)

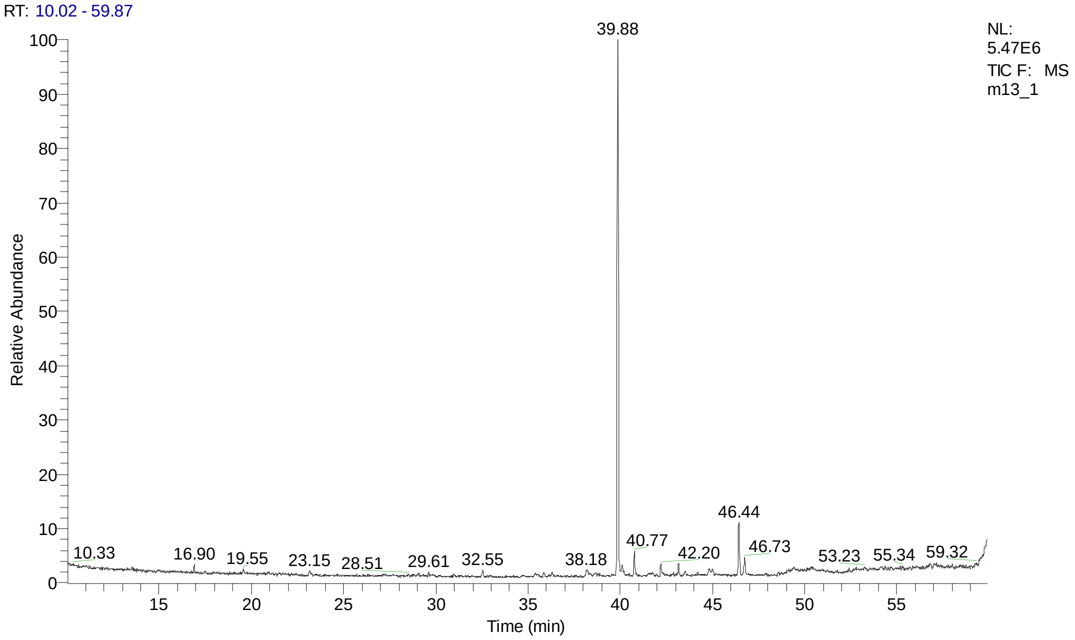

Figure 45 GC chromatogram of male small Indian civet secretion (13)

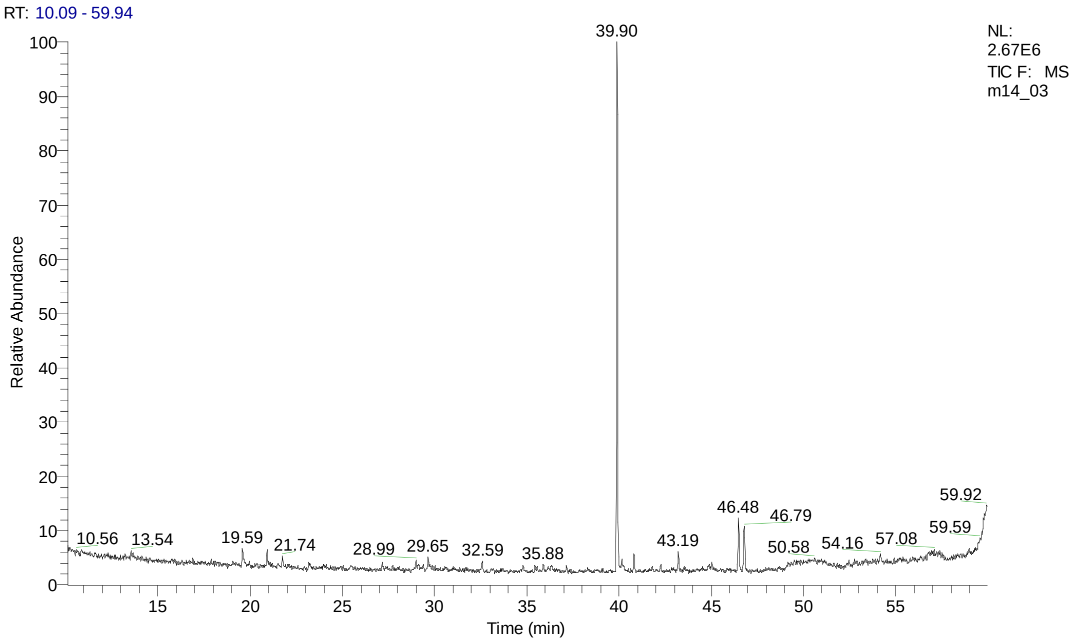

Figure 46 GC chromatogram of male small Indian civet secretion (14)

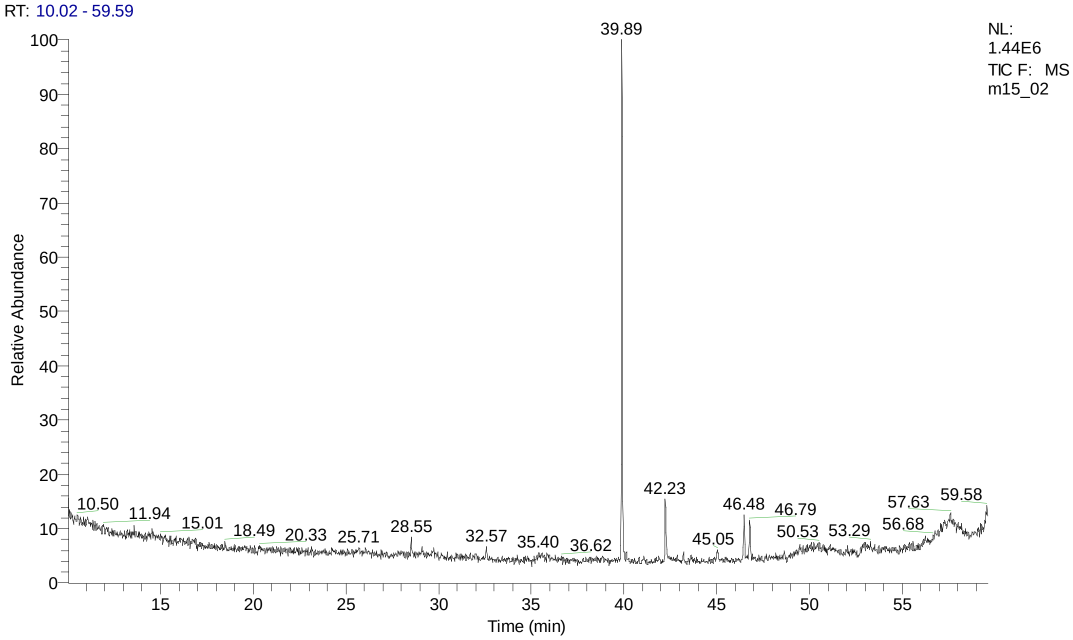

Figure 47 GC chromatogram of male small Indian civet secretion (15)

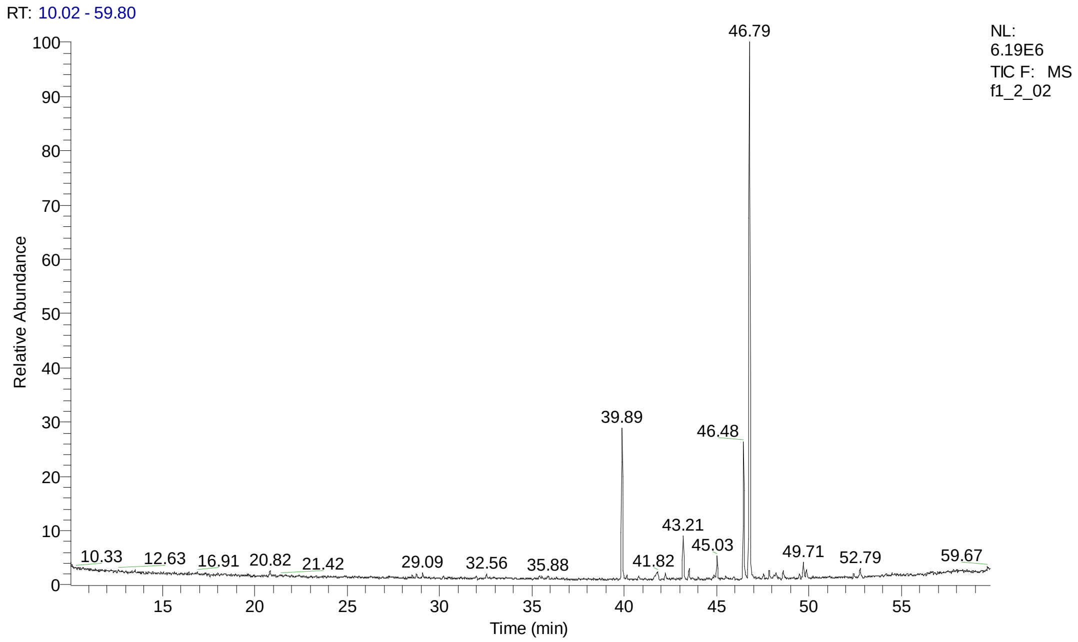

Figure 48 GC chromatogram of female small Indian civet secretion (1)

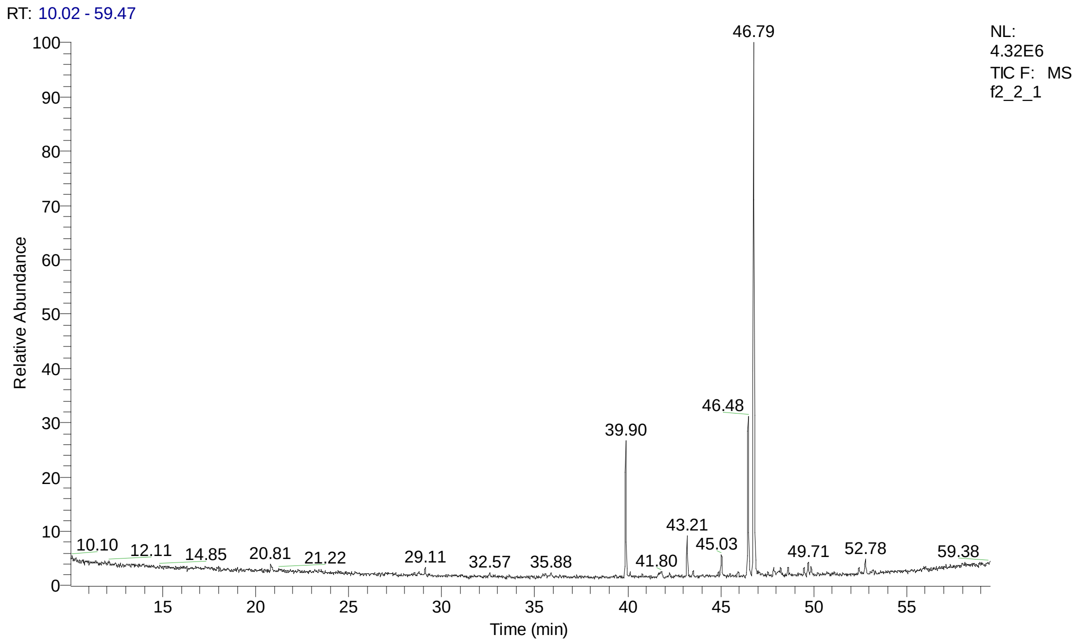

Figure 49 GC chromatogram of female small Indian civet secretion (2)

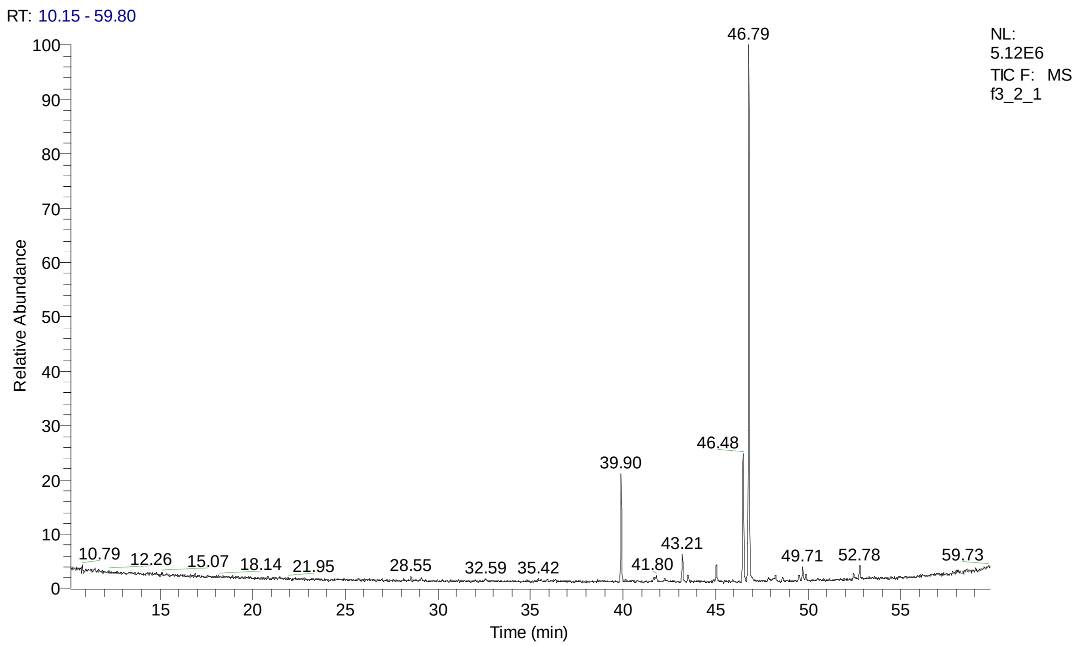

Figure 50 GC chromatogram of female small Indian civet secretion (3)

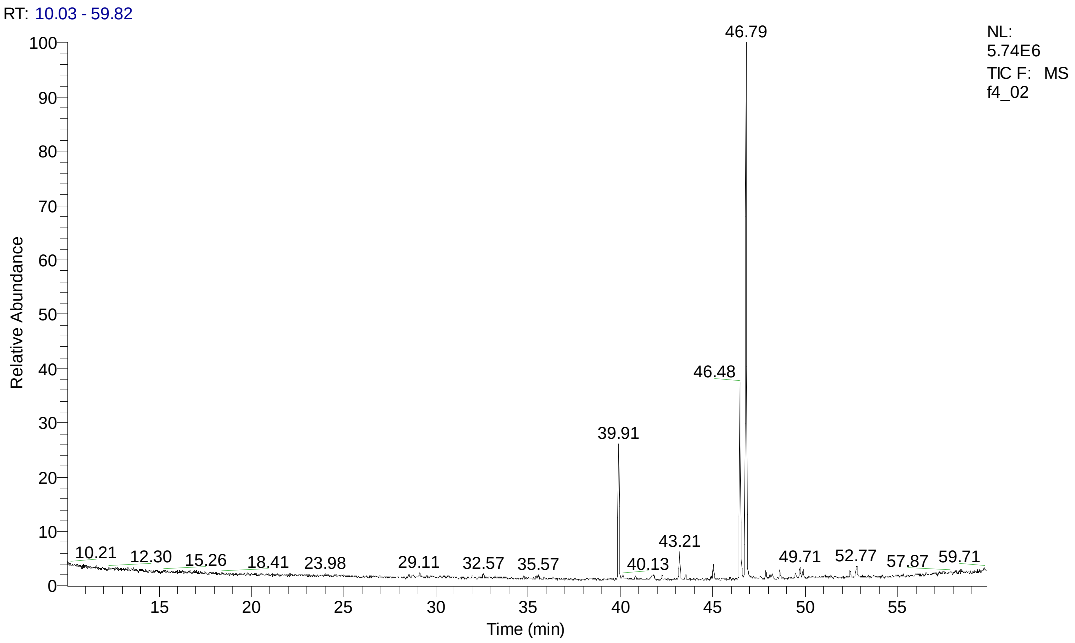

Figure 51 GC chromatogram of female small Indian civet secretion (4)

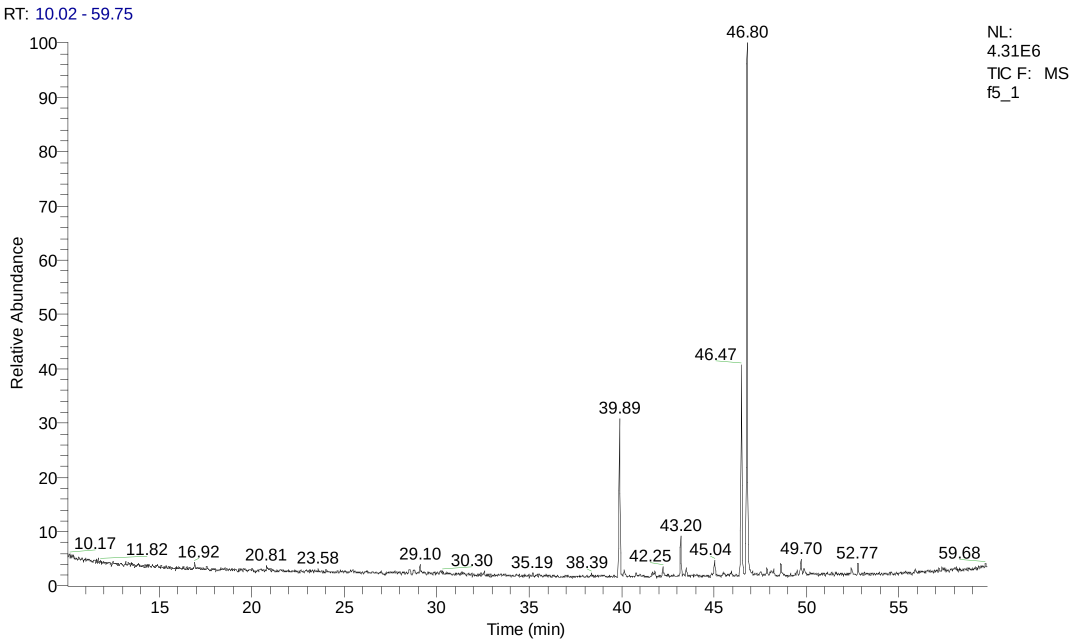

Figure 52 GC chromatogram of female small Indian civet secretion (5)

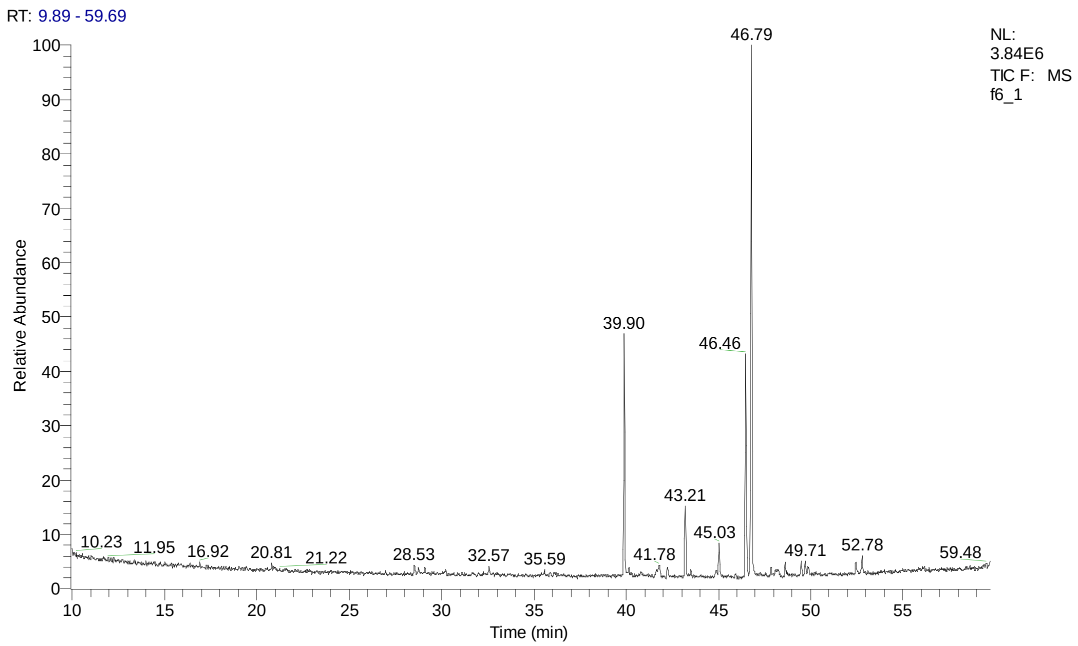

Figure 53 GC chromatogram of female small Indian civet secretion (6)

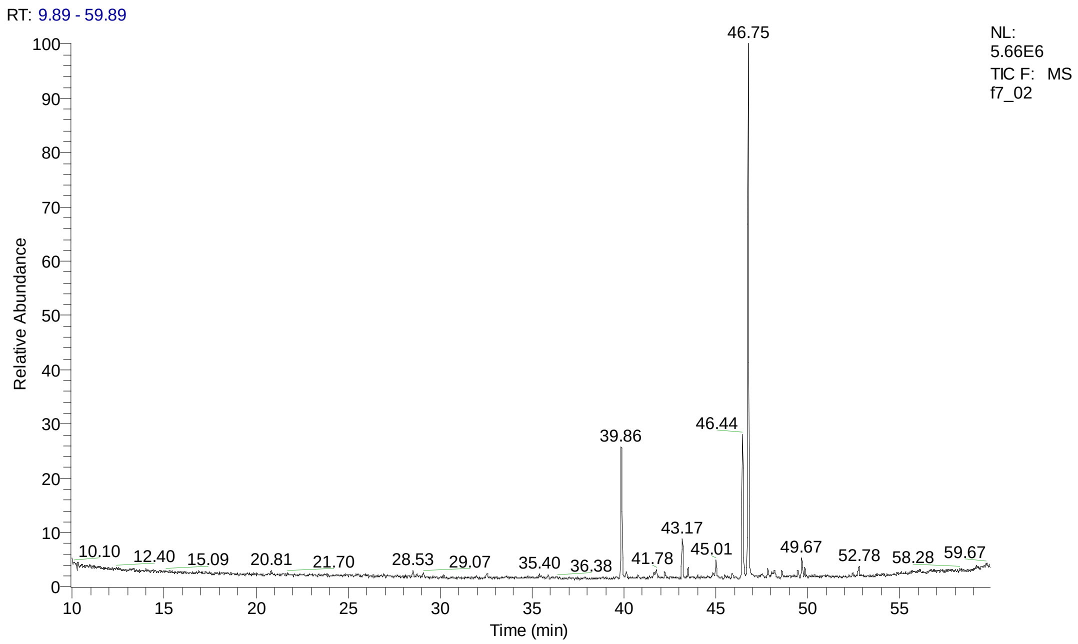

Figure 54GC chromatogram of female small Indian civet secretion (7)

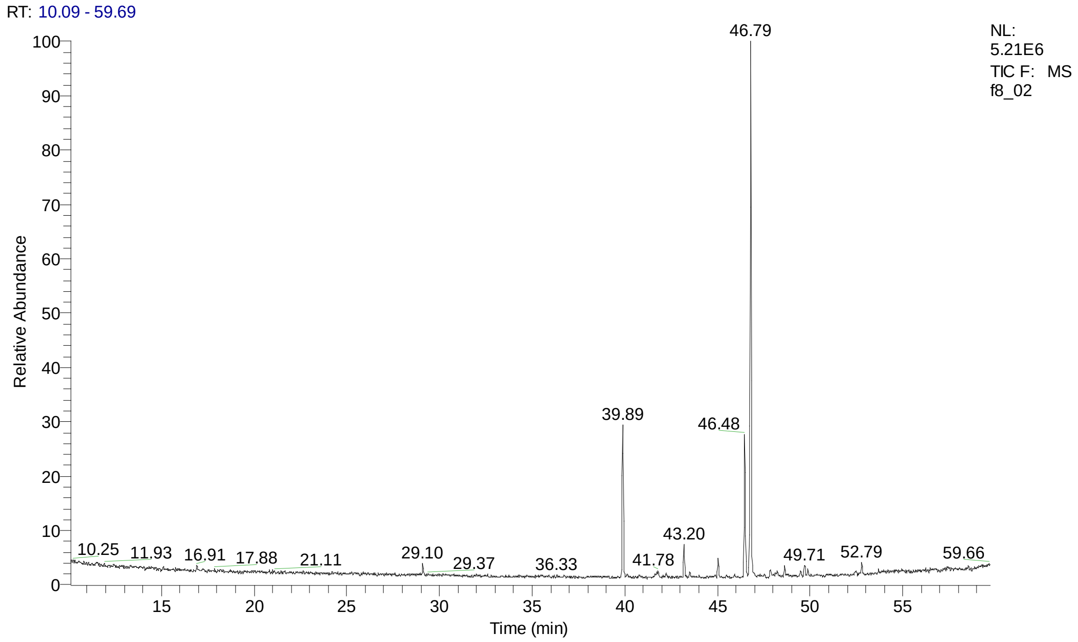

Figure 55 GC chromatogram of female small Indian civet secretion (8)

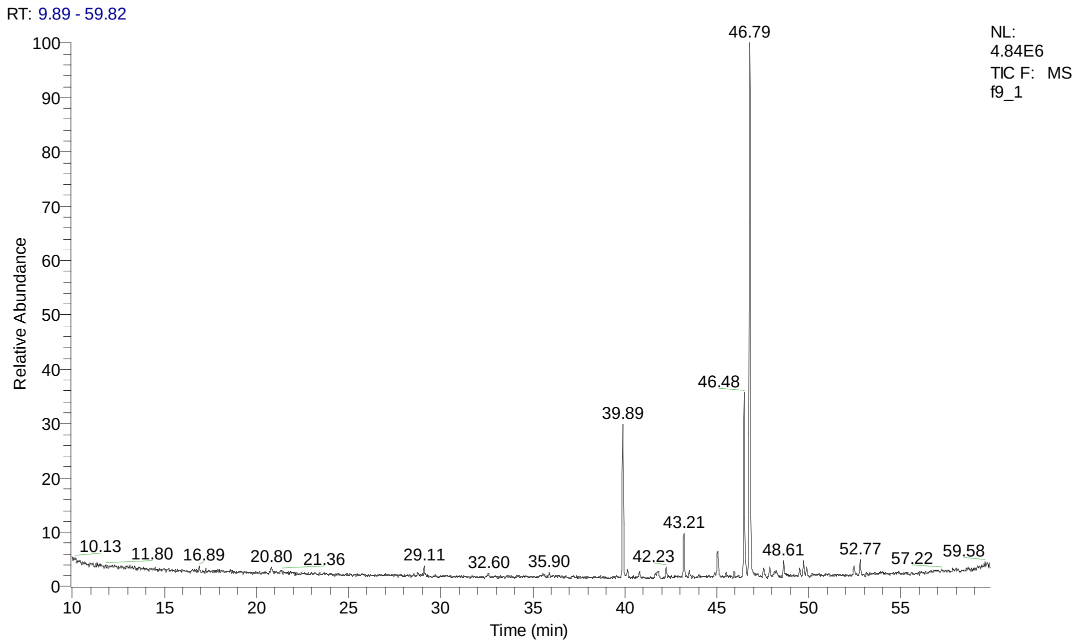

Figure 56 GC chromatogram of female small Indian civet secretion (9)

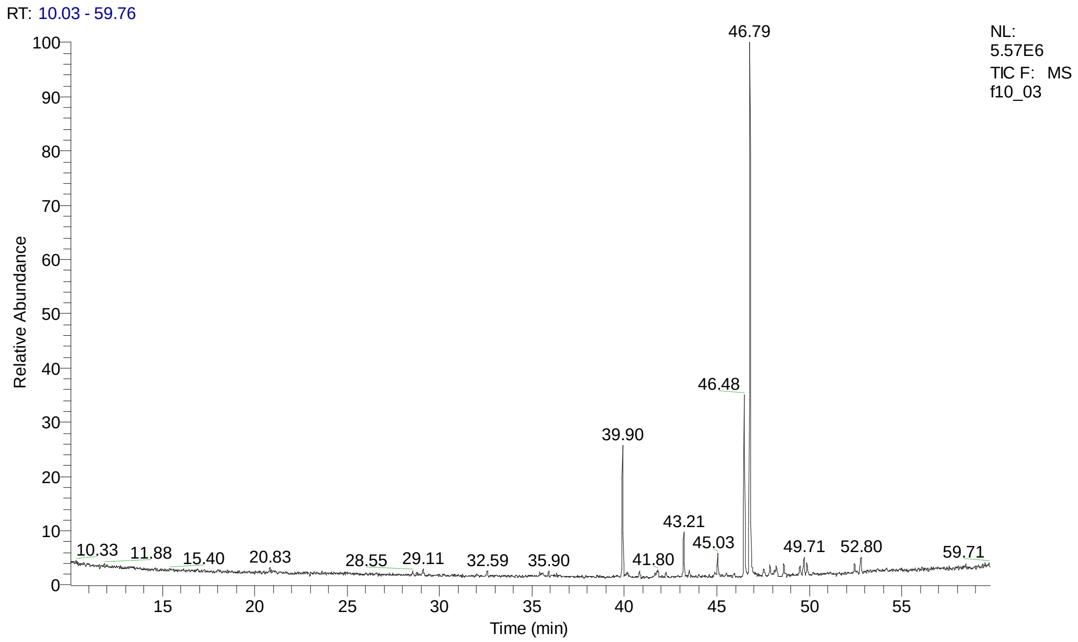

Figure 57 GC chromatogram of female small Indian civet secretion (10)

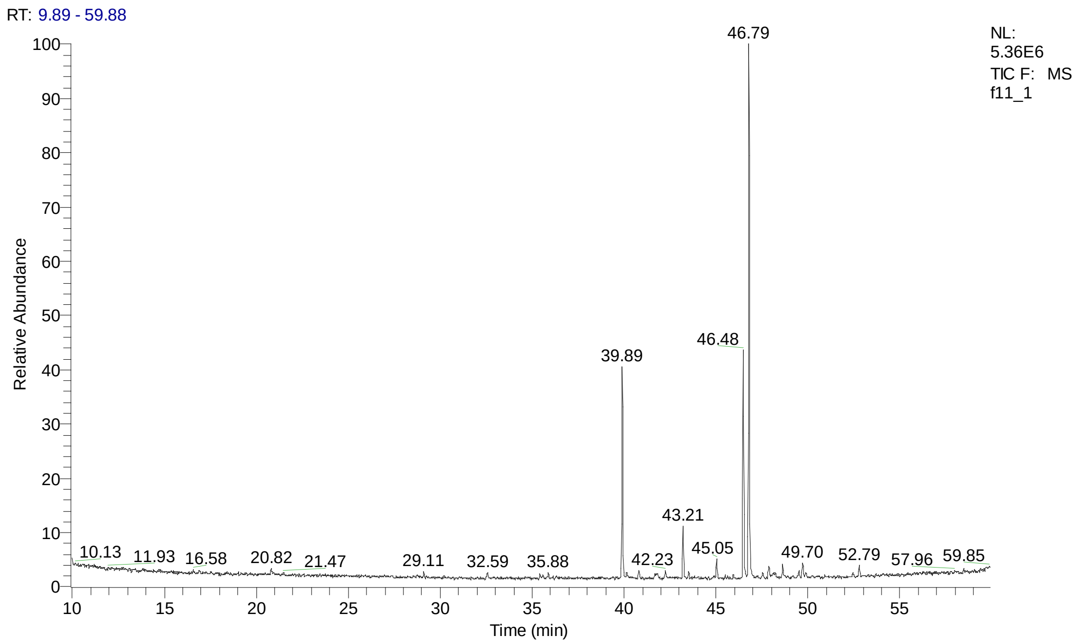

Figure 58 GC chromatogram of female small Indian civet secretion (11)

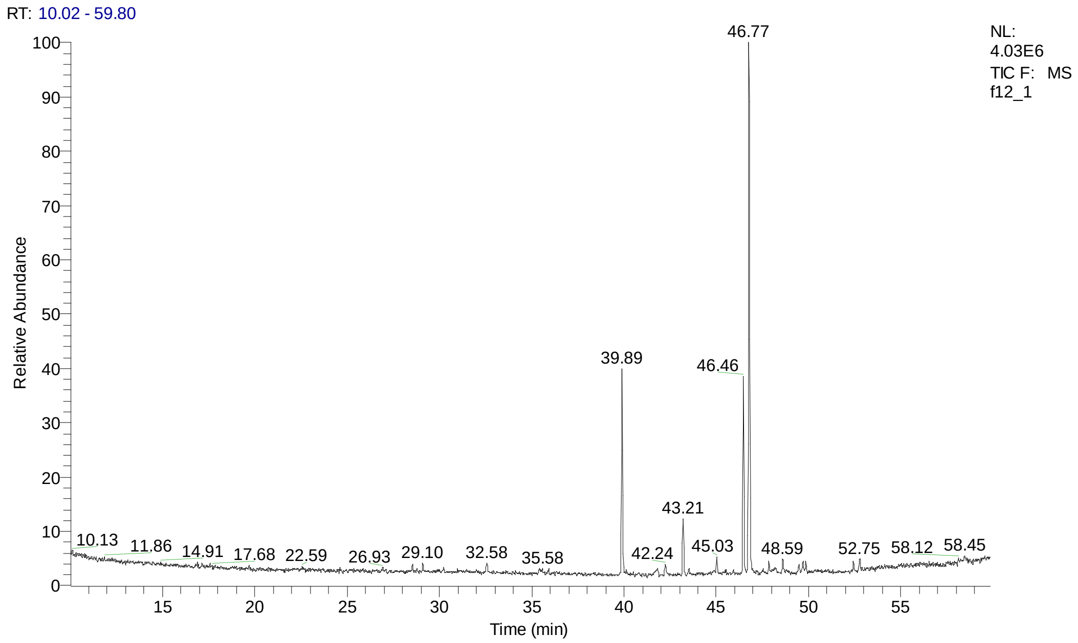

Figure 59 GC chromatogram of female small Indian civet secretion (12)

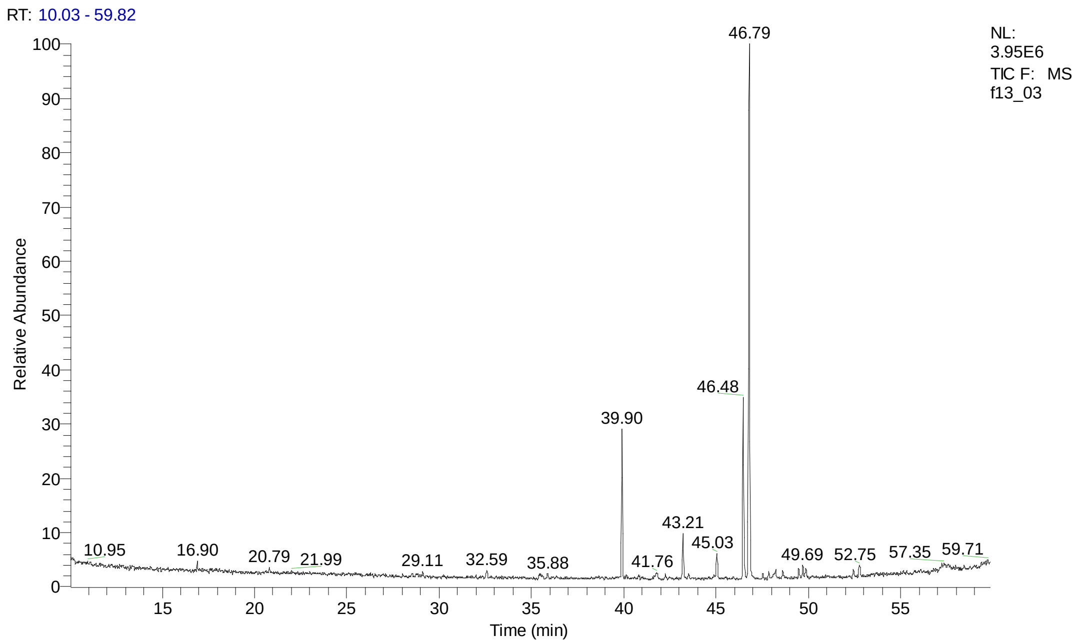

Figure 60 GC chromatogram of female small Indian civet secretion (13)

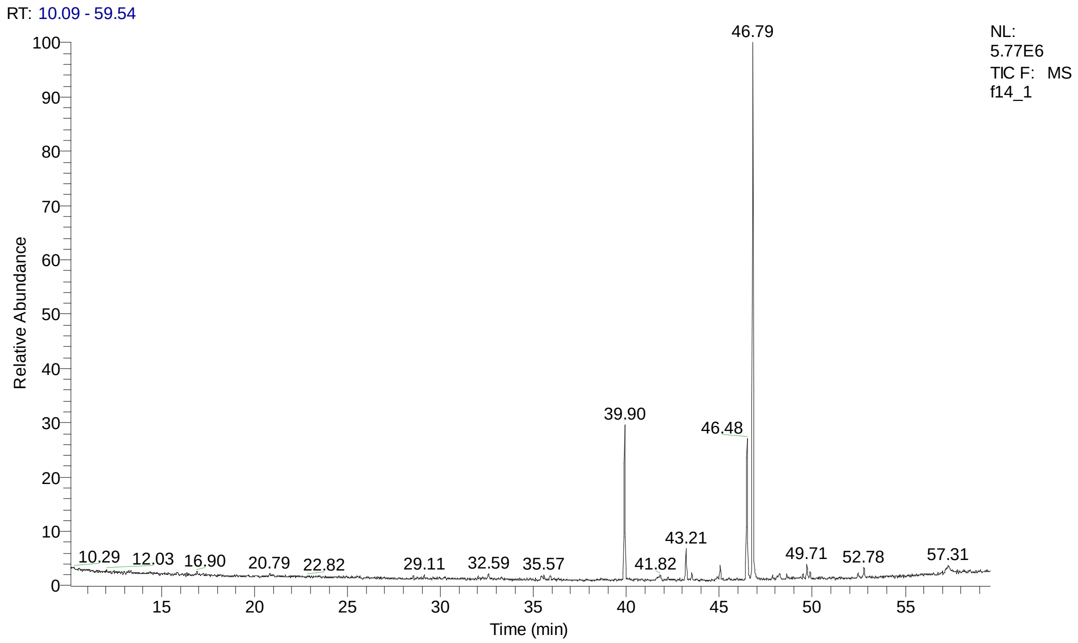

Figure 61 GC chromatogram of female small Indian civet secretion (14)

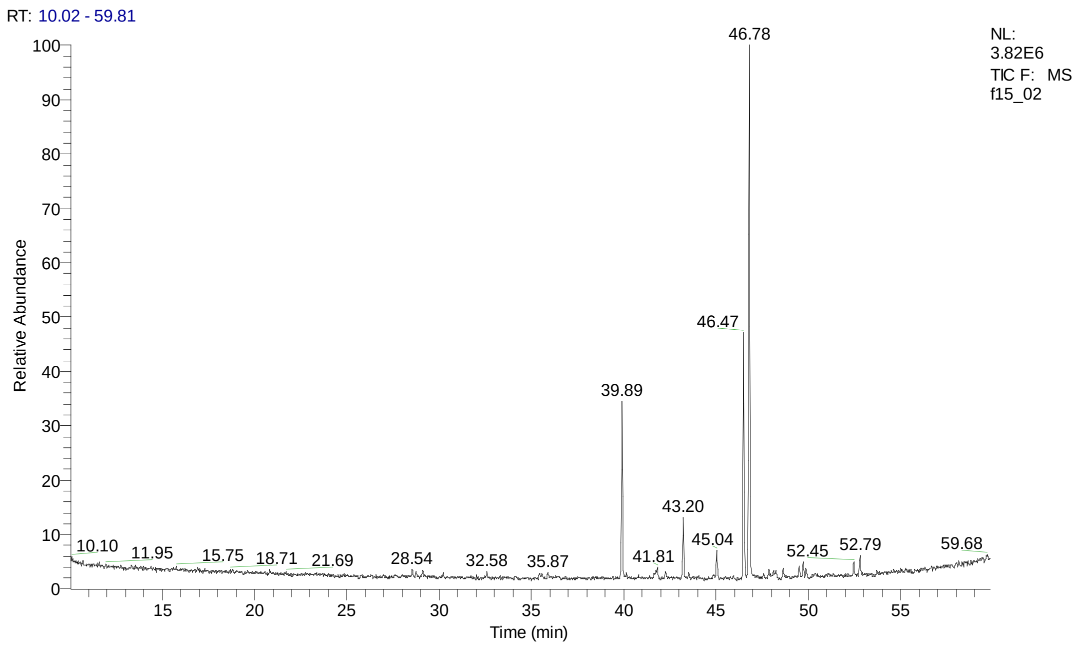

Figure 62 GC chromatogram of female small Indian civet secretion (15)

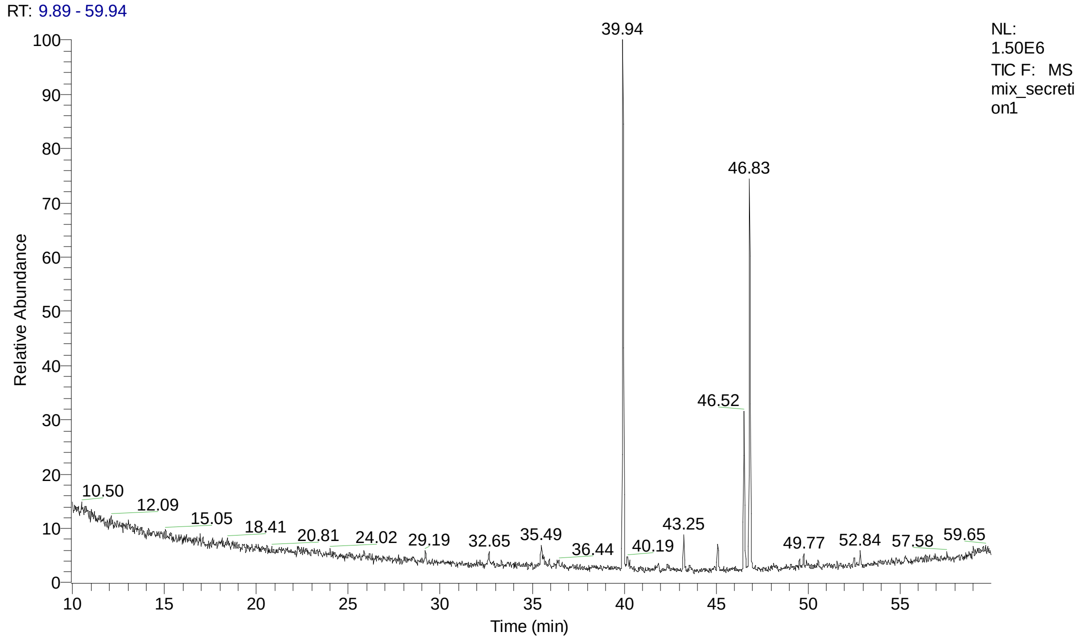

Figure 63 GC chromatogram of pooled small Indian civet secretion (1)

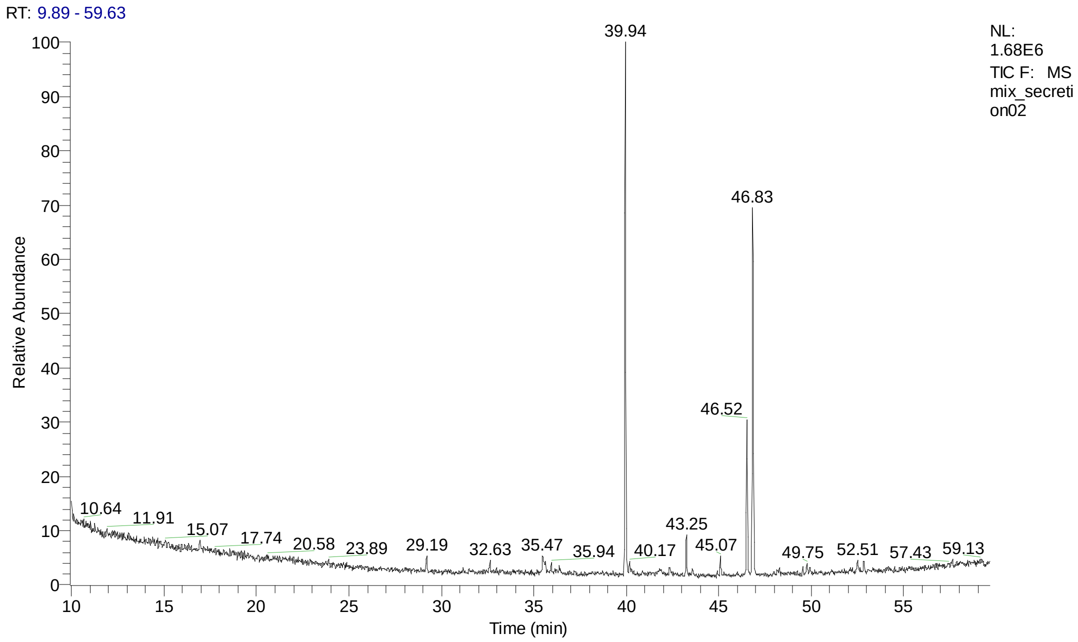

Figure 64 GC chromatogram of pooled small Indian civet secretion (2)

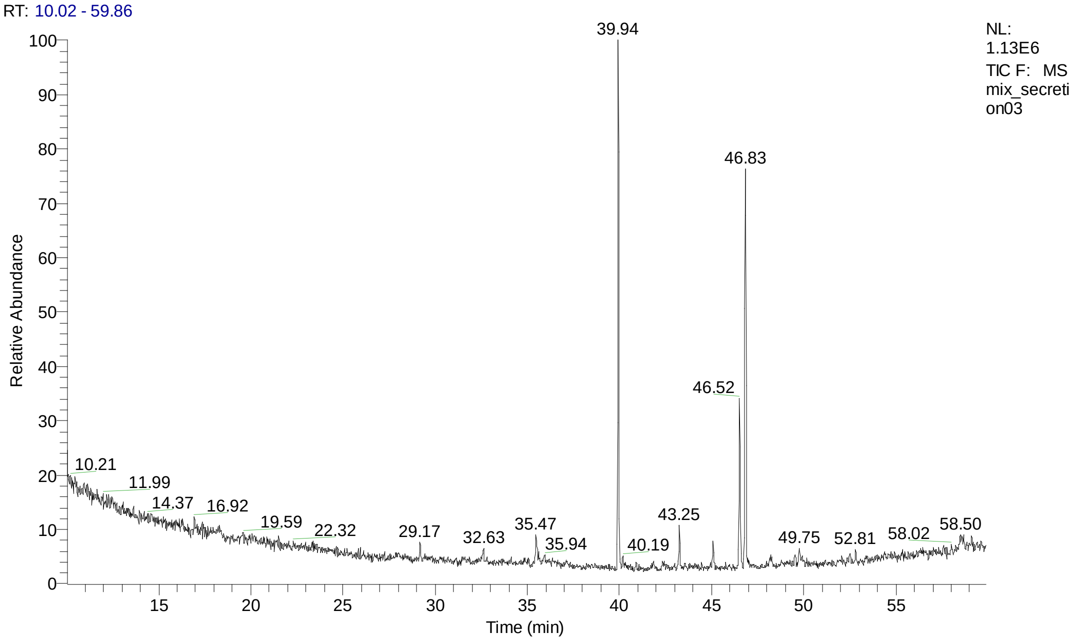

Figure 65 GC chromatogram of pooled small Indian civet secretion (3)

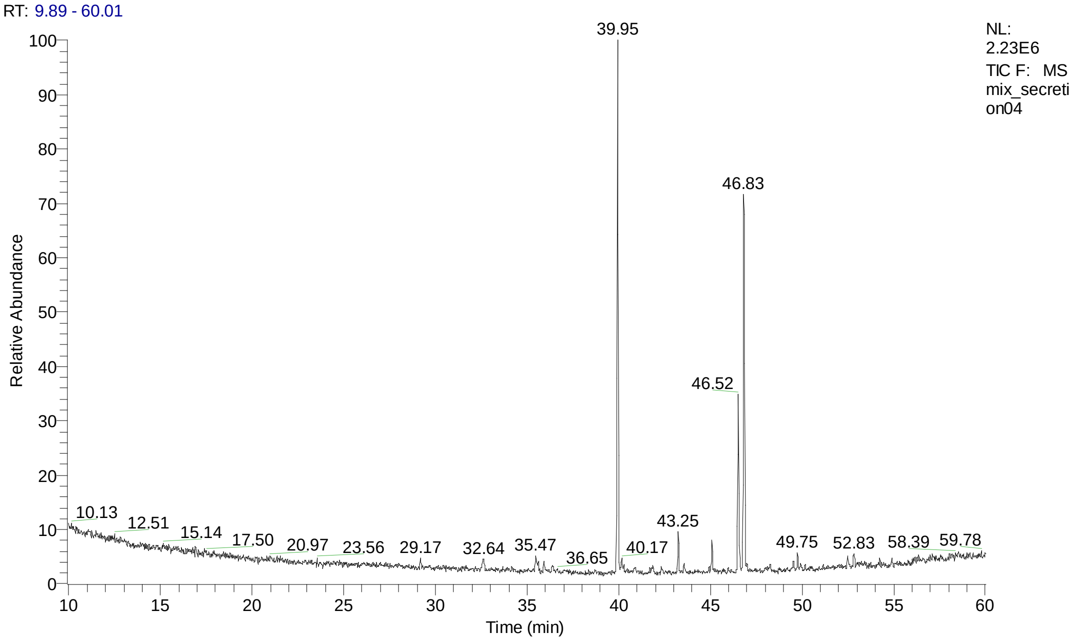

Figure 66 GC chromatogram of pooled small Indian civet secretion (4)

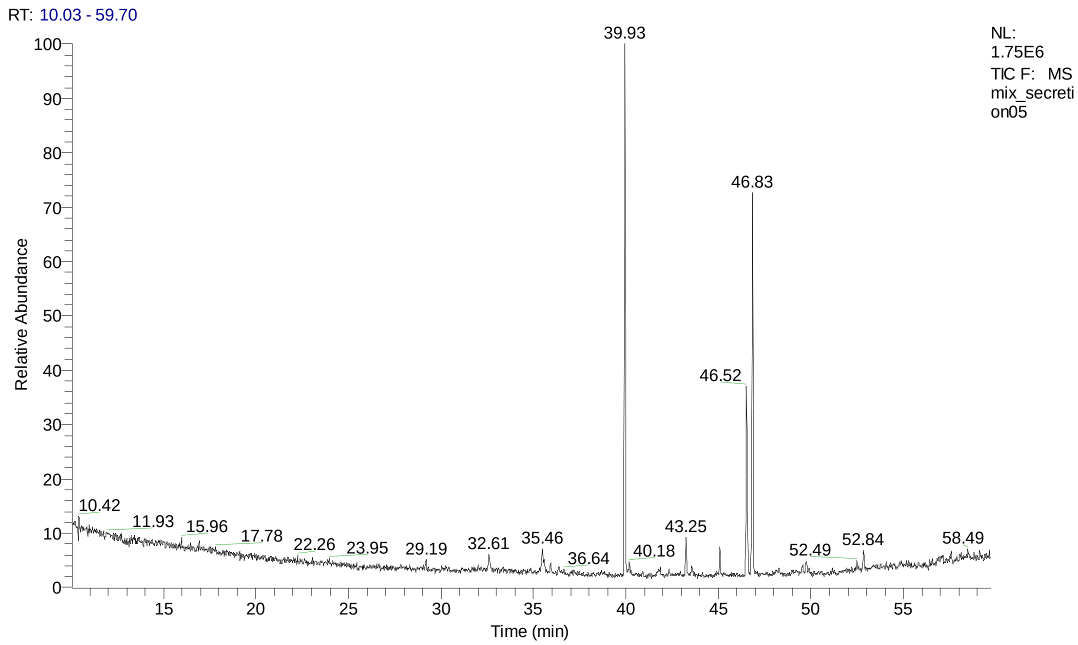

Figure 67 GC chromatogram of pooled small Indian civet secretion (5)

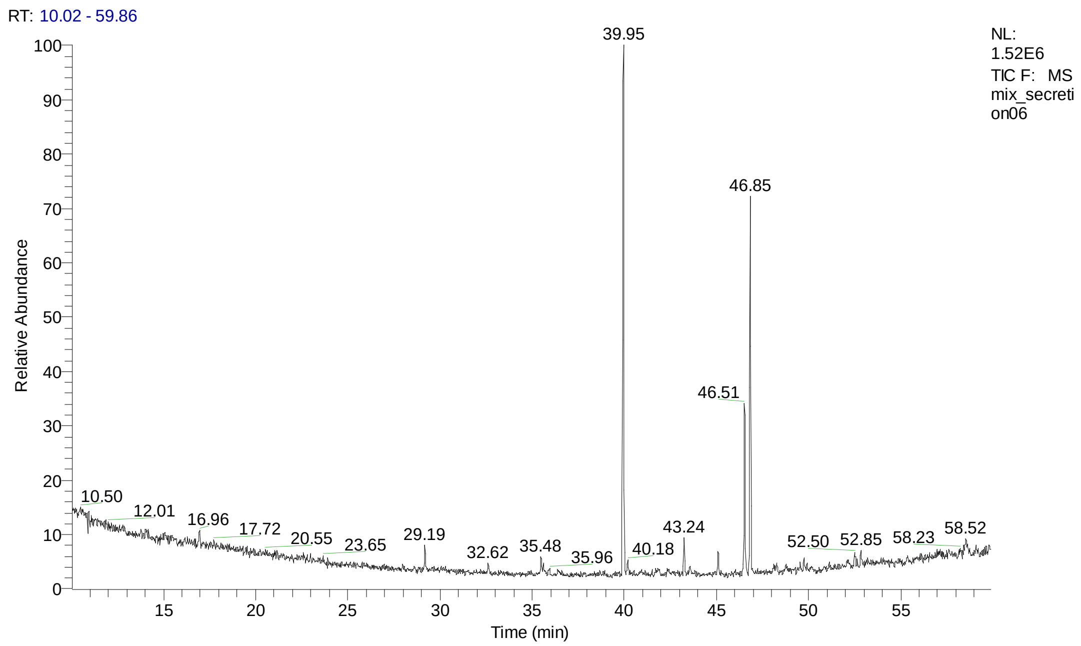

Figure 68 GC chromatogram of pooled small Indian civet secretion (6)

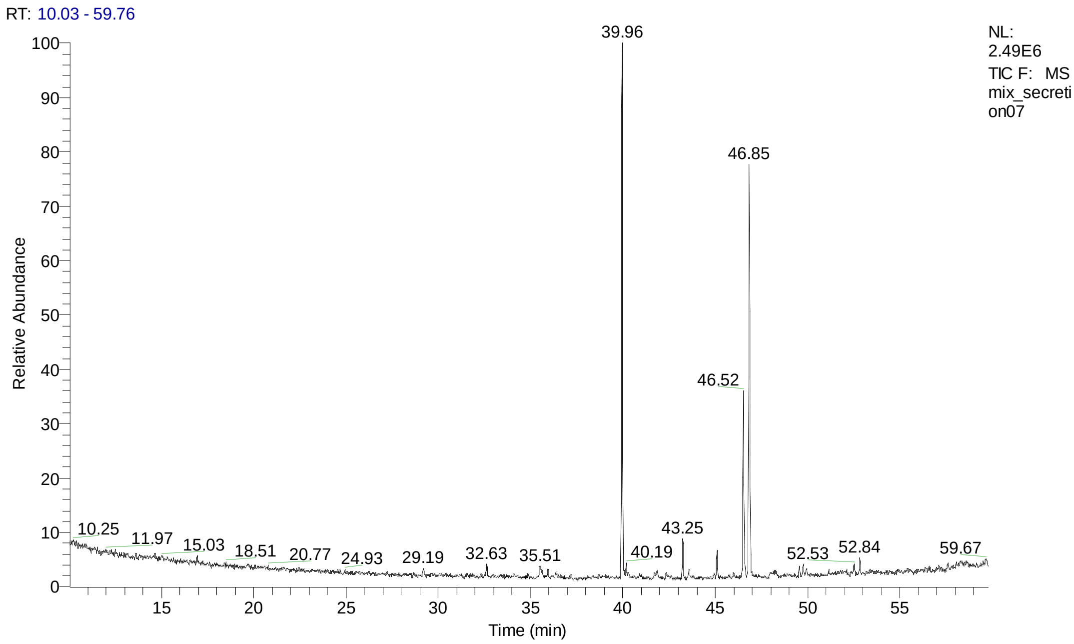

Figure 69 GC chromatogram of pooled small Indian civet secretion (7)

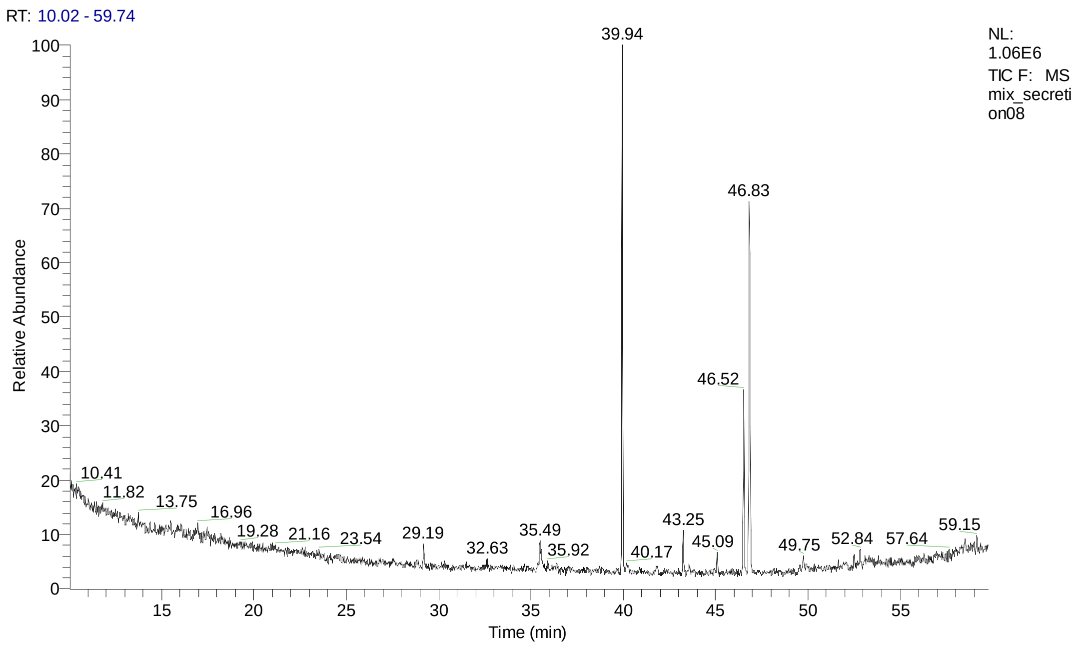

Figure 70 GC chromatogram of pooled small Indian civet secretion (8)

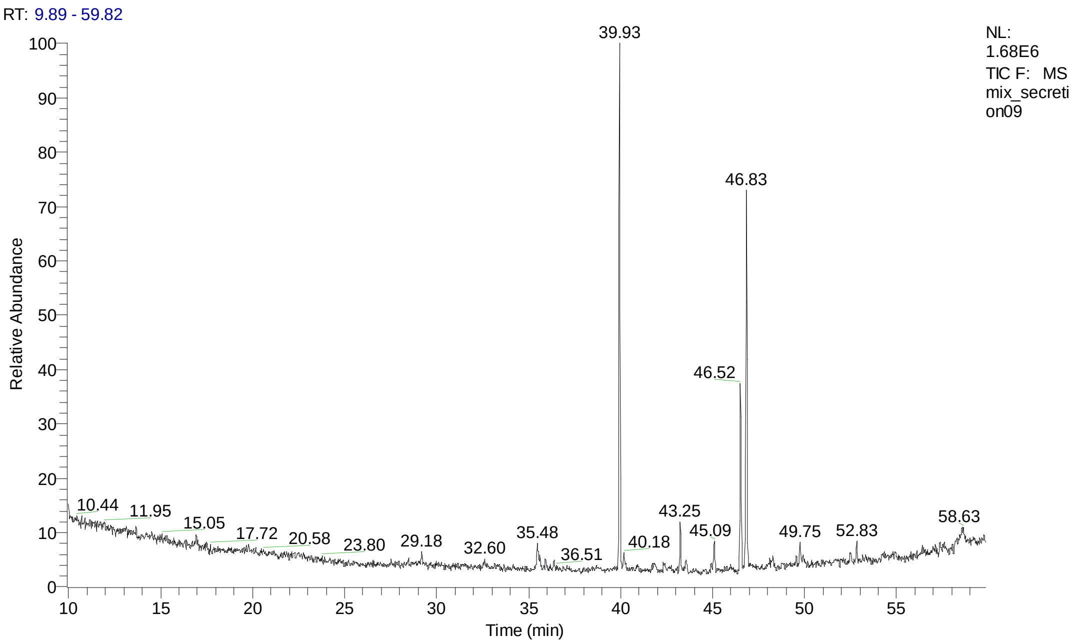

Figure 71 GC chromatogram of pooled small Indian civet secretion (9)

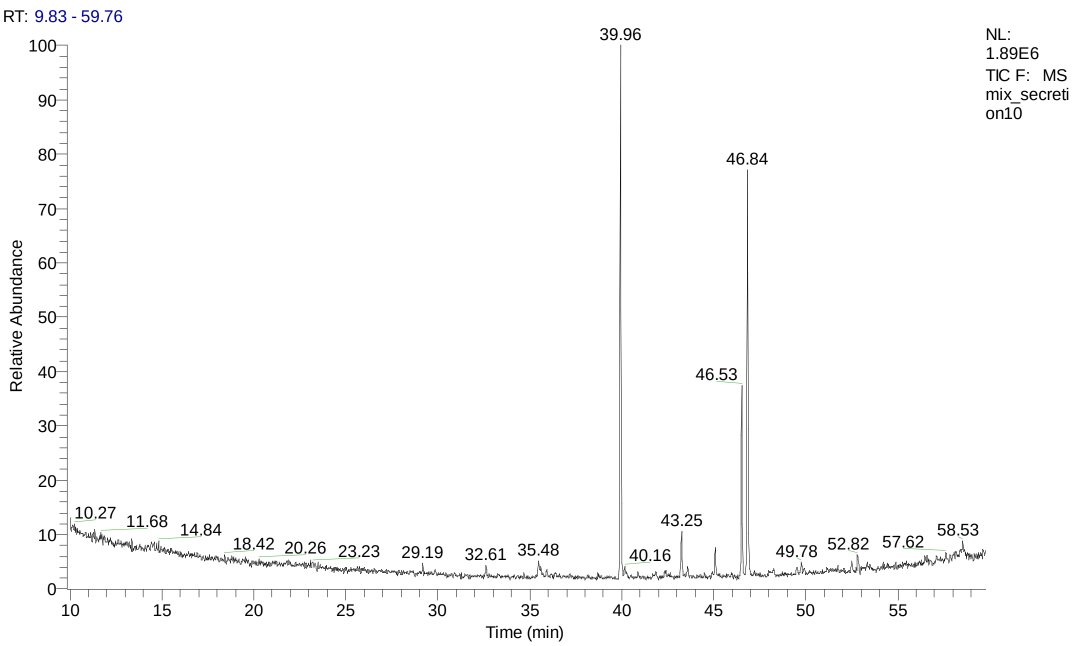

Figure 72 GC chromatogram of pooled small Indian civet secretion (10)

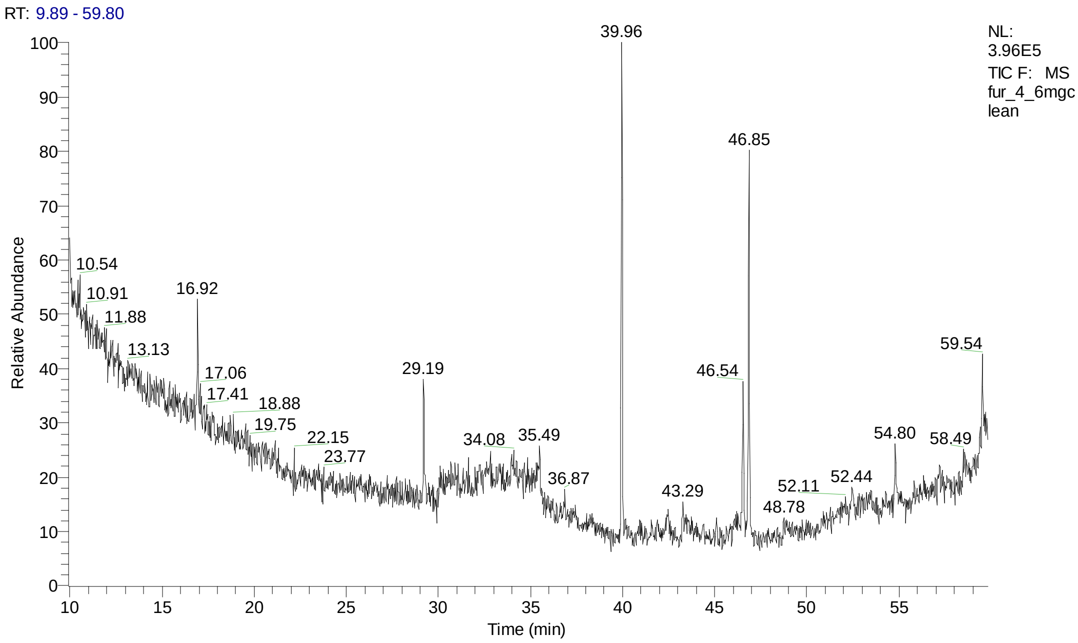

Figure 73 GC chromatogram of small Indian civet furs (1)

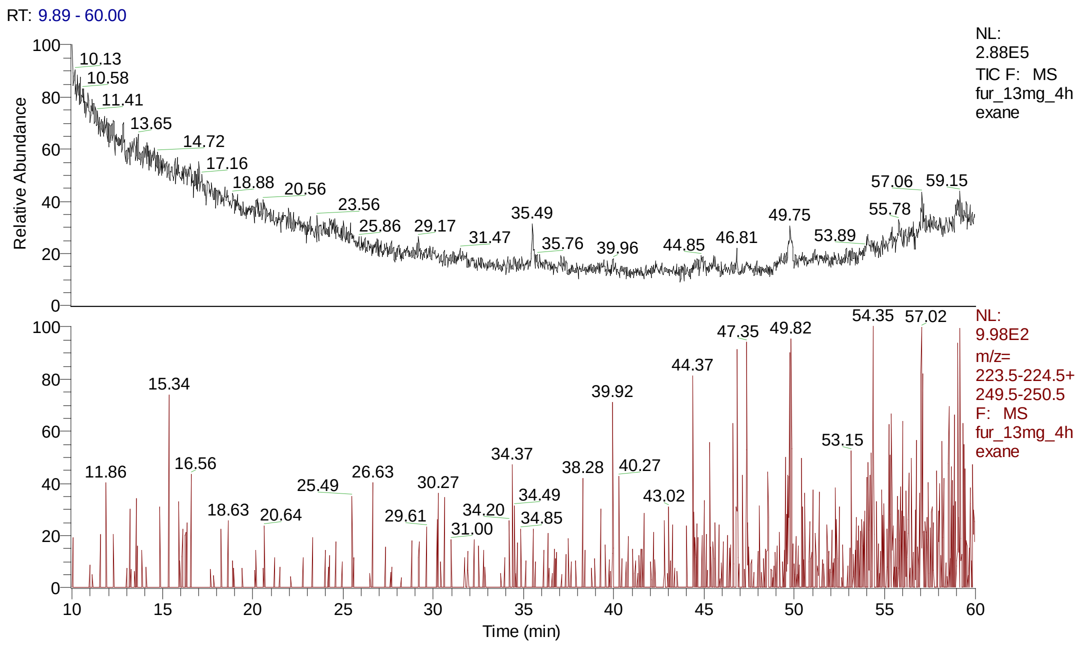

Figure 74 Full scan (upper) and SIM (lower) chromatogram of the last aliquot of hexane from civet furs washing (1)

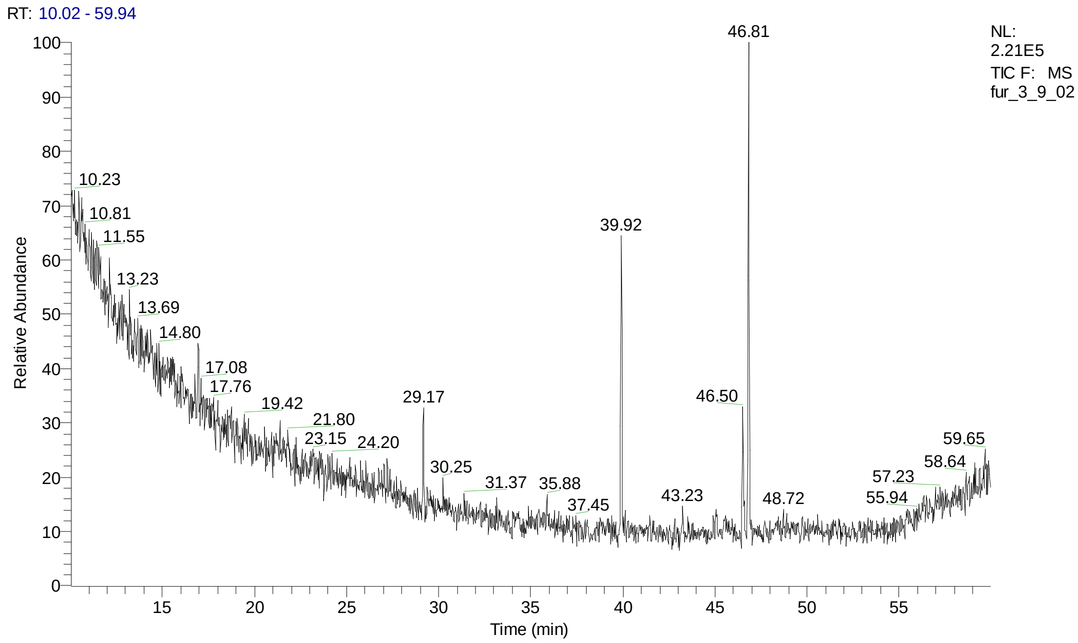

Figure 75 GC chromatogram of small Indian civet furs (2)

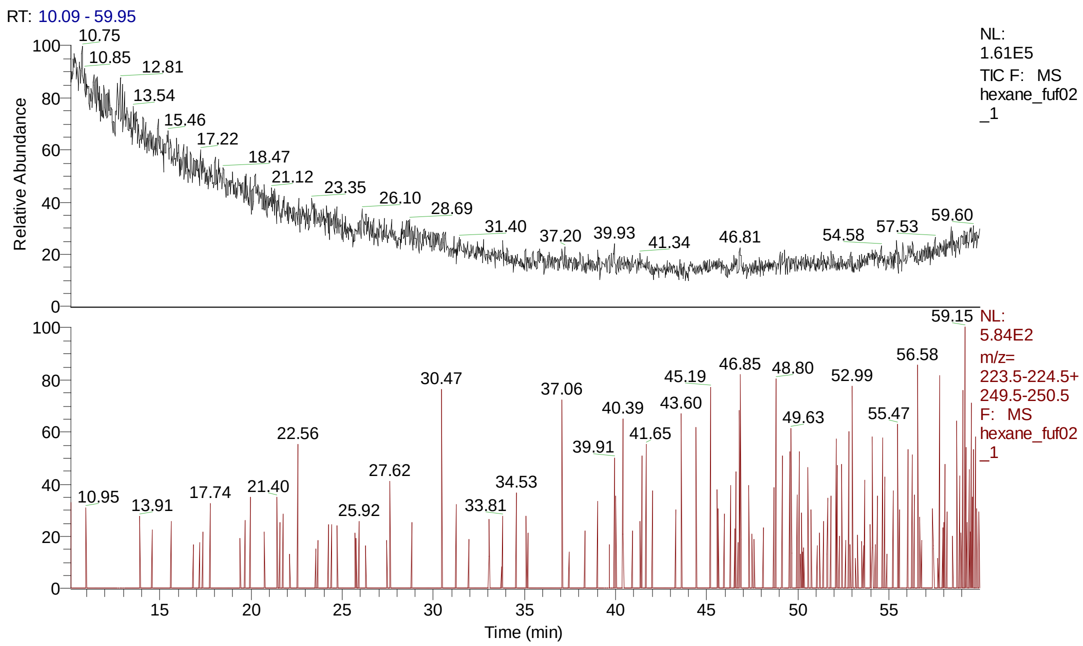

Figure 76 Full scan (upper) and SIM (lower) chromatogram of the last aliquot of hexane from civet furs washing (2)

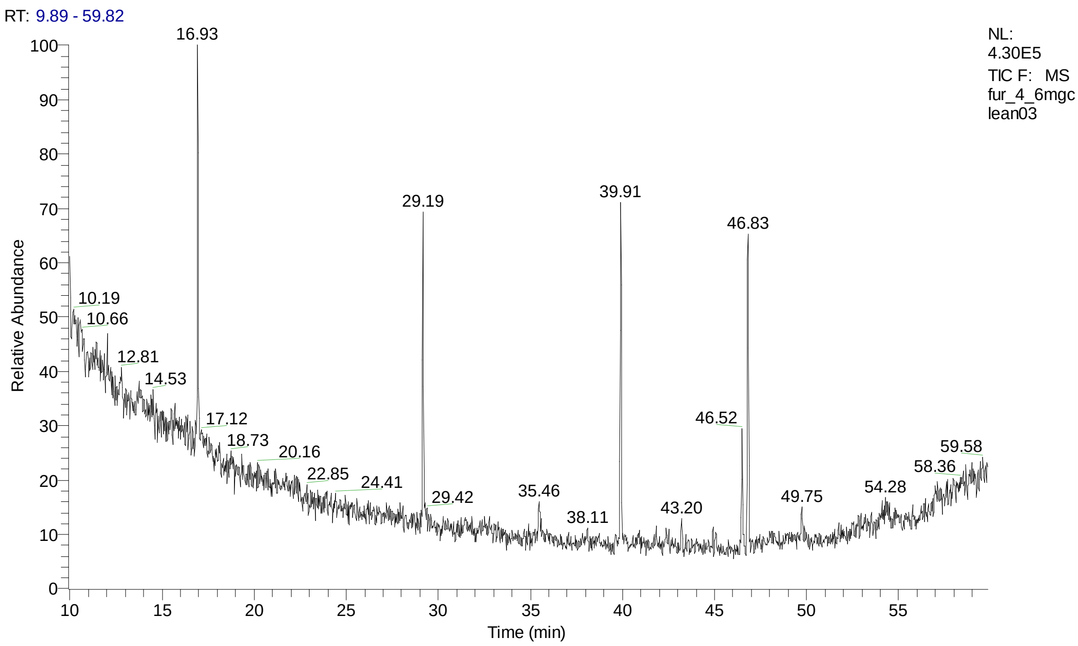

Figure 77 GC chromatogram of small Indian civet furs (3)

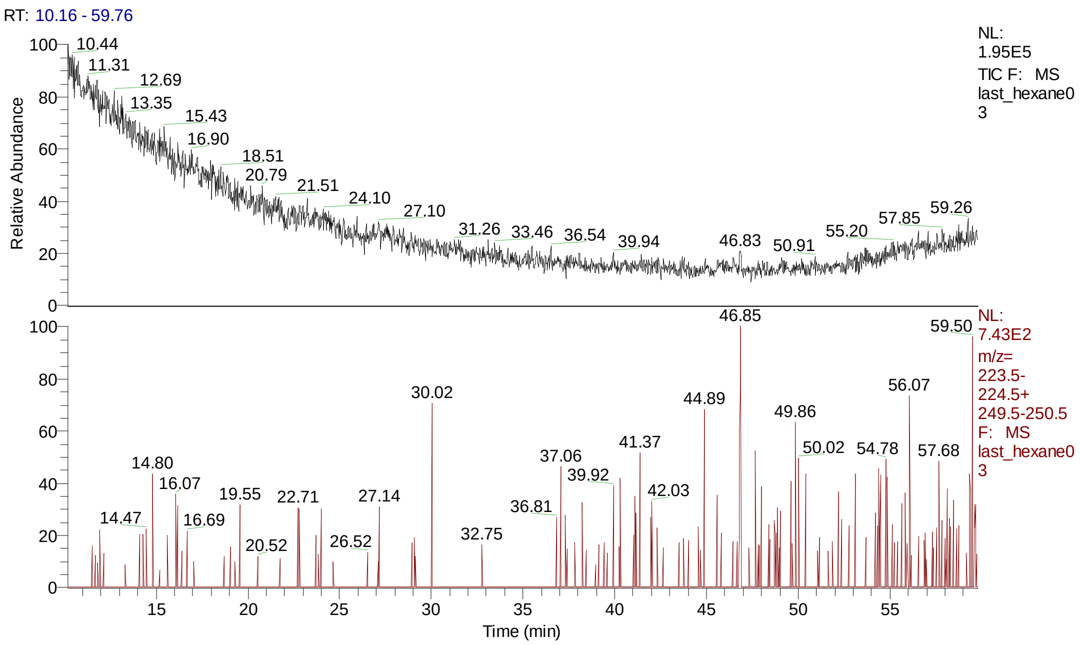

Figure 78 Full scan (upper) and SIM (lower) chromatogram of the last aliquot of hexane from civet furs washing (3)

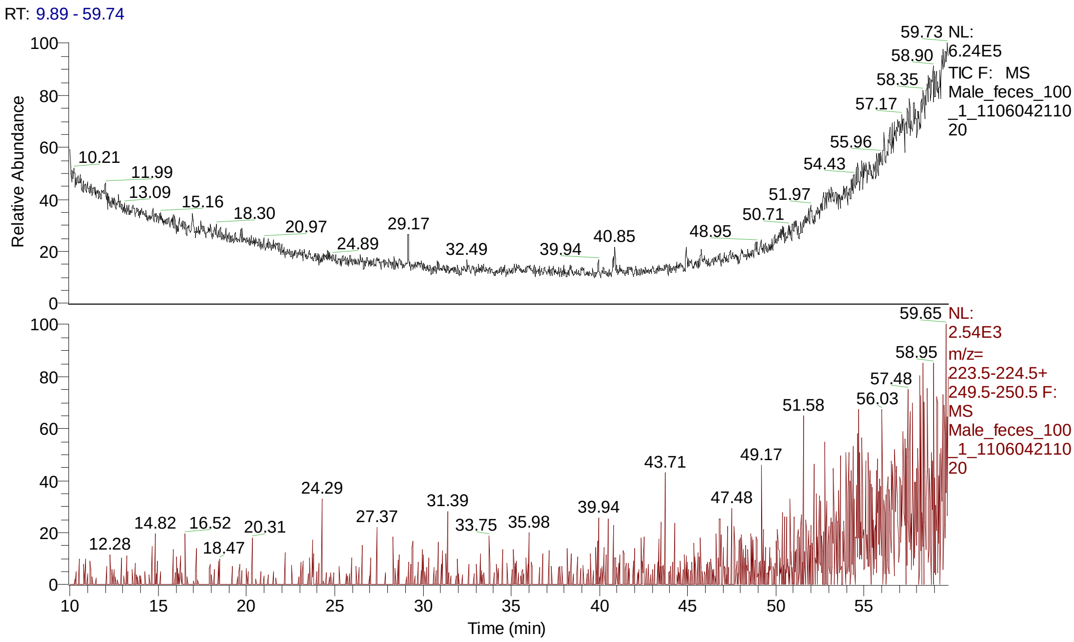

Figure 79 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (1)

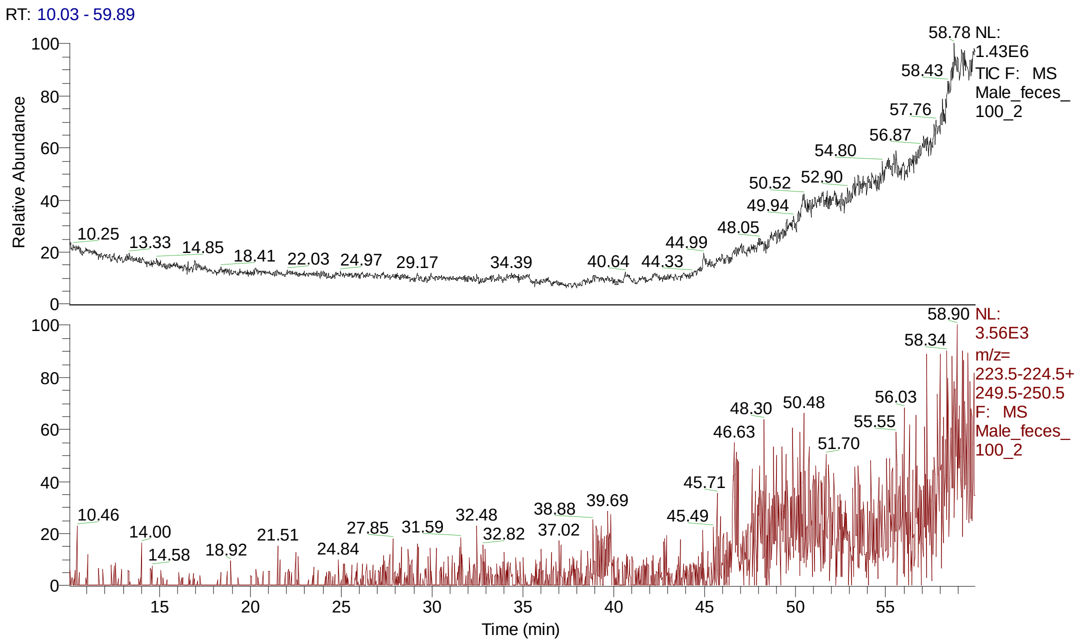

Figure 80 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (2)

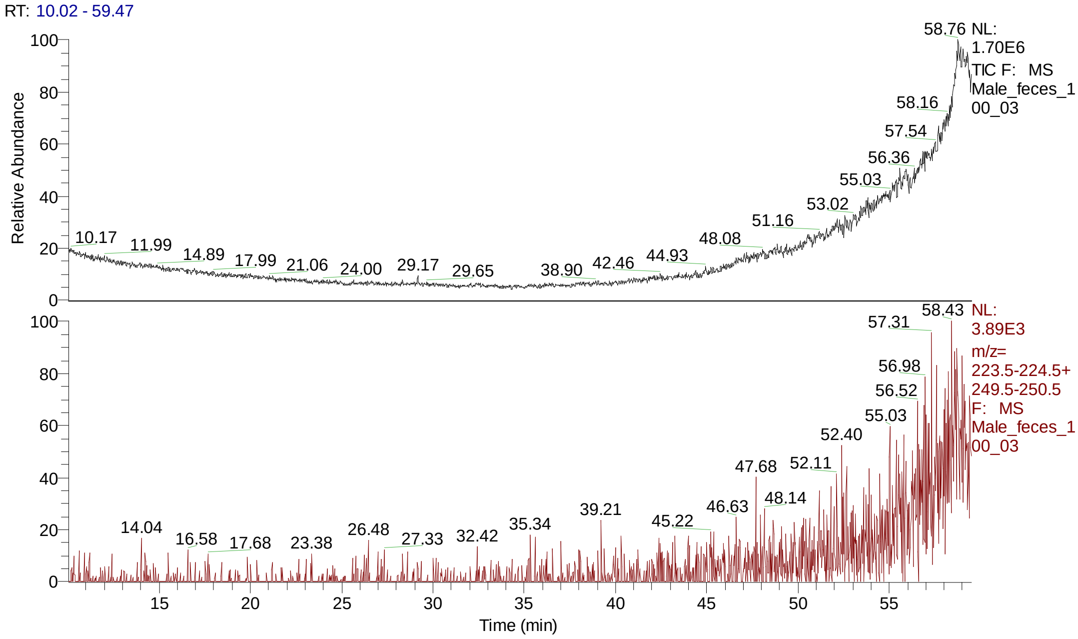

Figure 81 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (3)

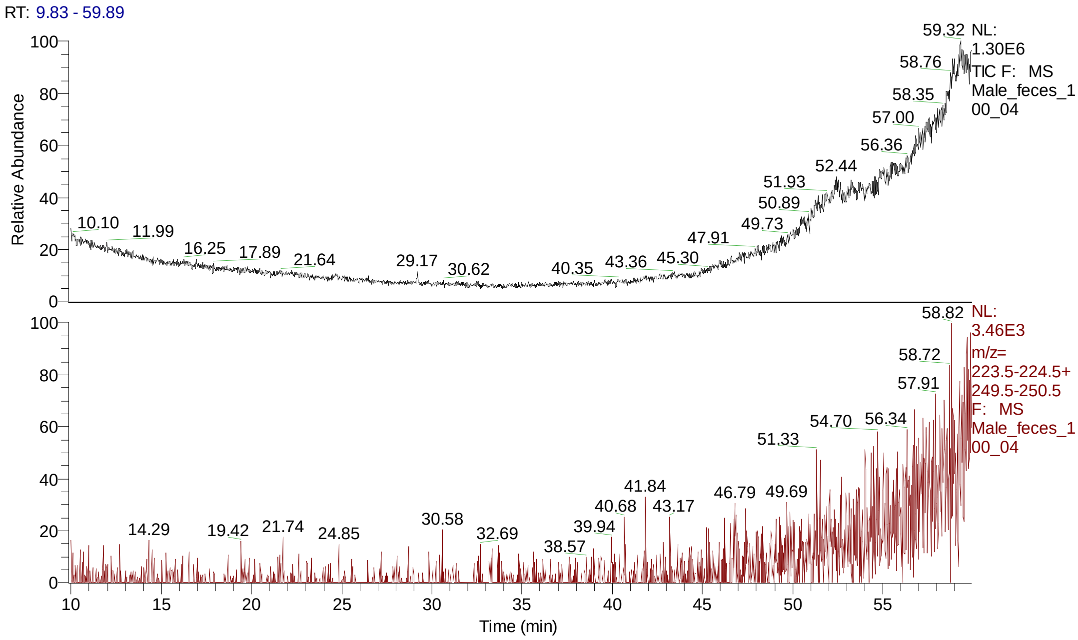

Figure 82 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (4)

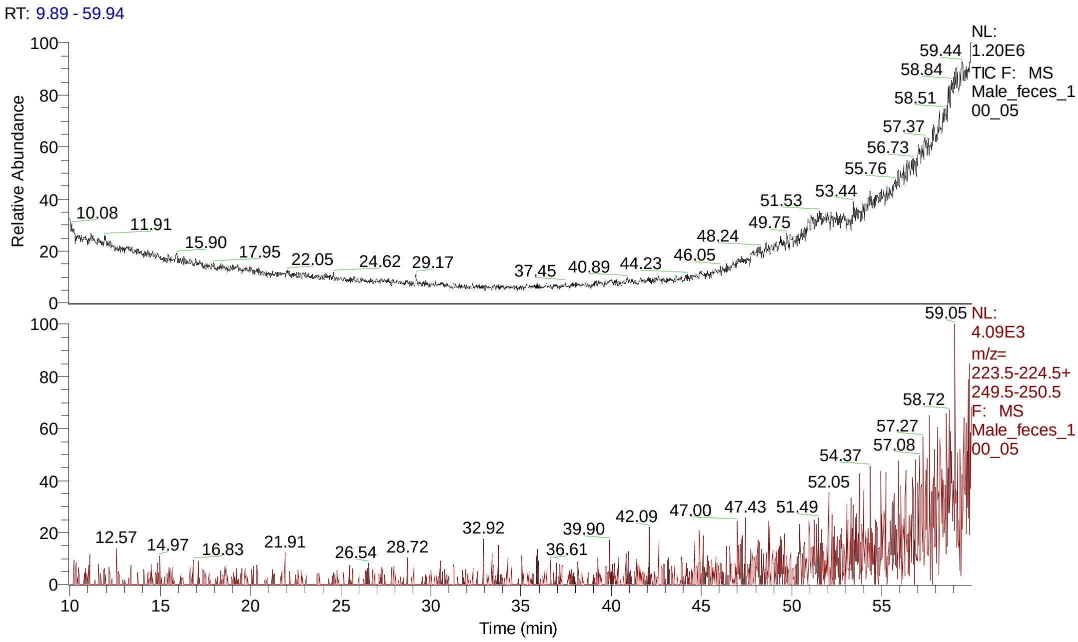

Figure 83 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (5)

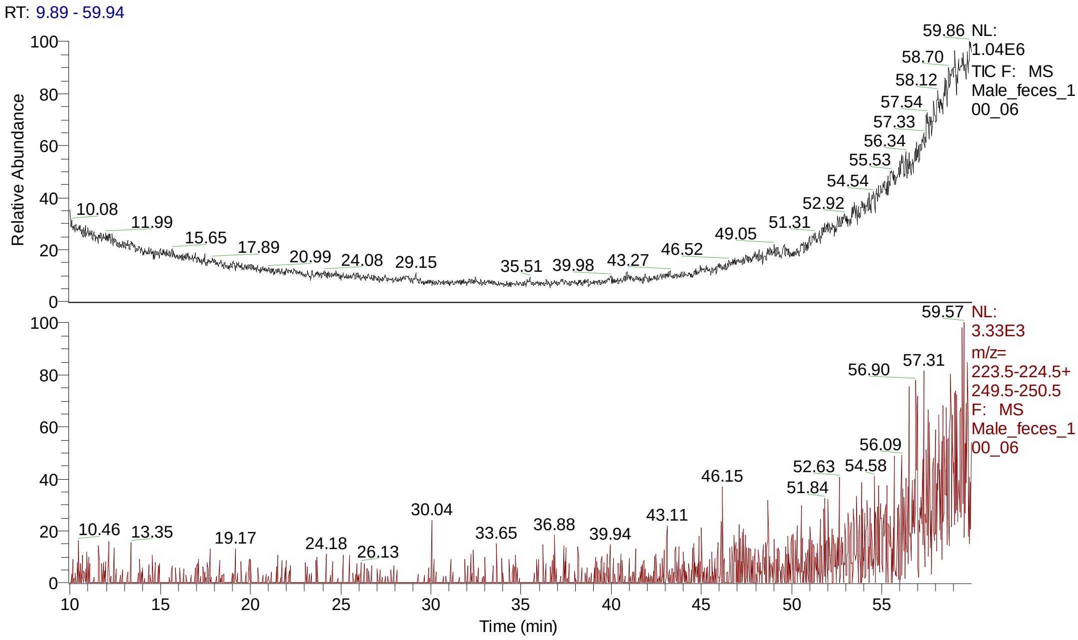

Figure 84 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (6)

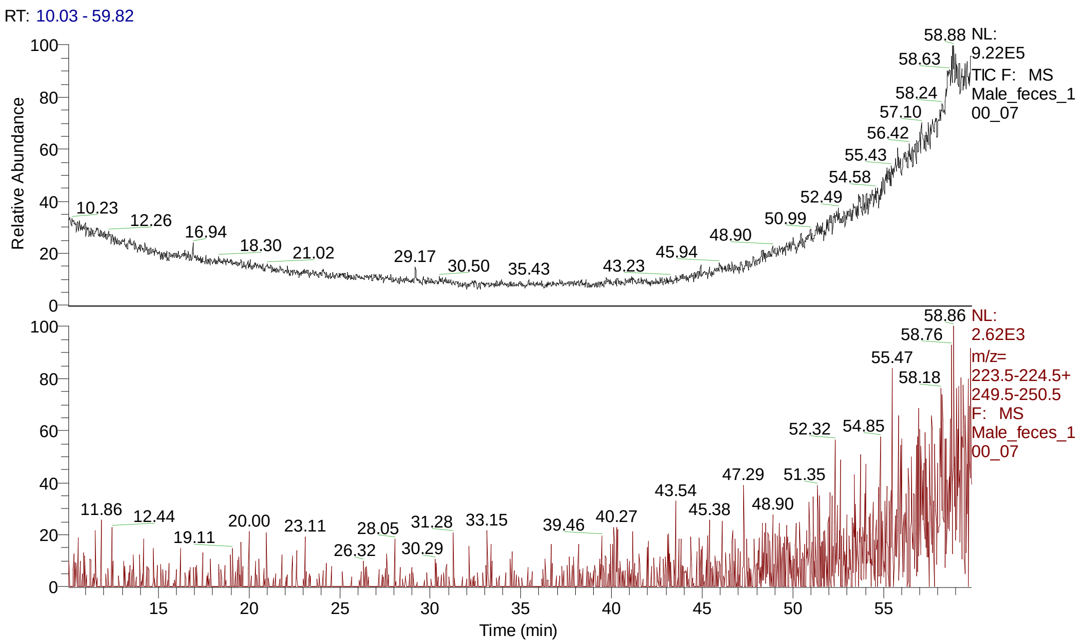

Figure 85 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (7)

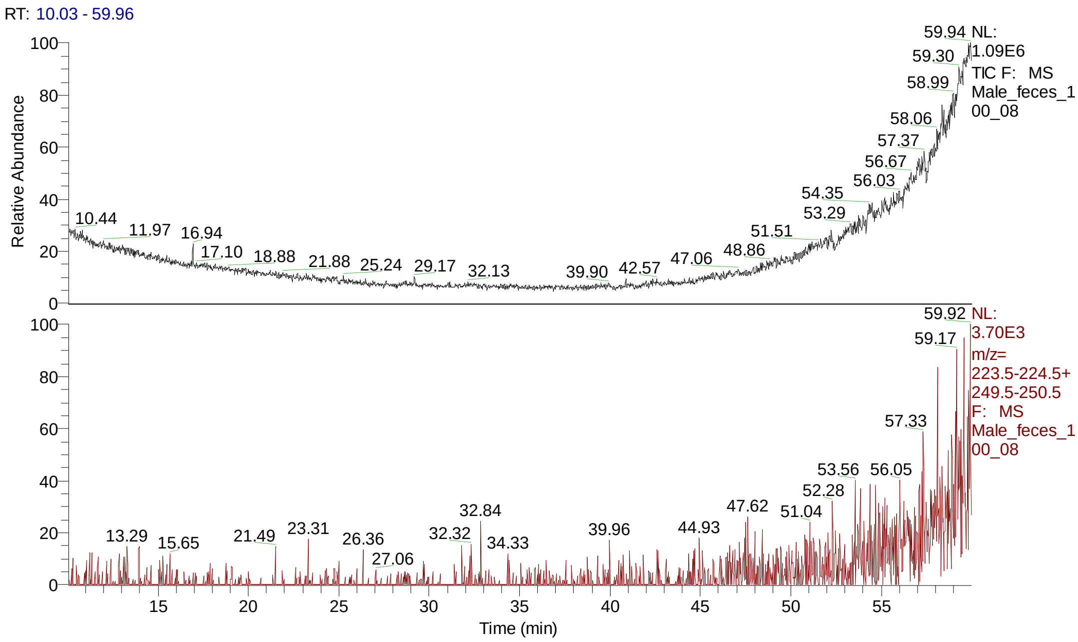

Figure 86 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (8)

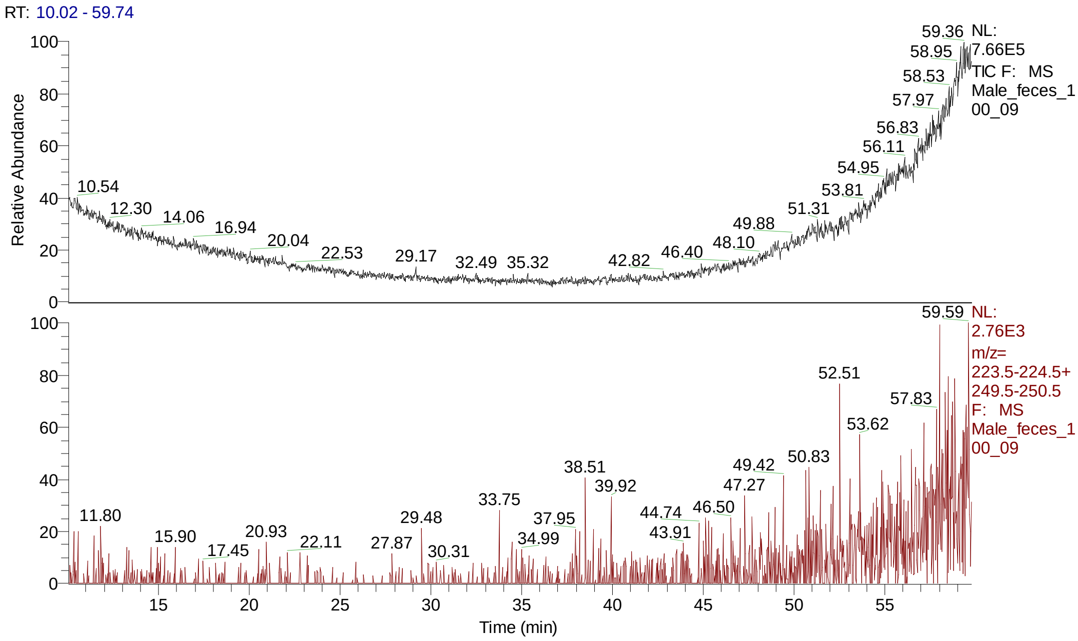

Figure 87 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (9)

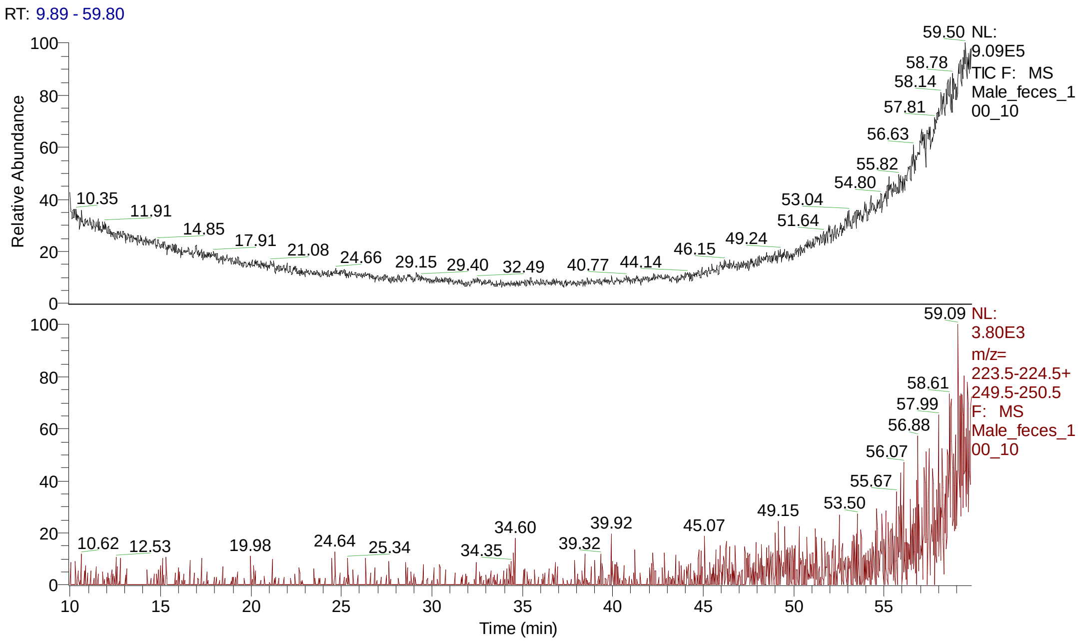

Figure 88 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (10)

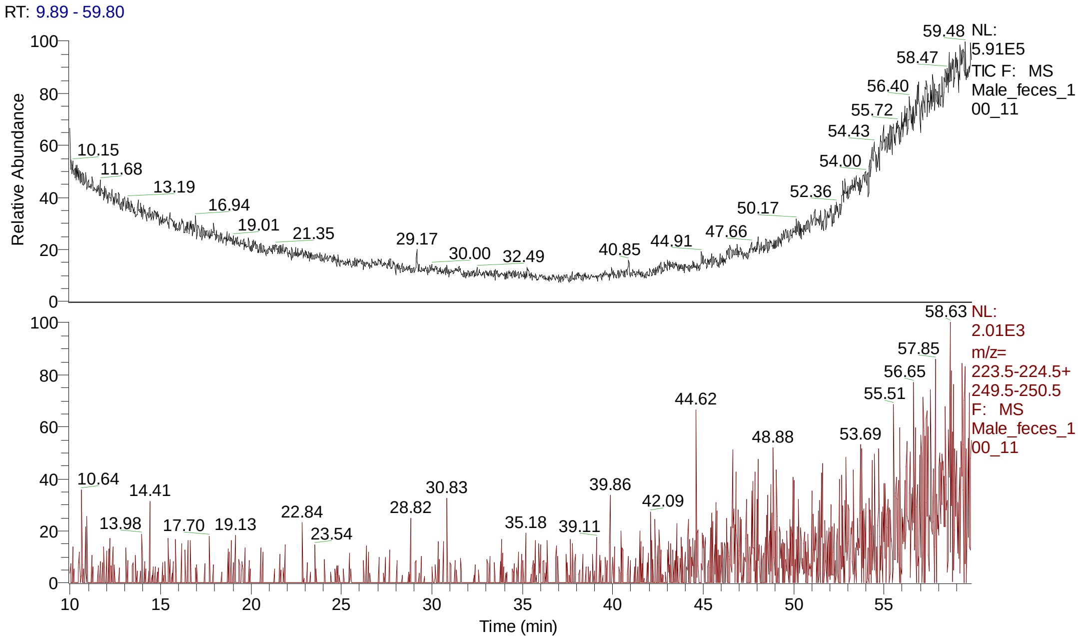

Figure 89 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (11)

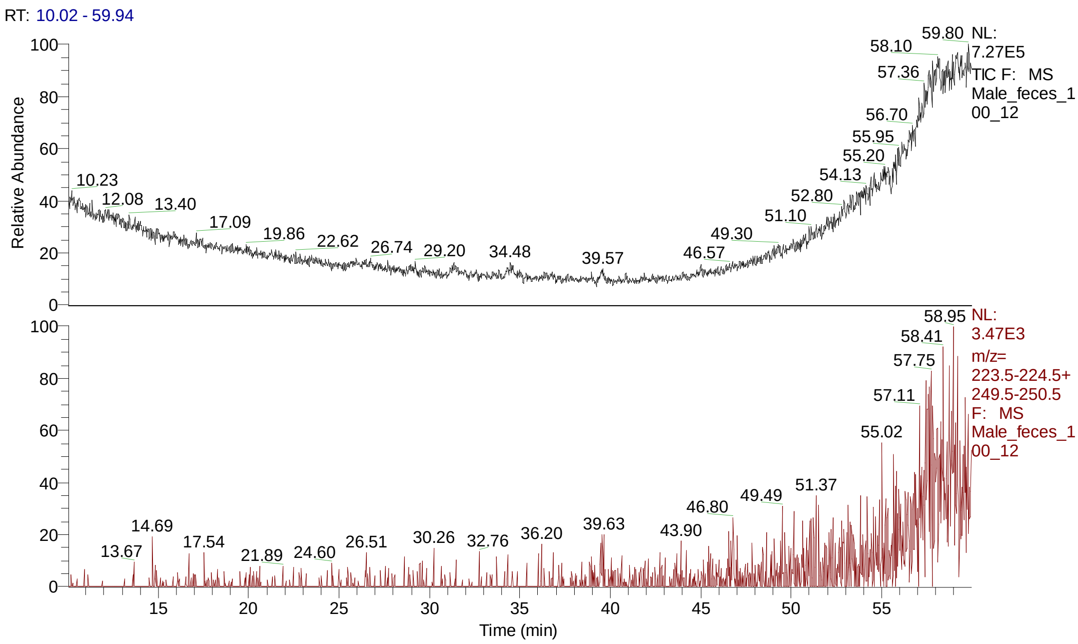

Figure 90 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (12)

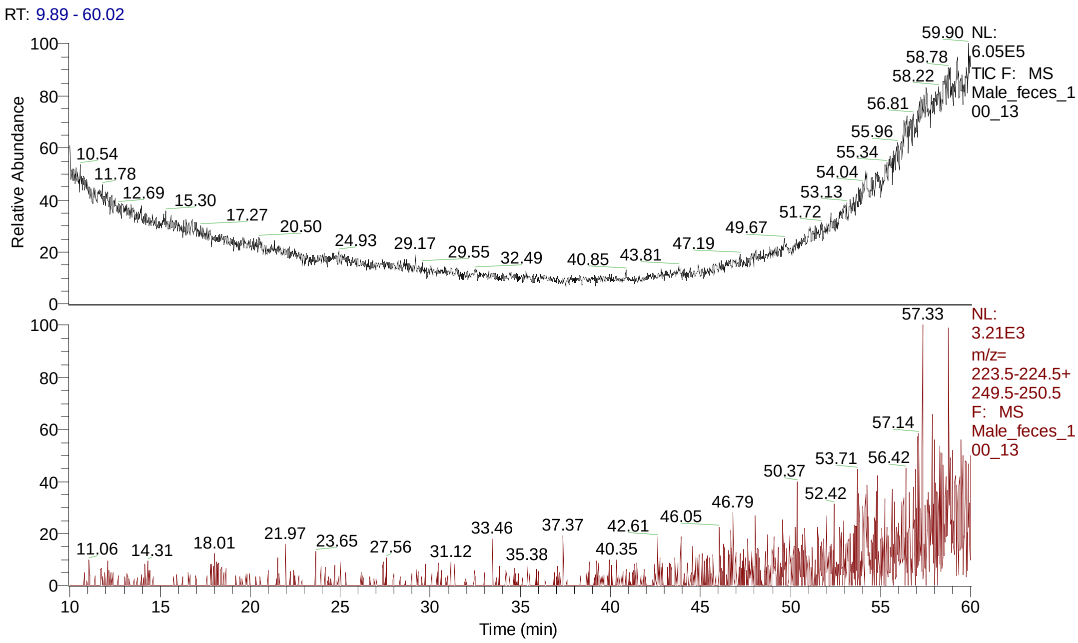

Figure 91 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (13)

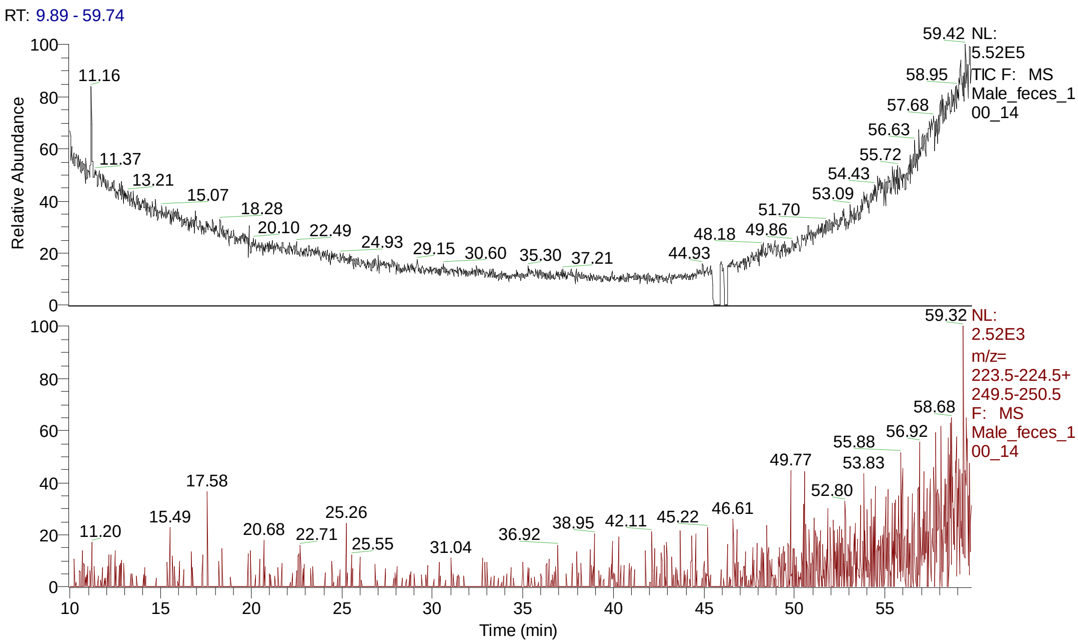

Figure 92 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (14)

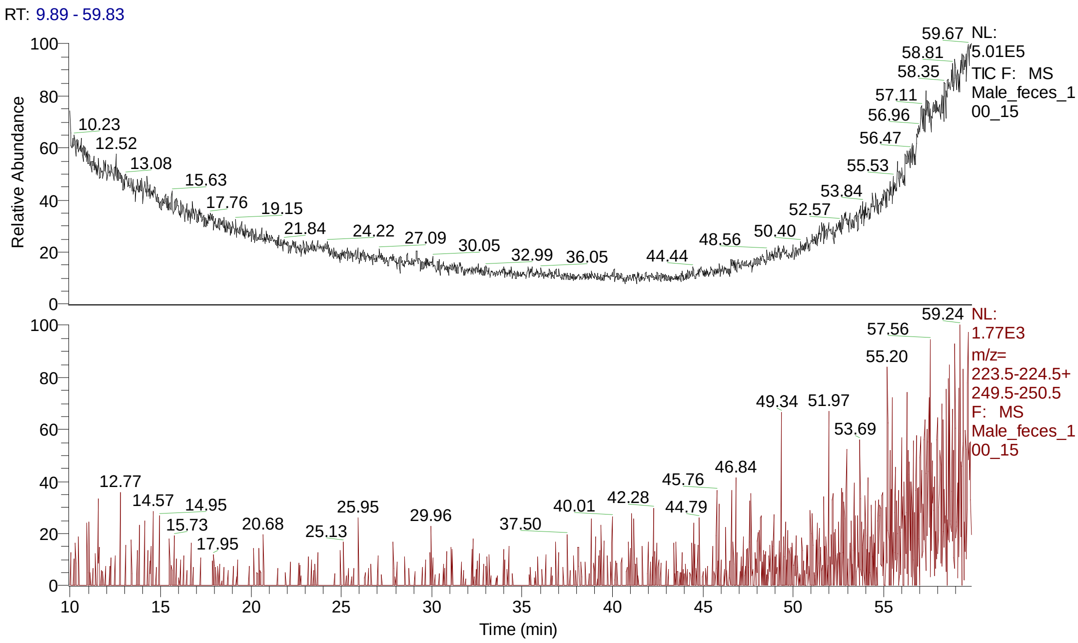

Figure 93 Full scan (upper) and SIM (lower) chromatogram of male small Indian civet feces (15)

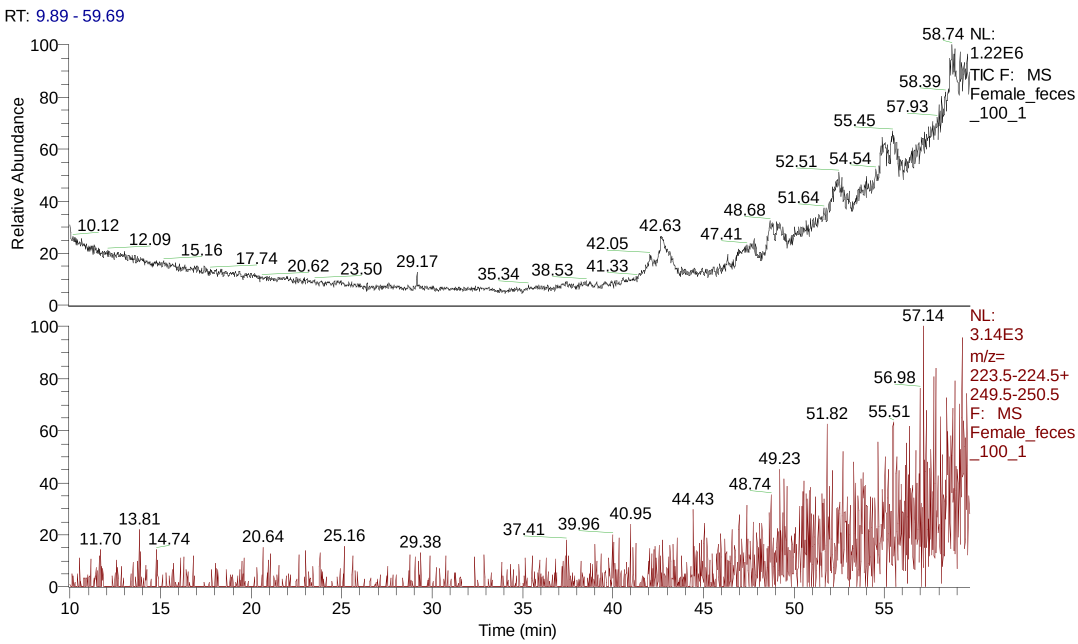

Figure 94 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (1)

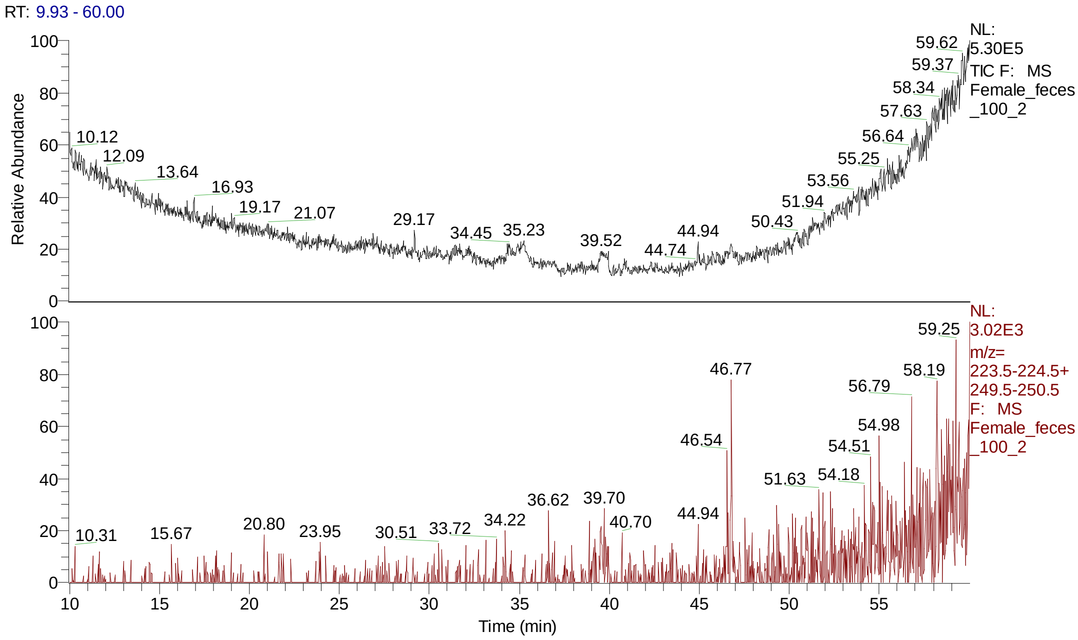

Figure 95 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (2)

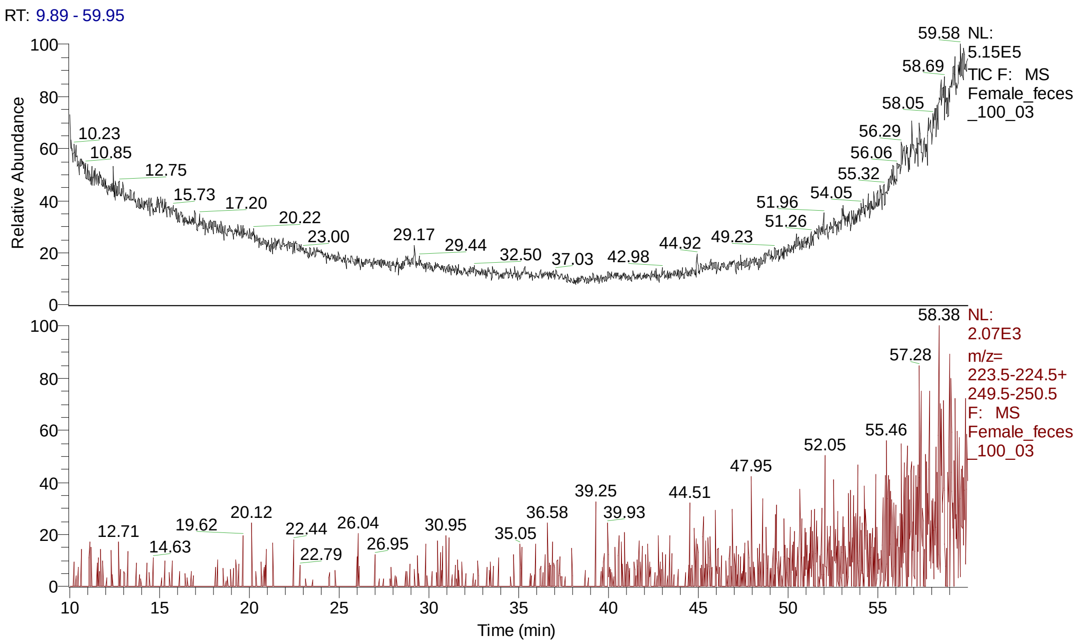

Figure 96 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (3)

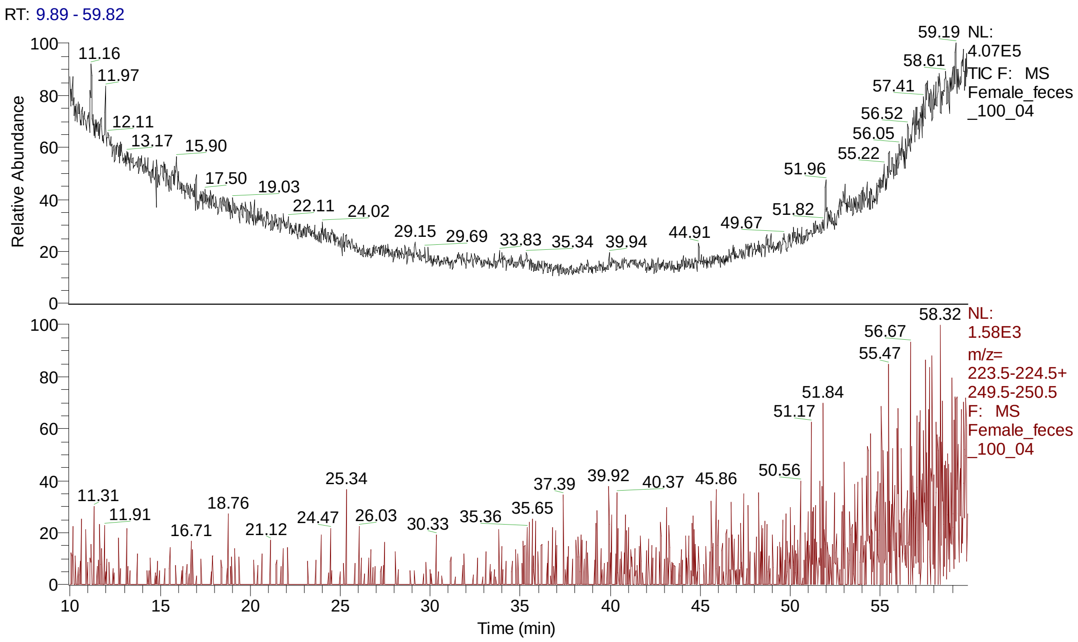

Figure 97 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (4)

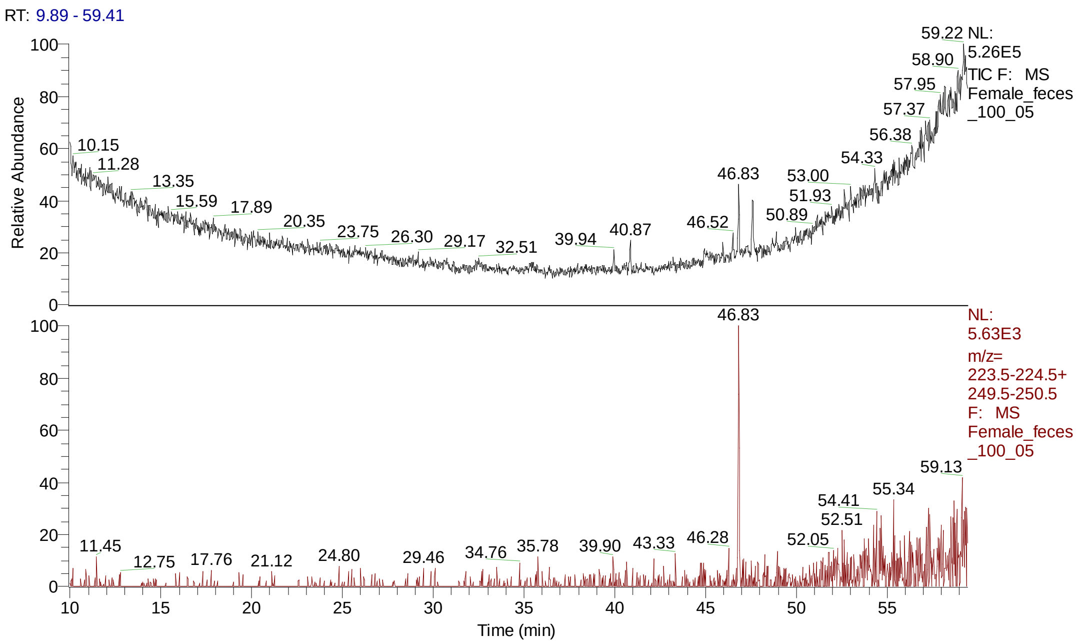

Figure 98 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (5)

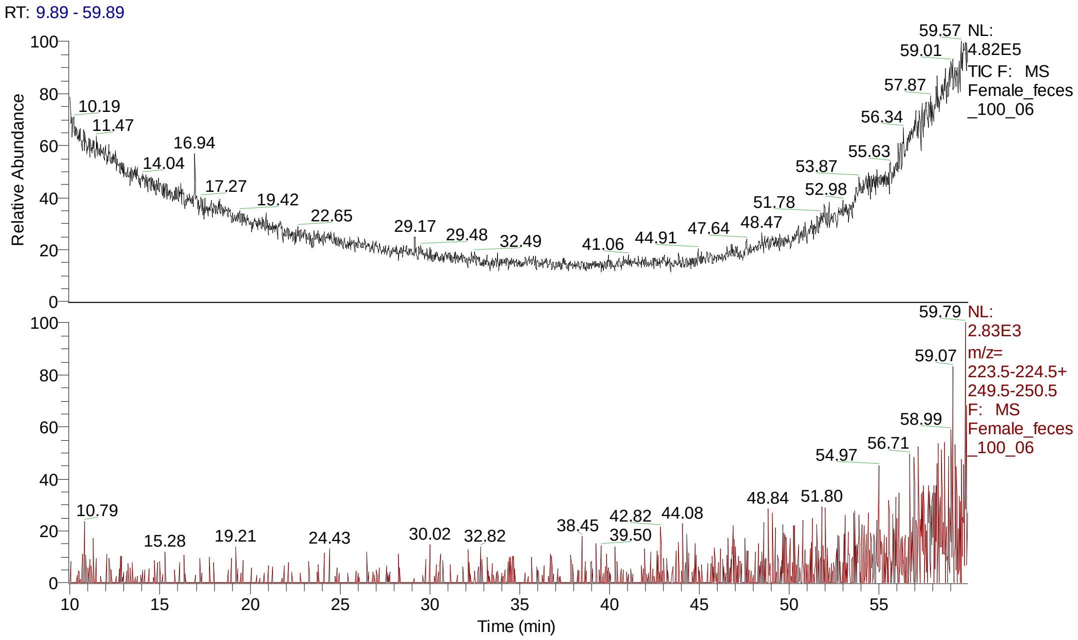

Figure 99 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (6)

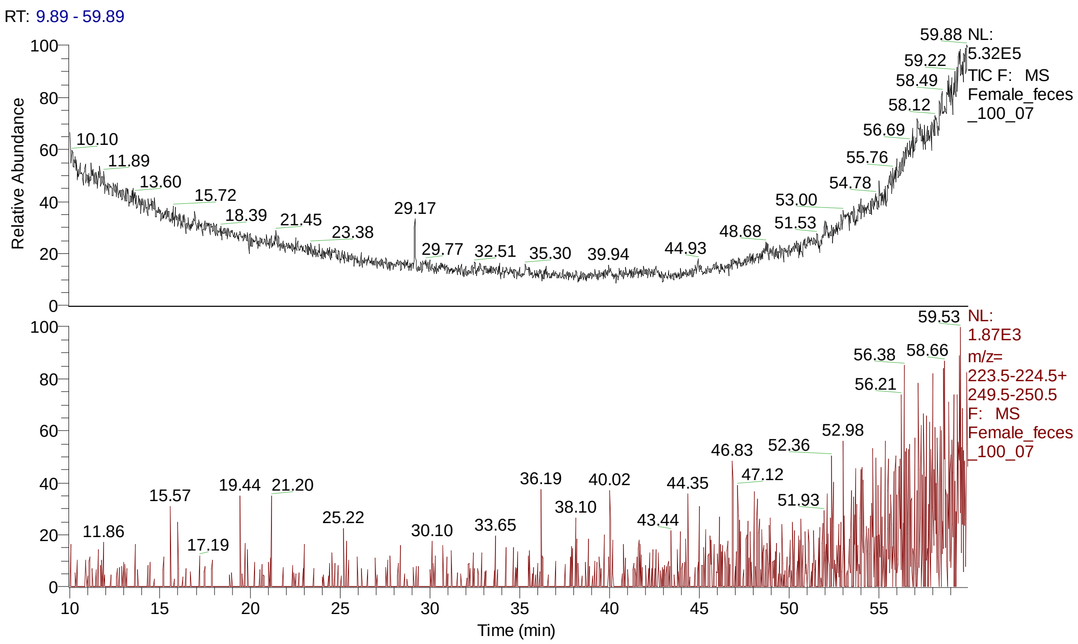

Figure 100 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (7)

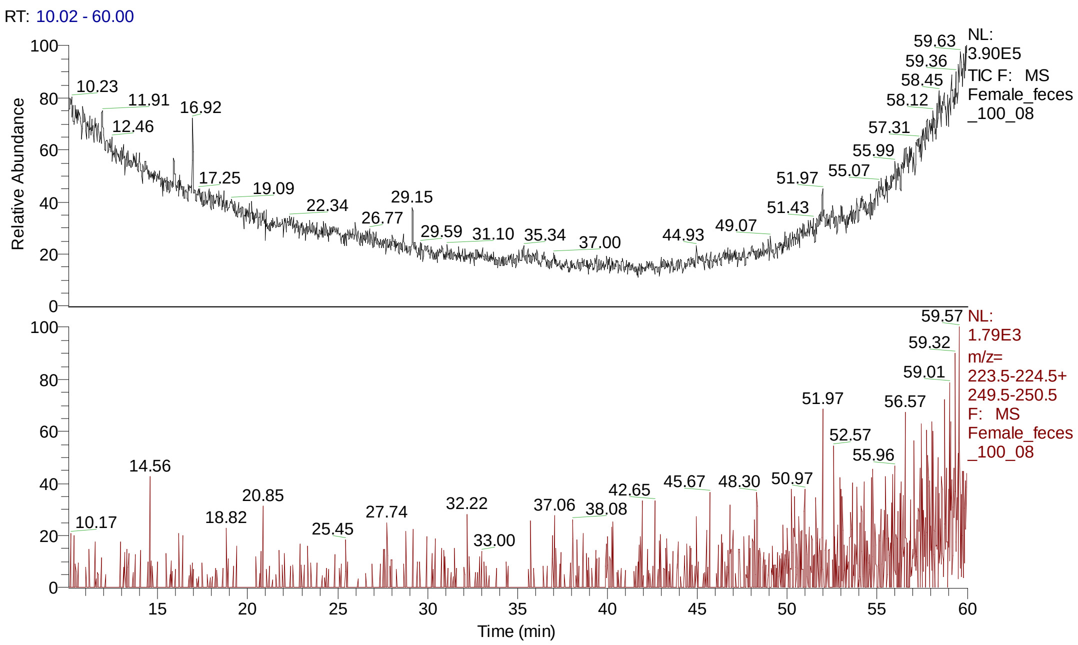

Figure 101 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (8)

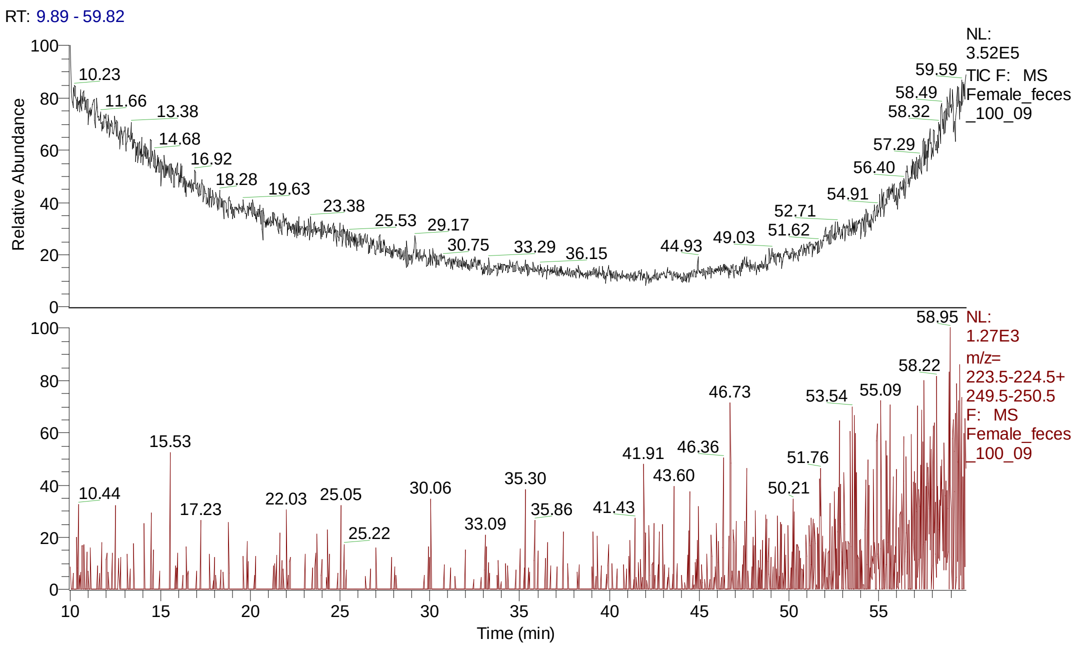

Figure 102 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (9)

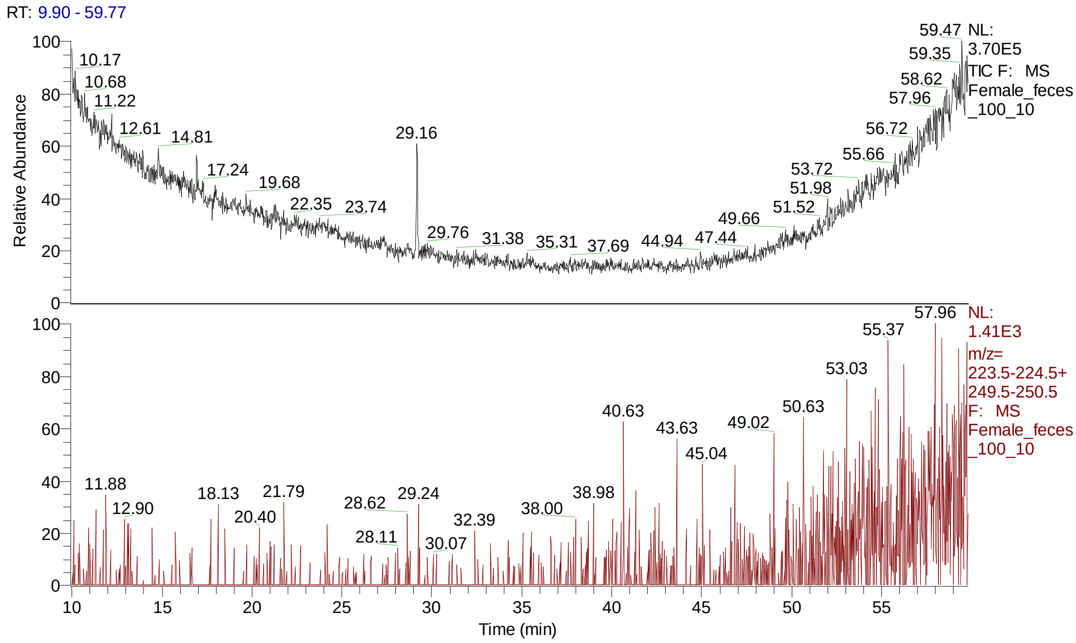

Figure 103 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (10)

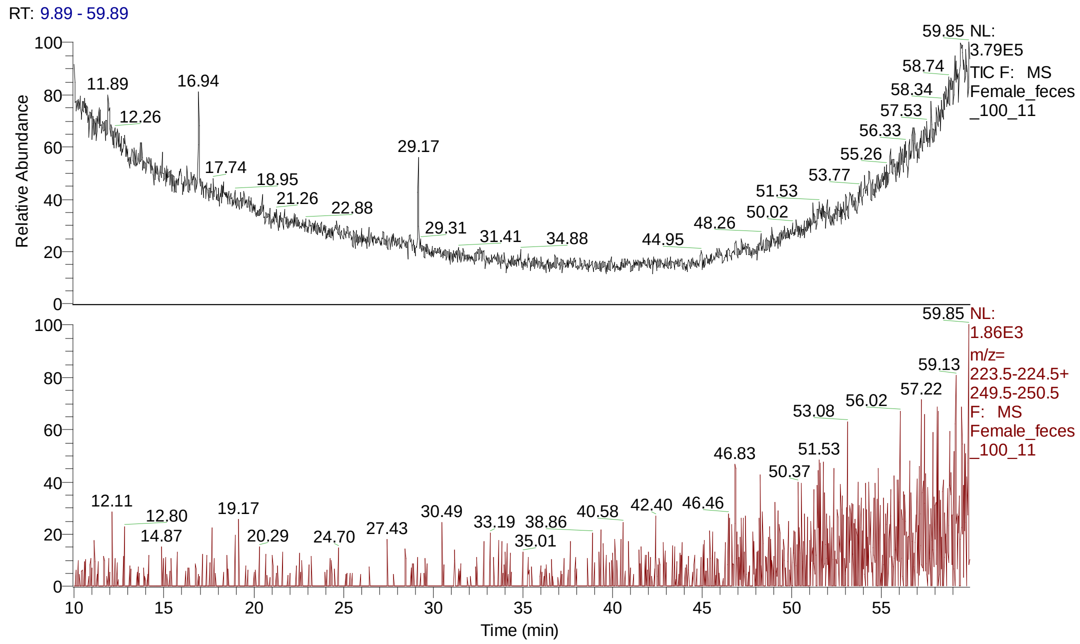

Figure 104 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (11)

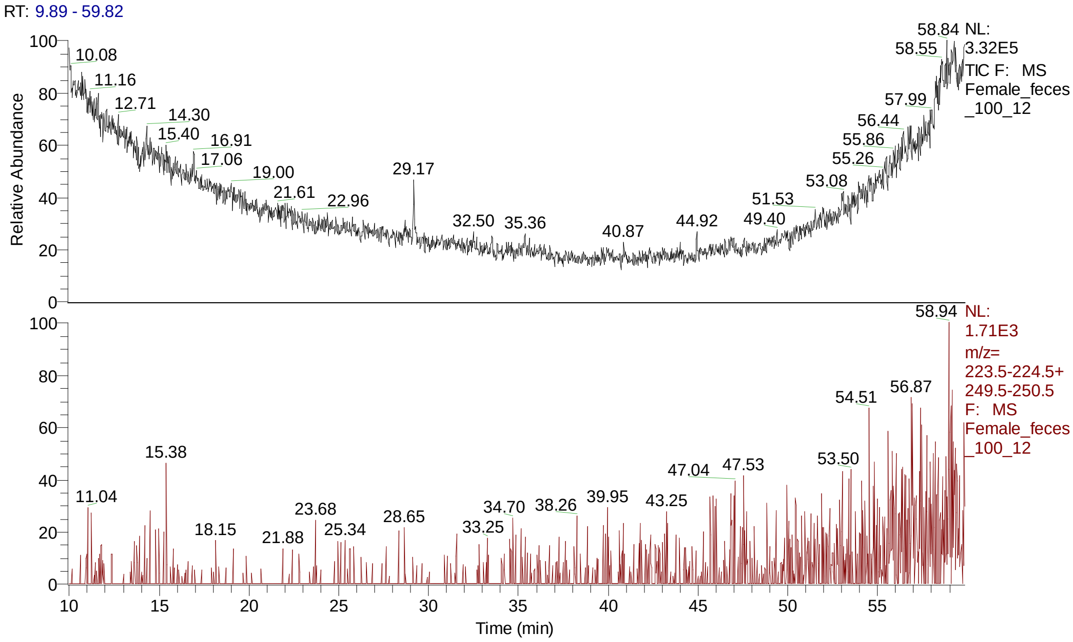

Figure 105 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (12)

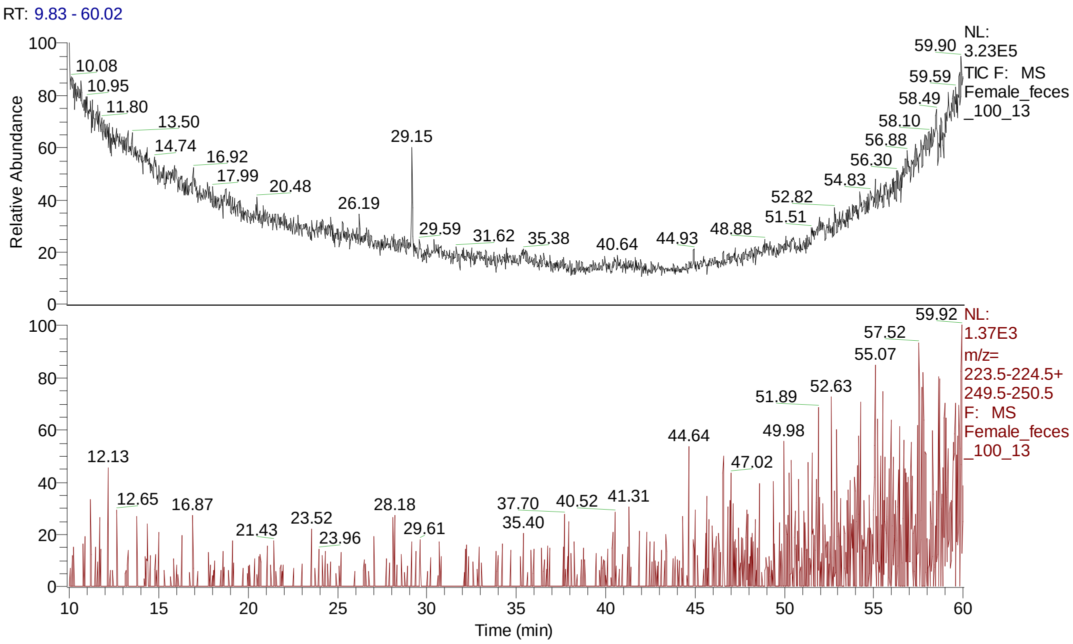

Figure 106 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (13)

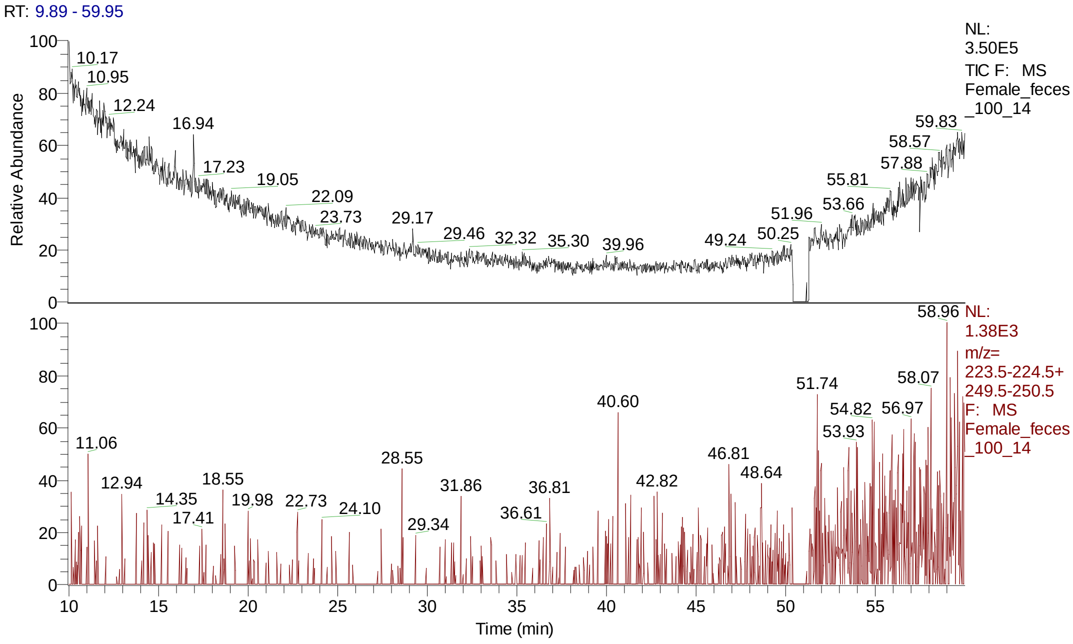

Figure 107 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (14)

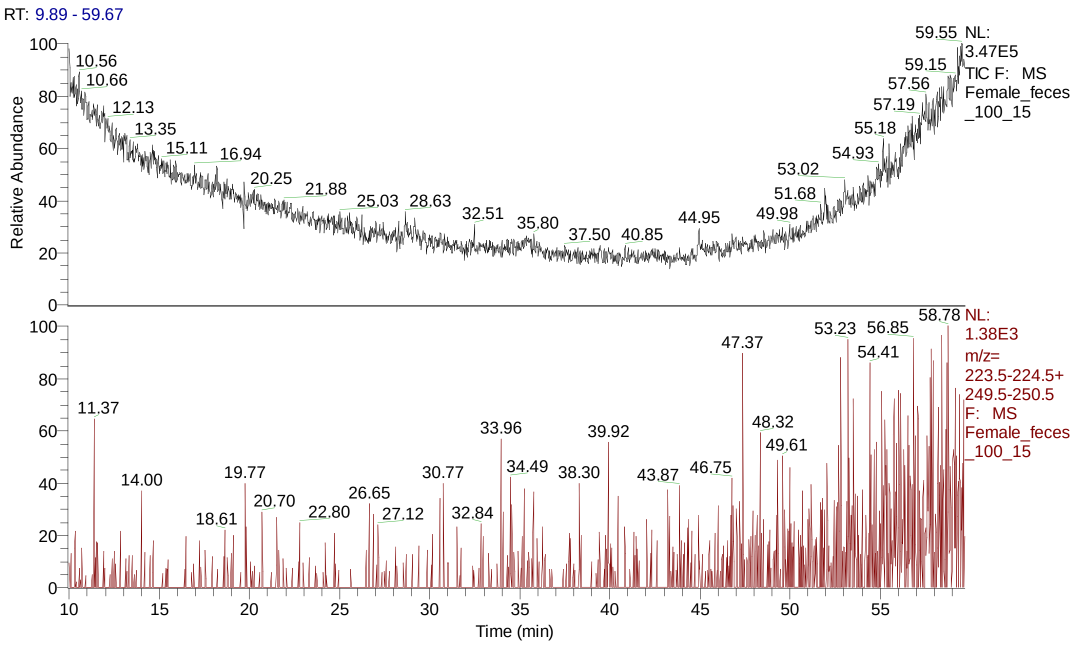

Figure 108 Full scan (upper) and SIM (lower) chromatogram of female small Indian civet feces (15)

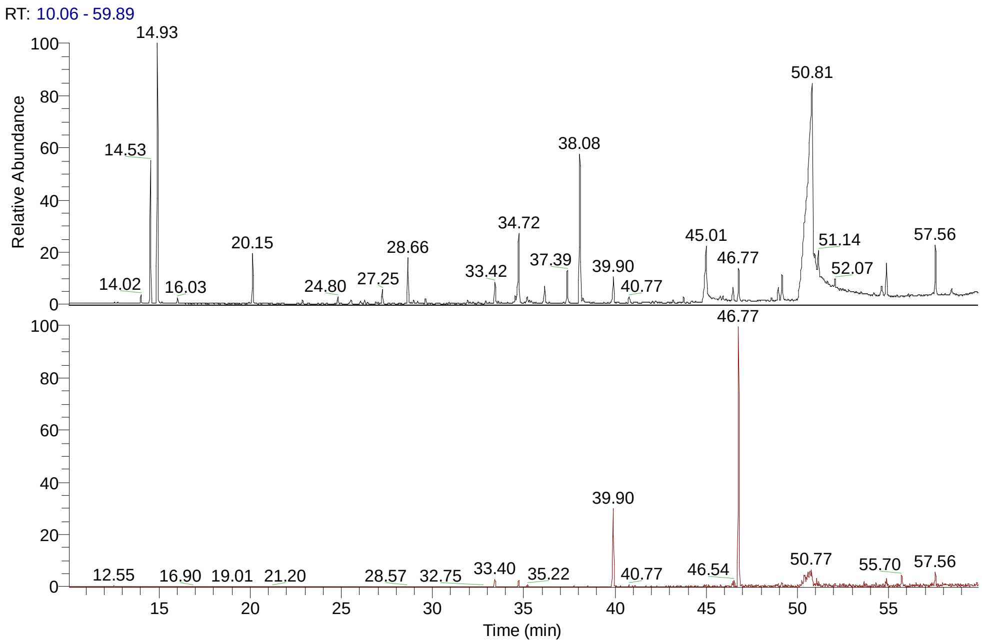

Figure 109 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy A (Lot No. 1)

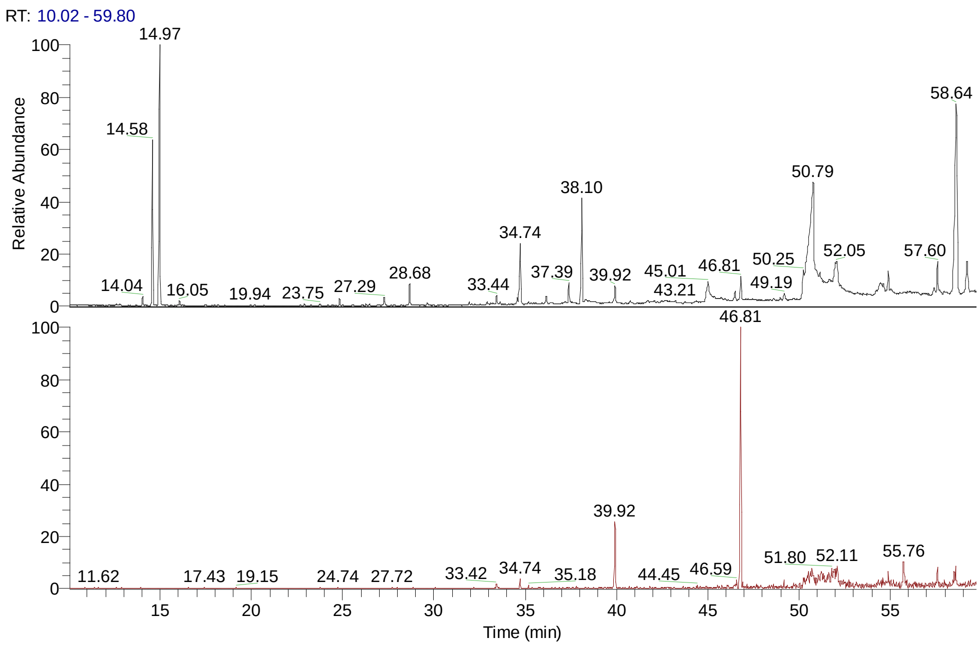

Figure 110 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy A (Lot No. 2)

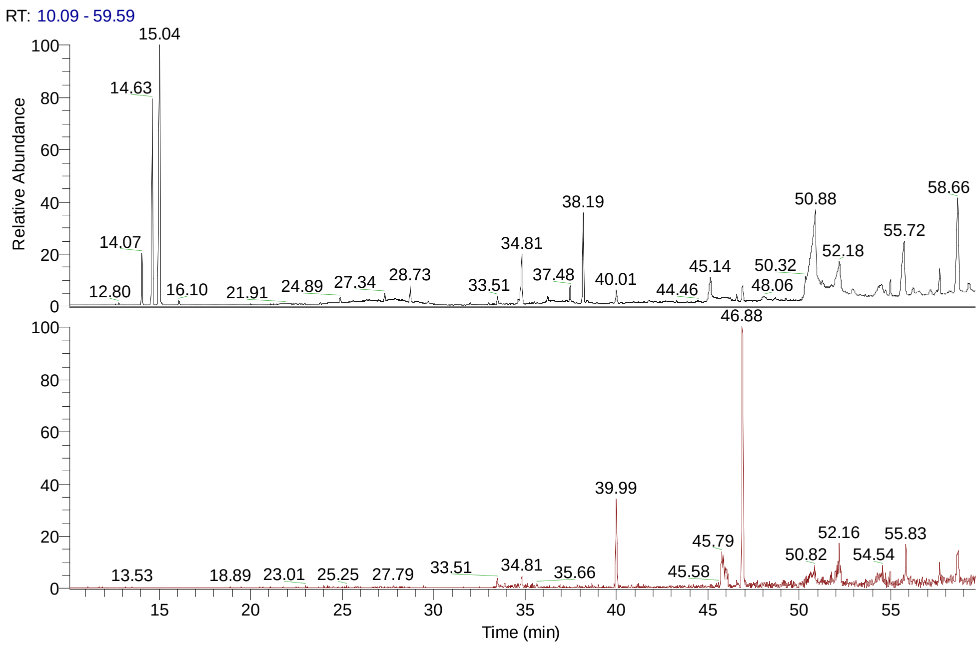

Figure 111 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy A (Lot No. 3)

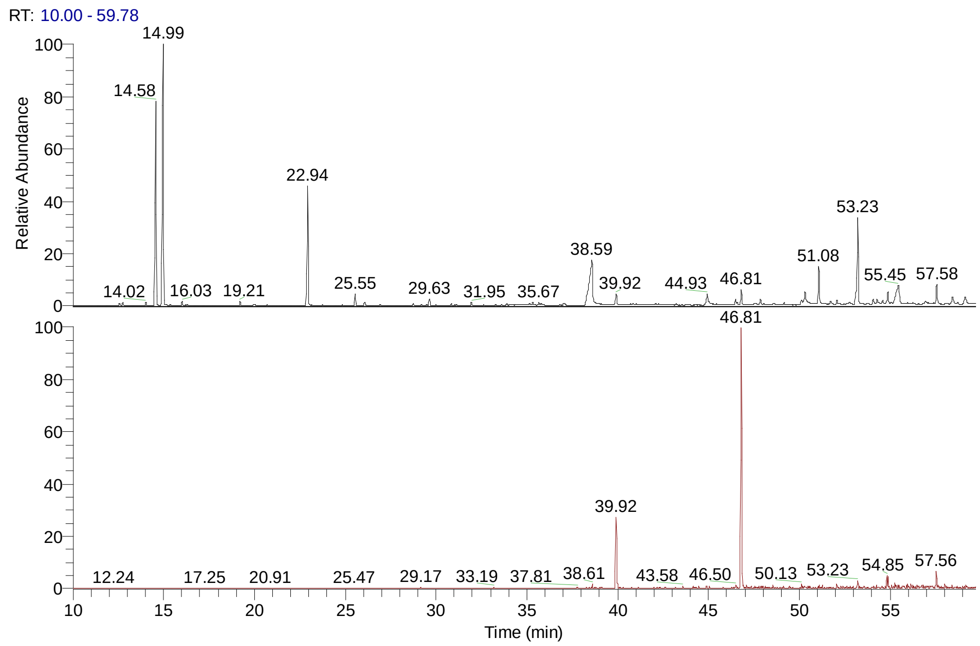

Figure 112 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy B (Lot No. 1)

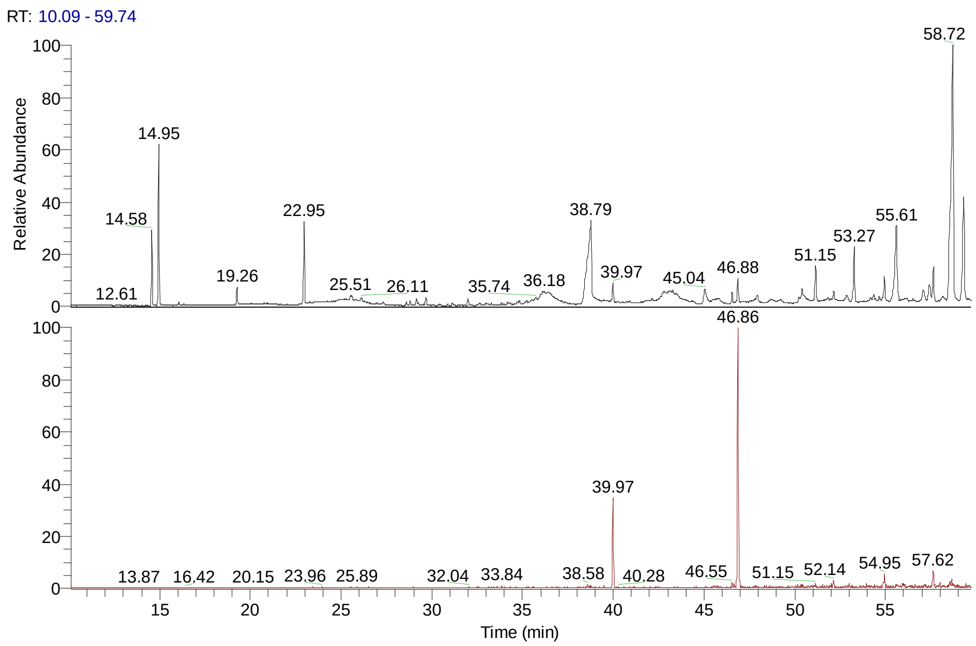

Figure 113 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy B (Lot No. 2)

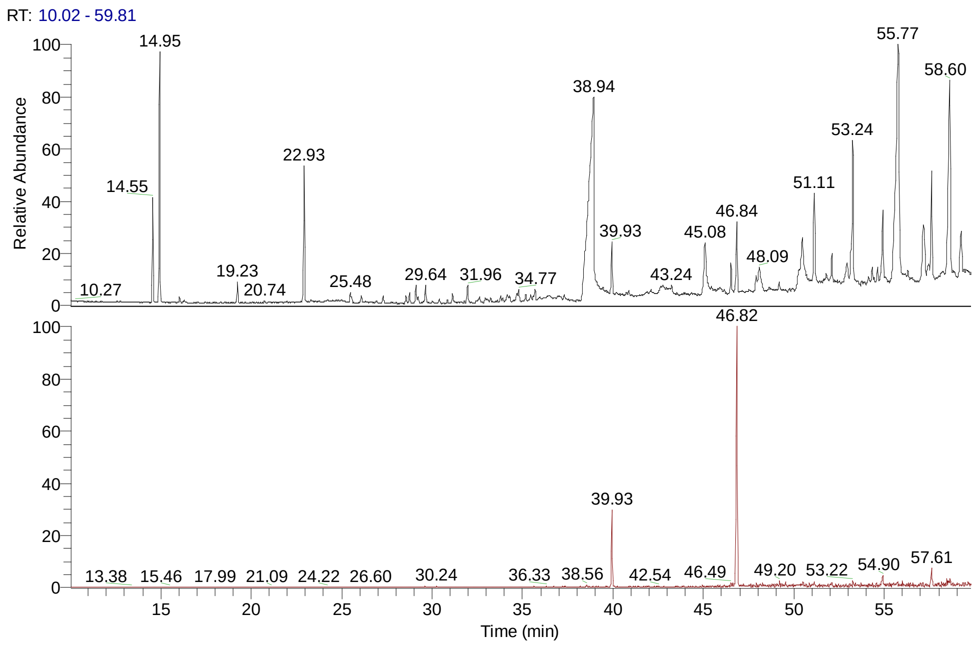

Figure 114 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy B (Lot No. 3)

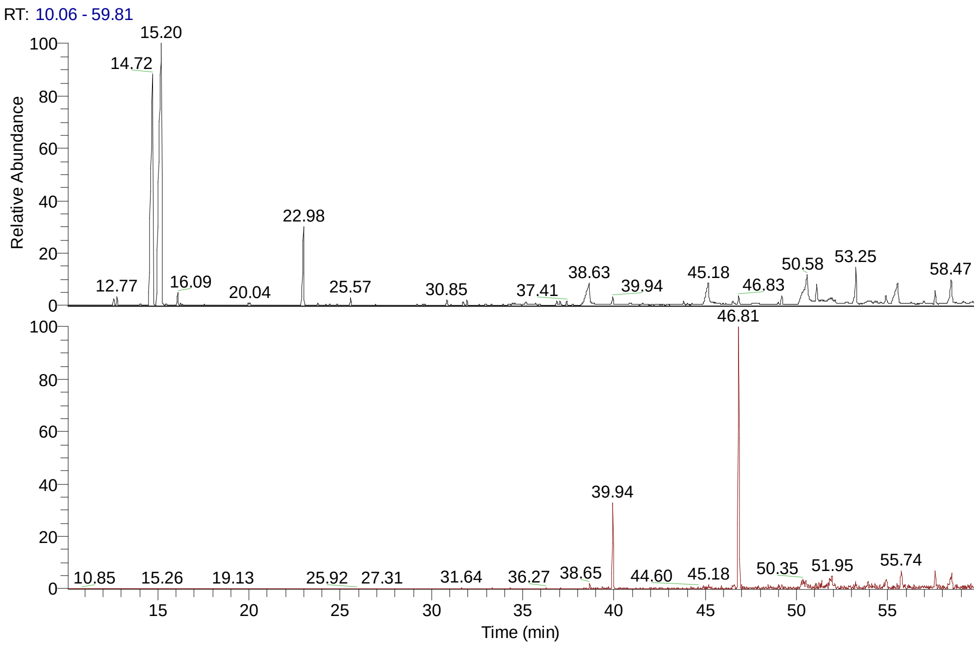

Figure 115 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy C (Lot No. 1)

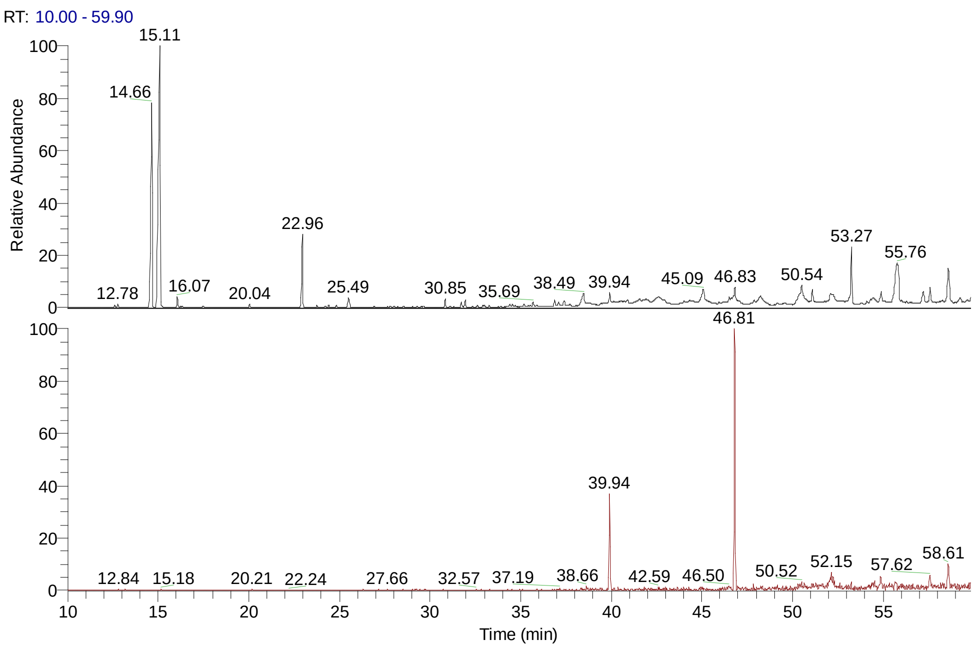

Figure 116 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy C (Lot No. 2)

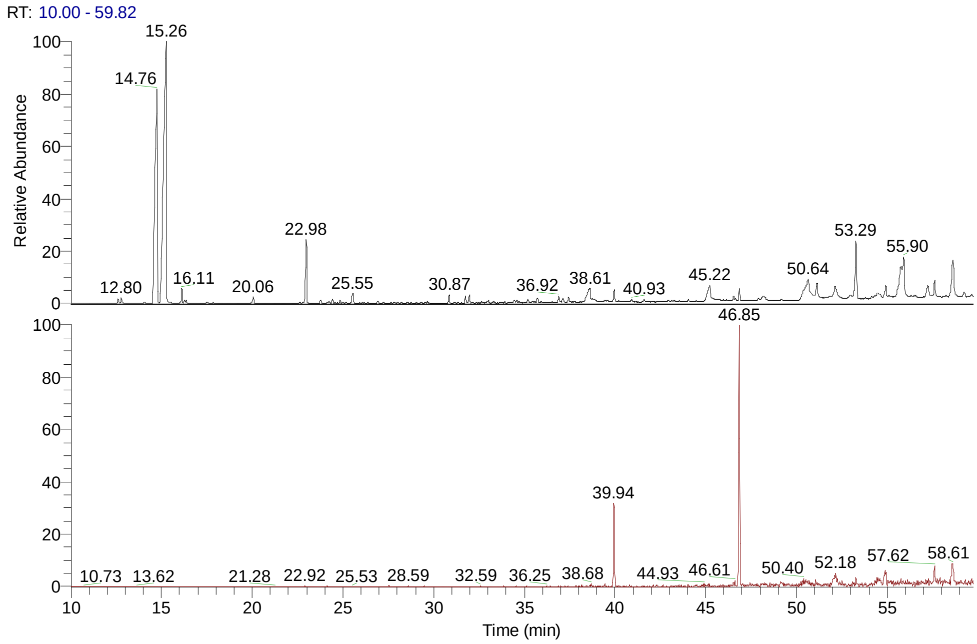

Figure 117 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy C (Lot No. 3)

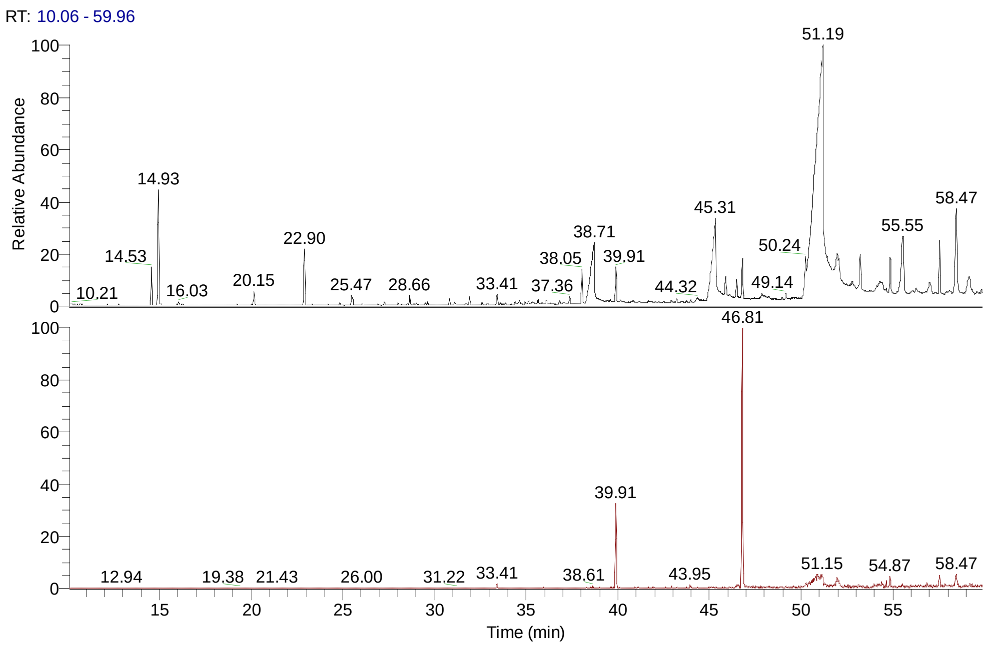

Figure 118 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy D (Lot No. 1)

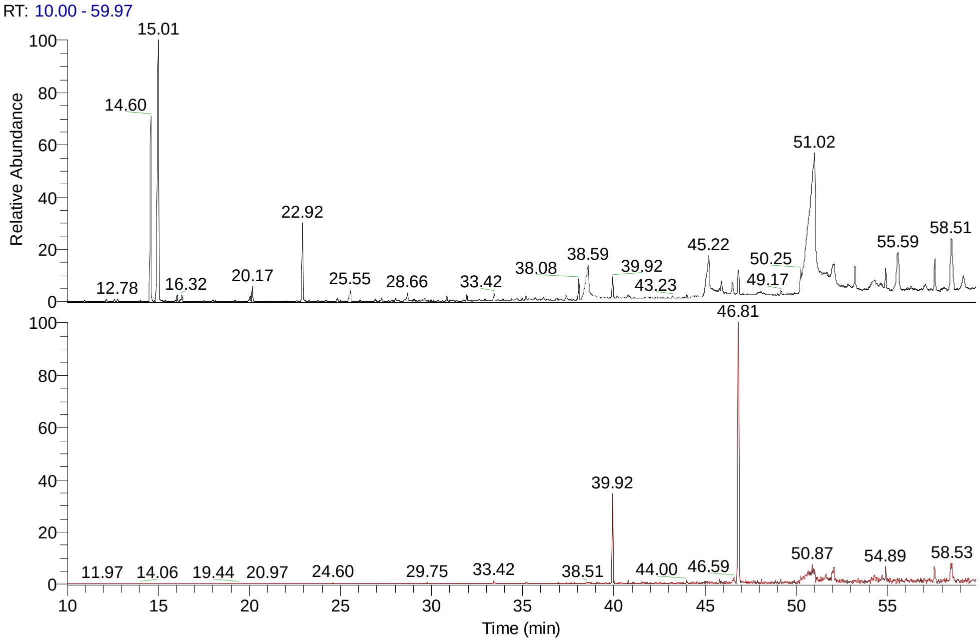

Figure 119 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy D (Lot No. 2)

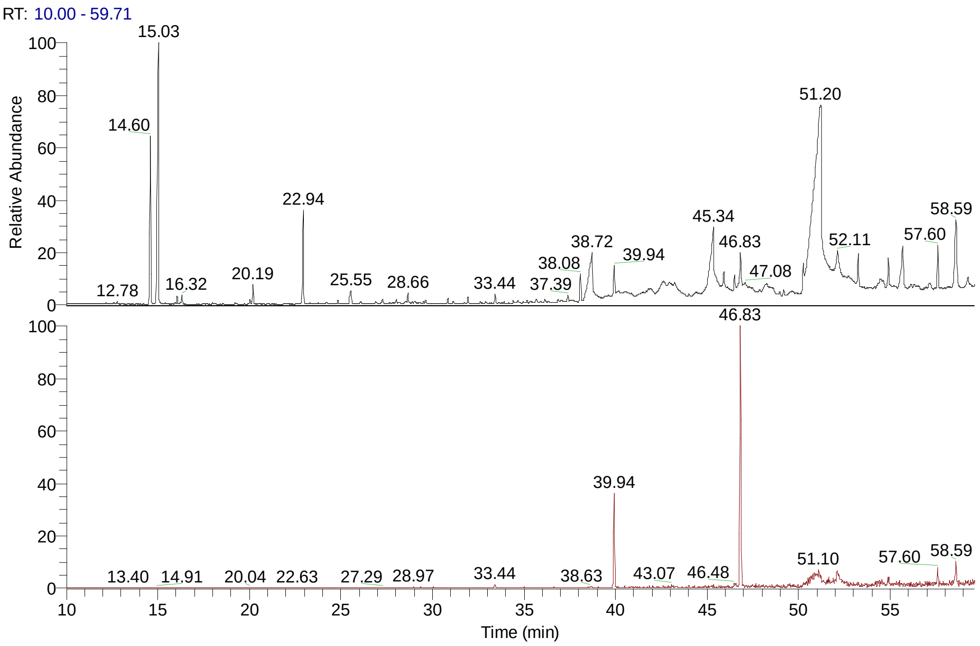

Figure 120 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy D (Lot No. 3)

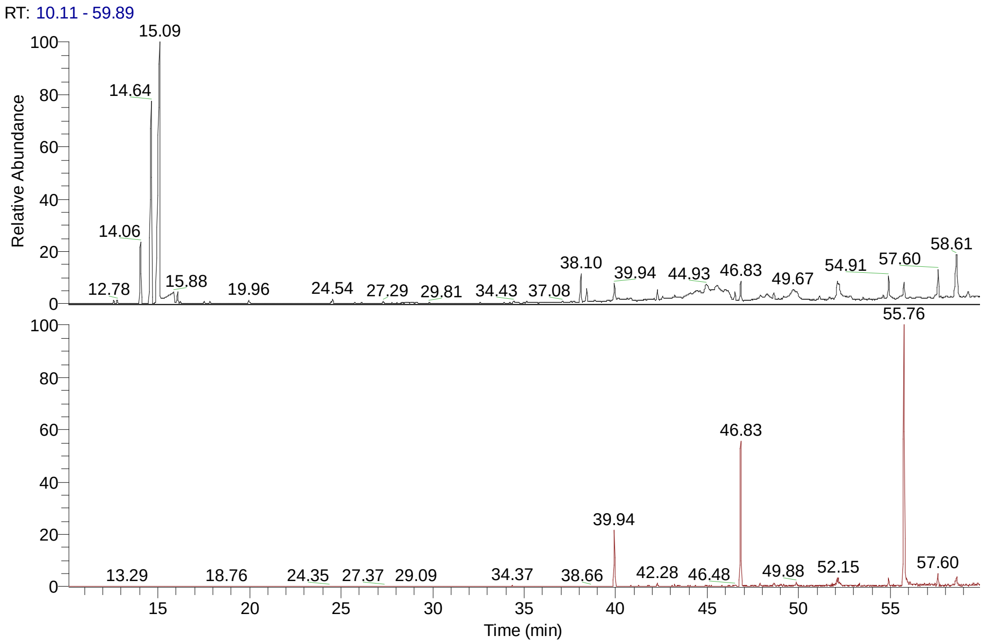

Figure 121 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy E (Lot No. 1)

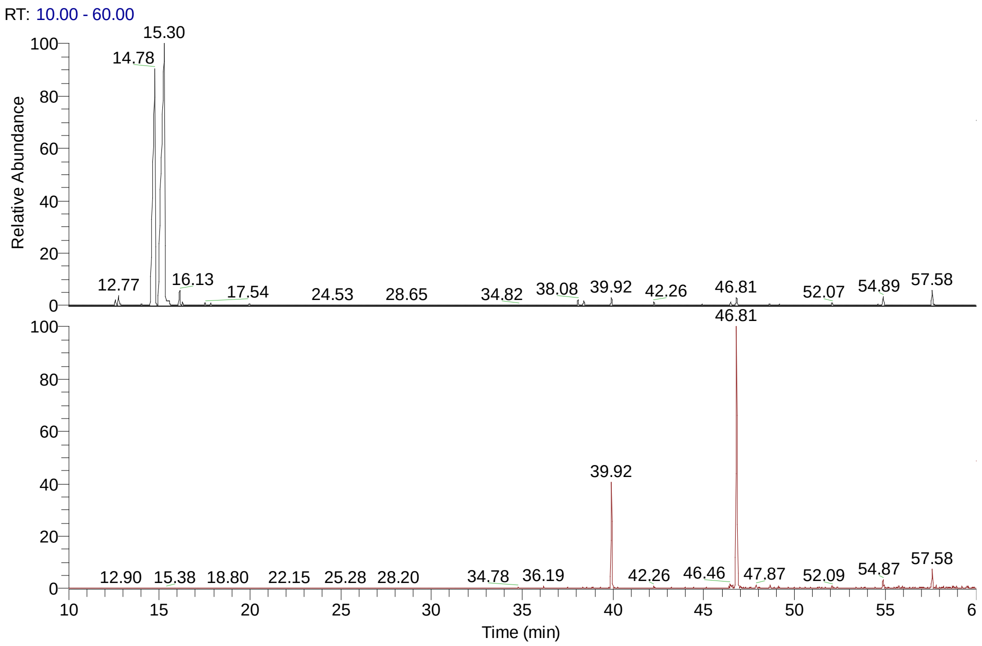

Figure 122 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy E (Lot No. 2)

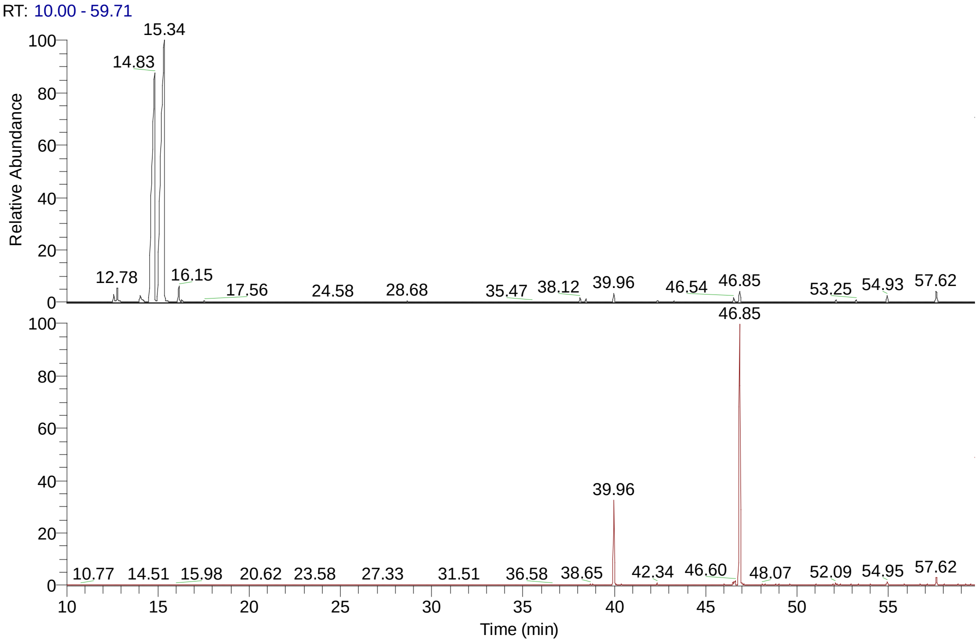

Figure 123 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy E (Lot No. 3)

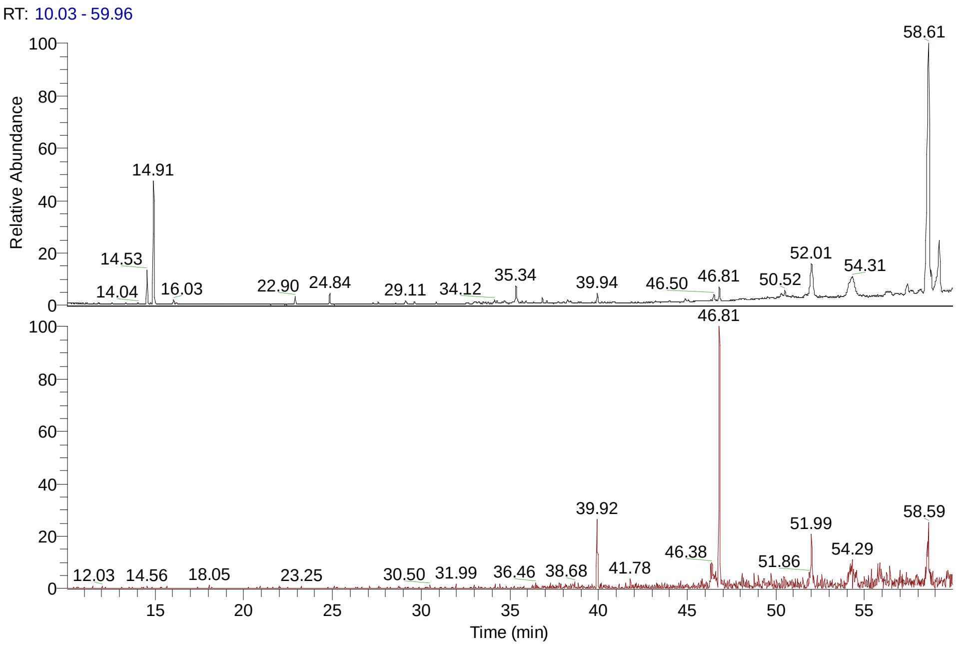

Figure 124 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy F (Lot No. 1)

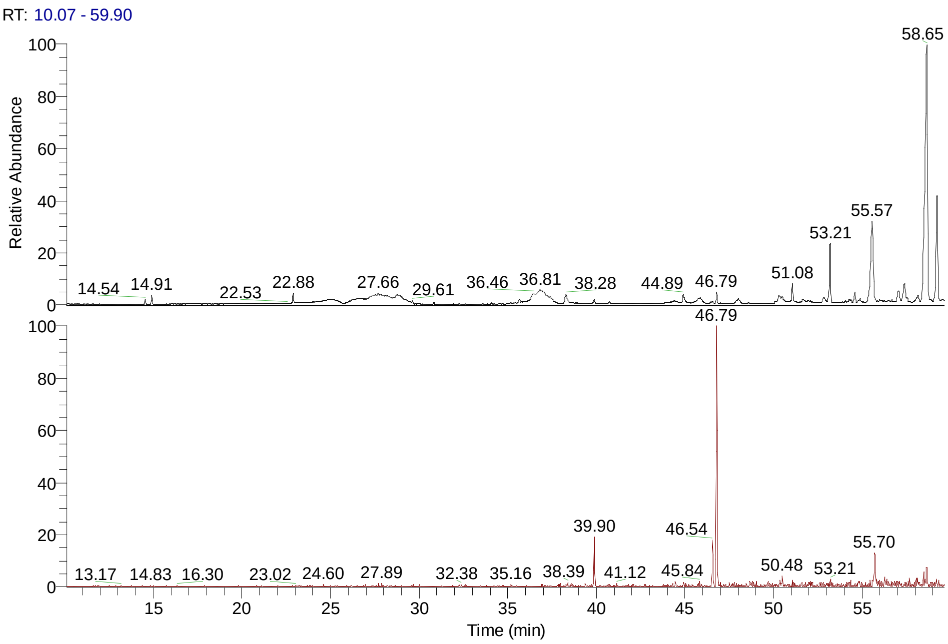

Figure 125 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy F (Lot No. 2)

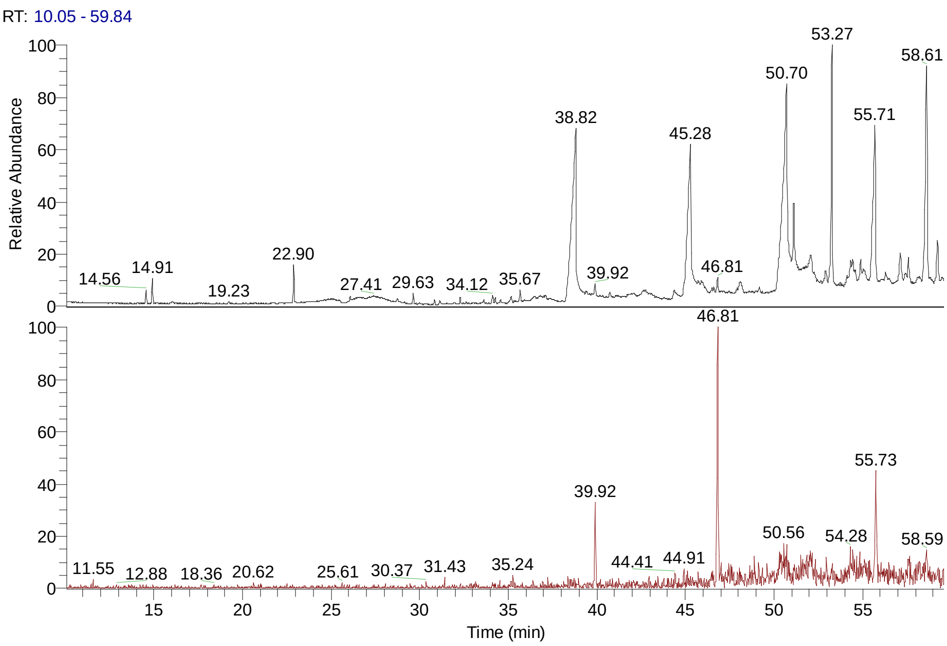

Figure 126 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy F (Lot No. 3)

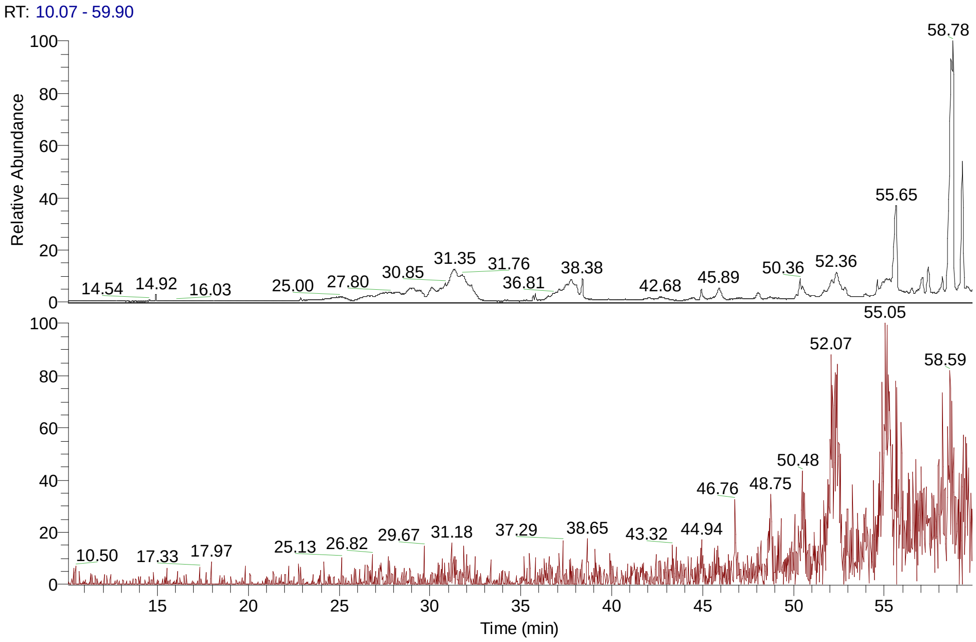

Figure 127 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy G (Lot No. 1)

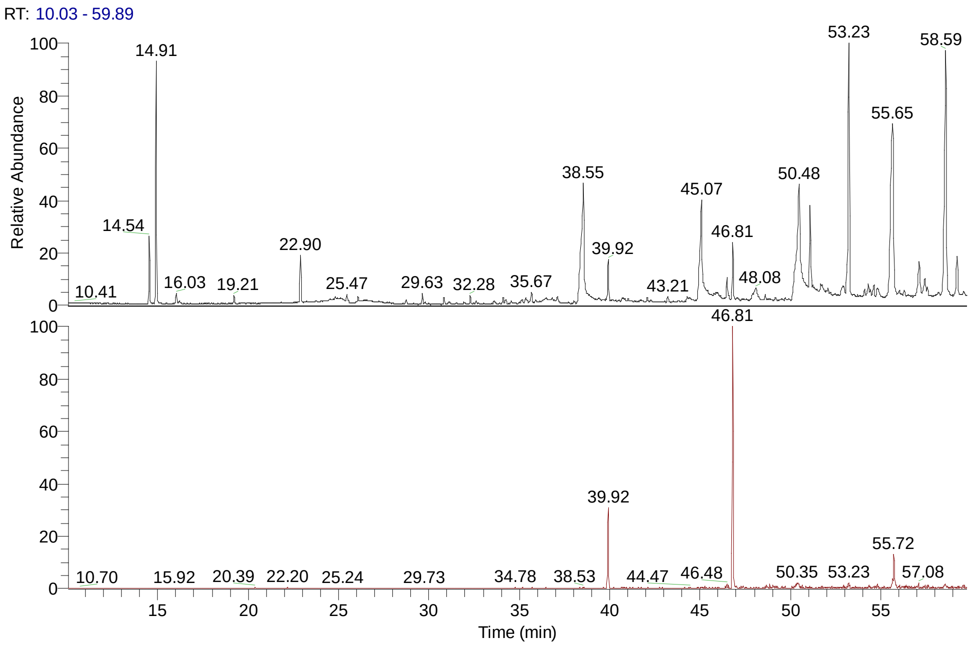

Figure 128 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy G (Lot No. 2)

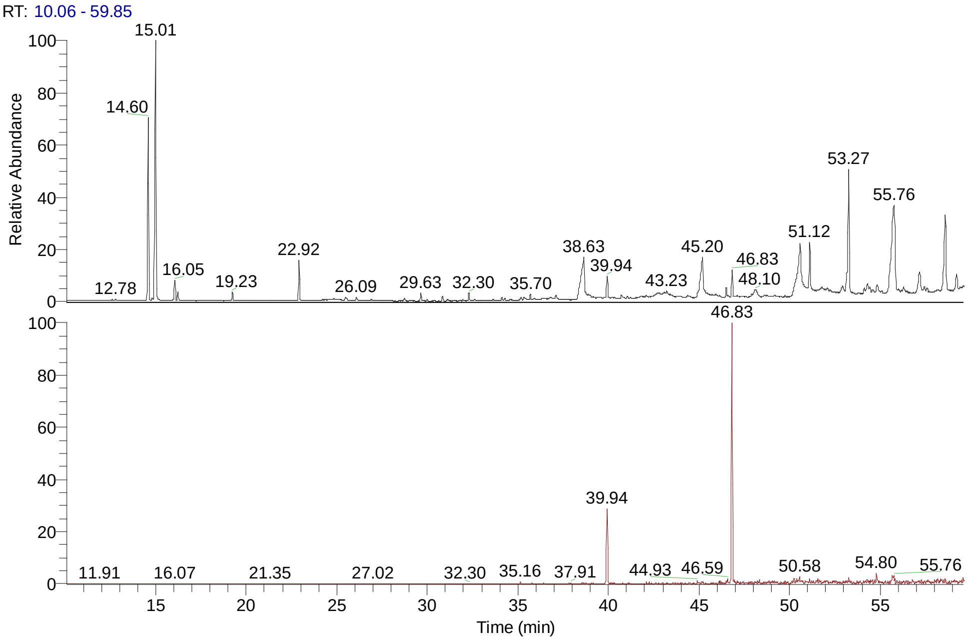

Figure 129 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy G (Lot No. 3)

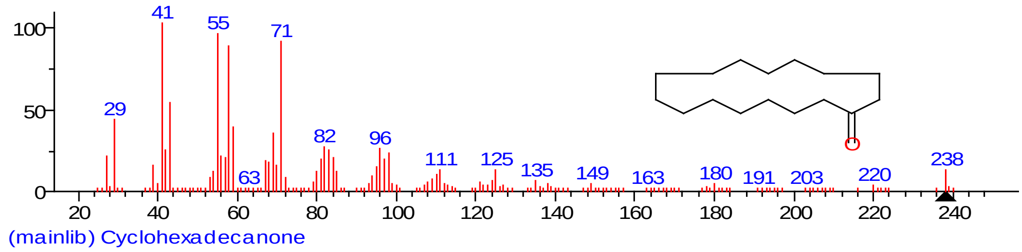

Figure 130 Mass spectrum of cyclohexadecanone

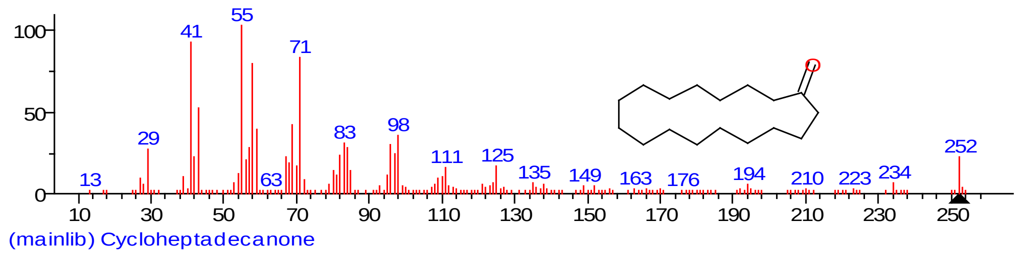

Figure 131 Mass spectrum of dihydrocivetone

APPENDIX C

Aromatic Remedies

Aromatic Remedy A

Important Ingredients

- โสมเกาหลี, Korean ginseng (*Panax ginseng* C.A. Mayer)
- พิมเสนเกล็ด, Borneo camphor (*Dryobalanops aromatica* Gaerth)
- ชะมดเช็ด, Small Indian civet secretion (*Viverricula indica* Desmarest)
- ใบพิมเสน, Patchouli (*Pogostemon cablin* (Blanco) Benth.)
- หญ้าฝรั่น, Saffron (*Crocus sativus* Linn.)

Use: relief of nausea, dizziness and fever

Direction: 5-7 tablets taken with water or stir one teaspoonful with warm water, when needed.

Aromatic Remedy B

Important Ingredients

- รากแฝกหอม, Vetiver root (*Vetiveria zizanioides* Linn.)
- อบเชยญวณ, Saigon cinnamon (*Cinamomum loureirii* Nees)
- เห็ดนมเสือ, (*Polyporus sacer* Fr.)
- หญ้าฝรั่น, Saffron (*Crocus sativus* Linn.)
- ชะมดเช็ด, Small Indian civet secretion (*Viverricula indica* Desmarest)
- โคโรค, Cow bezeoar (Calculas bovis)

Use: relief of tiredness, cough and phlegm

Direction: 3-5 tablets taken with water or stir one teaspoonful with warm water, when needed.

Aromatic Remedy C

Important Ingredients

- โสมเกาหลี, Korean ginseng (*Panax ginseng* C.A. Mayer)
- พิมเสนเกล็ด, Borneo camphor (*Dryobalanops aromatica* Gaerth)
- อำพันทอง, Ambergris (*Physeter macrocephalus* Linn.)
- หญ้าฝรั่น, Saffron (*Crocus sativus* Linn.)
- ชะมดเช็ด, Small Indian civet secretion (*Viverricula indica* Desmarest)
- คุลิก่า, Ku-li-ka

Use: for heart stimulating effect and relief of palpitate

Direction: one tablets taken with water or stir one teaspoonful with warm water, when needed.

Aromatic Remedy D

Important Ingredients

- โสมเกาหลี, Korean ginseng (*Panax ginseng* C.A. Mayer)
- พิมเสนเกล็ด, Borneo camphor (*Dryobalanops aromatica* Gaerth)
- หญ้าฝรั่น, Saffron (*Crocus sativus* Linn.)
- ชะมดเช็ด, Small Indian civet secretion (*Viverricula indica* Desmarest)
- เหง้าขิงแห้ง, Ginger (*Zingiber officinale* Roscoe)

Use: relief of nausea, vomiting and flatulence

Direction: 5-9 tablets taken with water or stir one teaspoonful with warm water, when needed.

Aromatic Remedy E

Important Ingredients

- กฤษณา, Agarwood (*Aquilaria agallocha* Roxb.)
- หญ้าฝรั่น, Saffron (*Crocus sativus* Linn.)
- ชะมดเช็ด, Small Indian civet secretion (*Viverricula indica* Desmarest)
- พิกุล, Bullet wood flower (*Mimusops elengi* Linn.)

Use: for heart stimulating effect, relief of nausea, vomiting, dizziness, tiredness, flatulence, and nuturing of pregnancy
Direction: 0.25 g stir one teaspoonful with warm water, 3 times a day.

Aromatic Remedy F

Important Ingredient

- จันทน์แดง, Red sandal wood (*Pterocarpus santalinus* Linn.)
- ชะเอมเทศ, Licorice (*Glycyrrhiza glabra* Linn.)
- ลูกจันทน์, Nutmeg (*Myristica fragrans* Houtt.)
- กานพลู, Clove (*Eugenia caryophyllata* Thunb.)
- อบเชย, Cinnamon (*Cinnamomum cassia* J. S. Presl)
- หญ้าฝรั่น, Saffron (*Crocus sativus* Linn.)
- ชะมดเช็ด, Small Indian civet secretion (*Viverricula indica* Desmarest)
- พิมเสน, Borneo camphor (*Dryobalanops aromatica* Gaerth)
- กฤษณา, Agarwood (*Aquilaria agallocha* Roxb.)

Use: for heart stimulating effect, relief of nausea, dizziness, tiredness, and nuturing of pregnancy
Direction: 2-3 tablets taken with water, when needed.

Aromatic Remedy G

Important Ingredient

- หญ้าฝรั่น, Saffron (*Crocus sativus* Linn.)
- พิมเสน, Borneo camphor (*Dryobalanops aromatica* Gaerth)
- ชะมดเช็ด, Small Indian civet secretion (*Viverricula indica* Desmarest)
- กฤษณา, Agarwood (*Aquilaria agallocha* Roxb.)
- กระลำพัก, Triangular Spurge (*Euphorbia antiquorum* Linn.)
- กานพลู, Clove (*Eugenia caryophyllata* Thunb.)
- ขอนดอก, Bullet wood (*Mimusops elengi* Linn.)
- จันทน์แดง, Red sandal wood (*Pterocarpus santalinus* L.f.)
- แฝกหอม, Vetiver (*Vetiveria zizanioides*)
- ชะเอม, Licorice (*Glycyrrhiza glabra* Linn.)
- อบเชย, Cinnamon (*Cinnamomum cassia* J. S. Presl)
- ทองคำเปลว, Gold leaf

Use: for heart stimulating effect, relief of nausea, dizziness, tiredness, thirst, and phlegm, and nuturing of pregnancy

Direction: 1-3 tablets taken with water, when needed.

Aromatic Remedy without Small Indian Civet secretion

Important Ingredient

- ตักเฮียง, Korean Mint (*Agastache rugosa* Fisch. et Mey.) O. Kuntze.
- อบเชย, Cinnamon (*Cinnamomum cassia* J. S. Presl)
- โสยเซ็ง, Soie-cheng (*Asarum sieboldii* Mig.)
- ชะเอม, Licorice (*Glycyrrhiza glabra* Linn.)
- กานพลู, Clove (*Eugenia caryophyllata* Thunb.)
- บักเฮียง, โกฐกระดูก, Kote-kra-dook (*Saussurea lappa* Clarke)
- กฤษณา, Agarwood (*Aquilaria agallocha* Roxb.)
- โกฐสอ, Angelica root (*Angelica anomala* Lallem)

Use: relief of nausea, dizziness, faint, tiredness, and stomach troubles
Direction: (For adult) Stir one teaspoonful with warm water, 3-4 times a day.
(For Children) one half of the adult dose.

APPENDIX D

Publications

NRCT-JSPS Core University Program on
Natural Medicine in Pharmaceutical Sciences

The 9th Joint Seminar

Natural Medicine Research for the Next Decade:
New Challenges and Future Collaboration

December 8-9, 2010
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Bangkok, Thailand

Jointly organized by

Faculty of Pharmaceutical Sciences, Chulalongkorn University
Chulabhorn Research Institute
National Research Council of Thailand (NRCT)
Institute of Natural Medicine, University of Toyama
Japan Society for the Promotion of Science (JSPS)

PROCEEDINGS

The 9th NRCT-JSPS Joint Seminar
*Natural Medicine Research for the Next Decade:
New Challenges and Future Collaboration*

PP 8

CHEMICAL CONSTITUENTS OF *VIVERRICULA INDICA* SECRETION

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KEYWORDS: civet cat secretion, civetone, normuscone, cyclohexadecanone, dihydrocivetone

INTRODUCTION

Civetone (9-Cycloheptadecen-1-one) is a macrocyclic ketone which is a pheromone sourced from civet cat (*Viverricula indica* Desmarest). It is a powerful musky odor that becomes pleasant at extreme dilutions (1). Civetone has been used for many famous perfumes as the important raw material. Secretion from civet cat is commonly used in Thai traditional medicine especially in aromatic remedy to relieve faint, vertiginous, and cough. This research attempted to investigate the chemical constituents in secretion of *V. indica* cultivated in Thailand.

MATERIALS AND METHODS

Thirty samples of secretion from *Viverricula indica* (male: female, 1:1) were collected from a civet cat farm in Phetchaburi, Thailand. One milligram of each sample was extracted with 1 ml of hexane and centrifuged at 10,000 rpm for 10 min at 25°C. One microliter of the extract was analyzed by gas chromatography/mass spectrometry. The analysis was performed using a finnigan trace GC ultra gas chromatograph (Thermo electron corporation, USA) equipped with ZB-5 capillary column (30m x 0.25mm x 0.25μm) and interfaced to a finnigan trace DSQ MS detector. The oven temperature was ramped from 60°C to 240°C at a constant rate of 3°C/min. The injection port was held at 180°C throughout the separation. The carrier gas was helium with a flow rate of 1ml/min and split ratio of 10:1. MS was performed by E1 positive mode at 70 eV ionization voltages. The constituents of the extract were identified by matching their mass spectra and retention time indicated with NIST05 MS library and the percentage compositions were computed from GC peak areas.

RESULTS

Civetone is a major chemical constituent in the female civet cat secretion while it is smaller amount in the male civet cat secretion. Three main chemical constituents of male civet cat secretion were normuscone, dihydrocivetone and civetone with the percent area of 73.4±7.0, 5.1±1.5, and 3.2±1.9 % respectively. The female civet cat secretion exhibited four main chemical constituents including civetone, dihydrocivetone, normuscone, and cyclohexadecanone with the percent area of 56.1±5.2, 16.8±2.9, 11.8±2.1, and 3.2±0.6 % respectively.

DISCUSSION

The results showed that the secretion of civet cat consisted of civetone, dihydrocivetone and normuscone as main components which were related to the previous study (2). Cyclohexadecanone was found only in the secretion of female civet cat. The results suggested that the content of chemical constituents was different between secretion of male and female civet cat. Civetone was dominated in female whereas mormuscone was dominated in male civet cat secretion. This finding was in accordance with the civet cats in China (3).

CONCLUSION

Sex dependent difference in chemical constituents of Thai *V. indica* secretion was demonstrated and should be concerned for their application.

PP 8

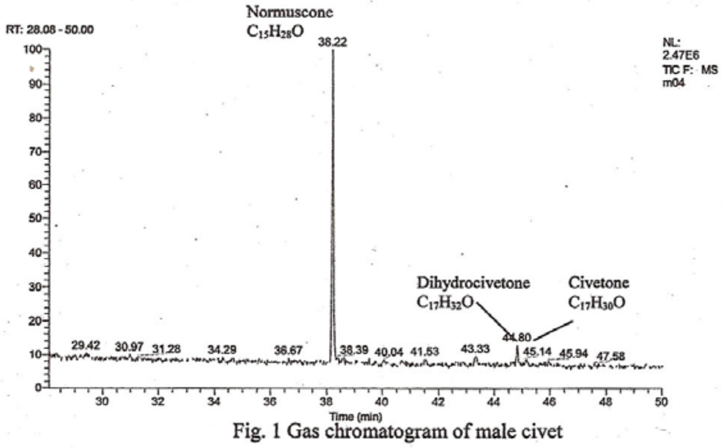

Fig. 1 Gas chromatogram of male civet

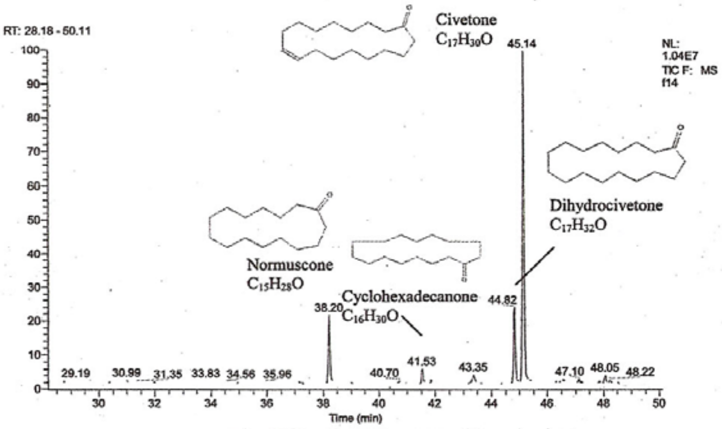

Fig. 2 Gas chromatogram of female civet

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3. He Lianghua and Lu Hauchong. 1983. Gas-liquid chromatography of civetone and its homologs. Fenxi Huaxue. 10(11): 781-783.

Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research

ISSN No: 0975-7384
CODEN(USA): JCPRC5

J. Chem. Pharm. Res., 2011, 3(5):196-204

Quantitative analysis of civetone and normuscone in secretion from *Viverricula indica* and in aromatic remedies by gas chromatography-mass spectrometry

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ABSTRACT

The quantitation of civetone and normuscone in small Indian civet (Viverricula indica Desmarest) secretion cultivated in Thailand was analyzed by gas chromatography- mass spectrometry (GC-MS). Three main chemical constituents of small Indian civet secretion were civetone, dihydrocivetone and normuscone. The contents of chemical constituents were different between male and female secretion. Civetone (23.6 ± 1.5 µg/mg of secretion) was a major chemical constituent in the female secretion while normuscone (52.1 ± 5.9 µg/mg of secretion) was a major in the male secretion. Both civetone and normuscone were found in civet fur but not in the feces. Aromatic remedies which claimed to use the small Indian civet secretion as an ingredient showed both civetone and normuscone. Linearity range of civetone was 0-50 µg/ml with a correlation coefficient of 0.9717, and of normuscone was 0-80 µg/ml with a correlation coefficient of 0.9965. The average recoveries were 97.3-98.0% in secretion and 91.4-105.7% in aromatic remedy for civetone. For normuscone, average recoveries in secretion and aromatic remedy were 98.5 % and 90.0-103.0% respectively. The intra-day and inter-day RSDs of the three components were less than 8%. Civetone and normuscone could be used as quantitatively marker for civet secretion ingredient in aromatic remedies. Civetone and normuscone contents in commercial civet secretion varied crop by crop and depended on male to female sex ratio of the small Indian civets.

Keywords: small Indian civet secretion, aromatic remedy, civetone, normuscone, gas chromatography-mass spectrometry

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INTRODUCTION

Small Indian civet is a mammal in a group of carnivores. It is found in Southeast Asia, Pakistan, India, Nepal, Bangladesh and South China [1]. Both male and female produce the strong smelling secretion from the perineal gland. Civetone is the main constituent of the secretion that produced from the civet of the genera Civettictis, Viverra and Viverricula [2]. Moreover, the civet secretion contains other macrocyclic ketones such as cyclohexadecanone, cycloheptadecanone, and 6-*cis*-cycloheptadecenone [3]. This secretion is widely used in perfume industry and in traditional medicine for a long time. Thai traditional medicine uses the secretion as an ingredient in aromatic remedy for relief of faint, dizziness, nausea and vomiting. In the local market, there are many aromatic remedies that claim to use the secretion as an ingredient in the remedies but it cannot prove that there is the secretion in the remedies. Nowadays, the secretion is adulterated with vaseline and petrolatum to increase the quantity because of the expensive secretion. In Thailand, there has been no report about the chemical constituents in secretion of small Indian civet. GC-MS is useful and applicable for qualitative and quantitative investigation of the chemical composition in complex mixtures for example the essential oil components as well as phytoconstituent analysis [4-7]. Hence, this study attempted to determine the chemical constituents of secretion from *V. indica* cultivated in Thailand and investigate *V. indica* secretion ingredient in aromatic remedies by GC-MS analysis.

EXPERIMENTAL SECTION

Chemicals and materials

Civetone (9-cyclohetadecon-1-one, CAS no 542-46-1) and normuscone (cyclopentadecanone, CAS no 502-72-7) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hexane was of AR grade (Lab-Scan Asia Co., LTD, Bangkok, Thailand). Polytetrafluoroethylene (PTFE) syringe membrane filter (0.45 µm) was from Chrom Tech, Inc (USA).

Sample collection

Male small Indian civet (*V. indica*) secretion (n =15), female small Indian civet secretion (n =15), pooled small Indian civet secretion (n = 10), small Indian civet feces (n = 15), and small Indian civet fur (n = 3) were collected from a civet farm in Petchaburi, Thailand. Each sample was kept in tightly capped vial and refrigerated until analysis.

Three different lot numbers of aromatic remedies with civet secretion ingredient in the label and one aromatic remedy not containing civet secretion ingredient were collected from the local markets. Each aromatic remedy was stored at ambient temperature until analysis.

Sample preparation

One milligram of secretion was mixed with 1 ml of hexane, vortex for 1 min, centrifuged at 10,000 rpm for 10 min at 25°C. One microliter of hexane supernatant was analyzed by GC-MS.
One hundreds milligrams of each feces was dissolved in 1 ml of hexane and vortex for 1 min. This solution was centrifuged at 10,000 rpm for 10 min at 25°C. One microliter of the hexane supernatant was analyzed by GC-MS.

Fifteen milligrams of small Indian civet fur was washed in aliquots of 2 ml hexane until exhaustion. Washing hexane aliquots were kept for further analysis. The fur was removed, dried and cut into fine pieces. Five milligrams of the washed fine pieces of small Indian civet fur was mixed with 1 ml of hexane and sonicated at 30°C for 15 min at 53 KHz. Then, it was centrifuged at 10,000 rpm for 10 min at 25°C. Hexane extract as well as washing hexane aliquots were analyzed by GC-MS.

One hundreds milligrams of each aromatic remedy was mixed with 1 ml of hexane and vortex for 1 min. Then, it was filtered through 0.45 µm PTFE membrane filter and evaporated. After this, the extract was adjusted to 250 µl of hexane and vortex again. The solution was analyzed by GC-MS.

Each sample was performed in triplicates.

Instruments and chromatographic conditions

The analysis was performed using a Finnigan trace GC ultra gas chromatography (Thermo Fisher Scientific Inc., USA) equipped with ZB-5 capillary column (30m x 0.25mm x 0.25µm) and interfaced to a Finnigan trace DSQ MS detector. The oven temperature was ramped from 60°C to 240°C at a constant rate of 3°C/min. The injection port was held at 180°C throughout the separation. The carrier gas was helium with a flow rate of 1ml/min and split ratio of 10:1. MS was performed by electron ionization (EI) mode at 70 electron volts.

Identification and determination of compounds

The chemical constituents in the secretion extract were identified by matching their mass spectra and retention time indicated with Adams Essential Oils Mass Spectral library and NIST 05 Mass Spectral library. The contents of civetone and normuscone in secretion were determined by comparing the area under peak with the calibration. The average contents were expressed as grand mean ± pooled standard deviation in µg / mg of secretion.

Method validation

- Calibration curve and linearity

Stock solution of civetone (1mg/ml) was prepared by dissolving 1.1 µl of civetone (density = 0.917 at 33°C) in 1 ml of hexane. Stock solution of normuscone (1mg/ml) was prepared by dissolving 1 mg of normuscone in 1 ml of hexane. The stock solutions were diluted at various concentrations for calibration curves and linearity range.

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

LOD and LOQ determination were based on the standard deviation of the blank. The triplicates of 1 mg/ml of aromatic remedy without civet secretion ingredient (blank sample) were prepared and analyzed. LOD and LOQ were calculated as follow [8]:

LOD = mean of blank sample + 3SD

LOQ = mean of blank sample + 10SD

- Precision

The precision of the method was assessed with intra-day and inter-day analyses. For repeatability, different concentration levels (3 concentrations / triplicate) which covered the specified range were analyzed on day 1 and this were repeated on 3 consecutive days. Relative standard deviation (RSD) was used to measure precision [8].

- Recovery

The extraction efficiency method was used for recovery evaluation of civet secretion by re-extracting the residue until exhaustion [9] and determining civetone and normuscone by GC-MS. The extraction of civetone and normuscone was performed at two concentrations of the secretion (1 and 2 mg/ml). The percentage of recovery was calculated as follow: % recovery = (Concentration in the first filtrated x 100)/ Concentration in sum of filtrated.

Recovery of aromatic remedy was carried out by spiking three concentrations of standard solution. Recovery (%) = $(A_s\text{-}A)/A_a$ x 100. A_s refers to the amount of civetone or normuscone that found after spiking of the standard solution whereas A refers to the amount of those found that before spiking and A_a refers to the amount of reference standards actually added to the sample. The average recoveries of every spiking concentration were calculated.

RESULTS AND DISCUSSION

Small Indian civet secretion constituents

Three main chemical constituents of male small Indian civet secretion (Figure 1) were normuscone, dihydrocivetone and civetone with the percent area of 73.4 ± 7.0, 5.1 ± 1.5, and 3.2 ± 1.9 % respectively. The female small Indian civet secretion exhibited four main chemical constituents (Figure 2) including civetone, dihydrocivetone, normuscone, and cyclohexadecanone with the percent area of 56.1 ± 5.2, 16.8 ± 2.9, 11.8 ± 2.1, and 3.2 ± 0.6 % respectively.

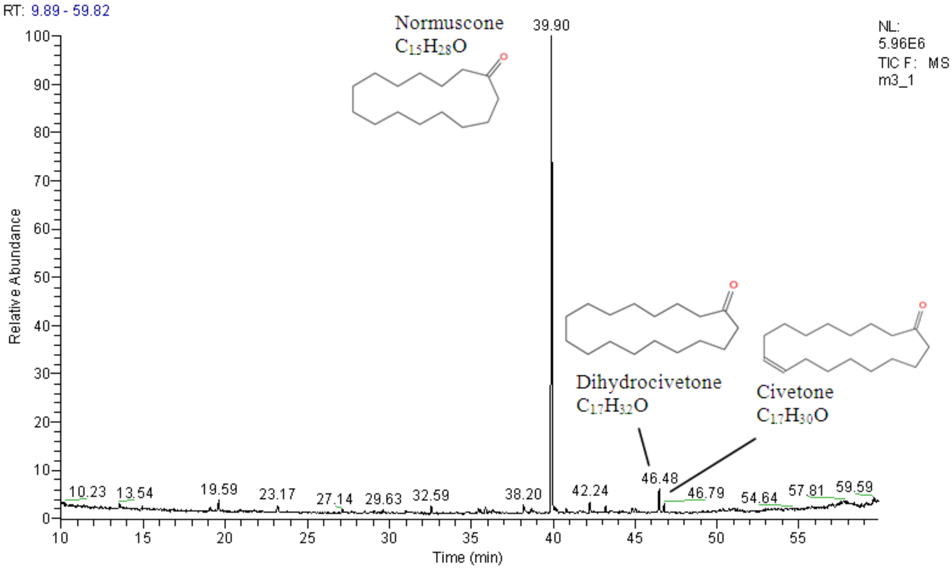

Figure 1 GC chromatogram of male small Indian civet secretion

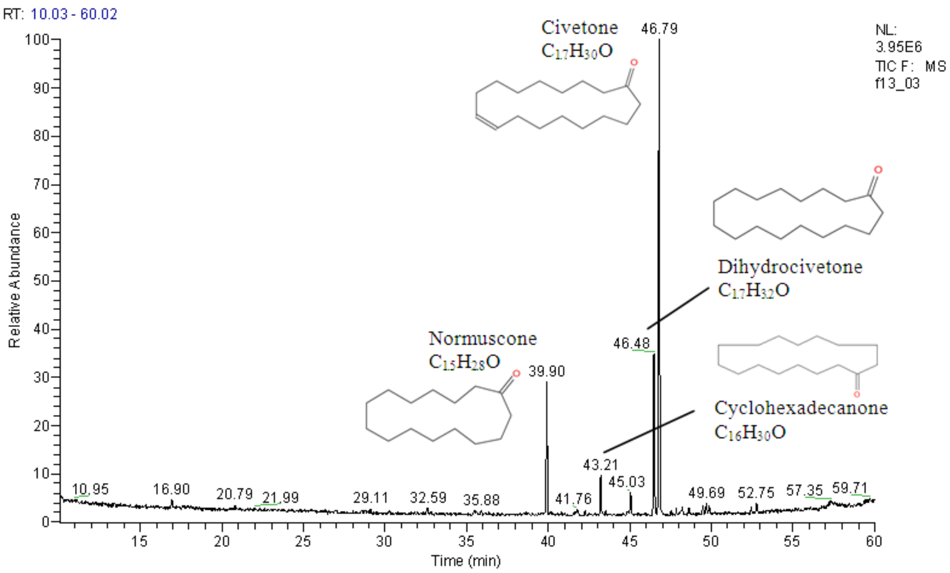

Figure 2 GC chromatogram of female small Indian civet secretion

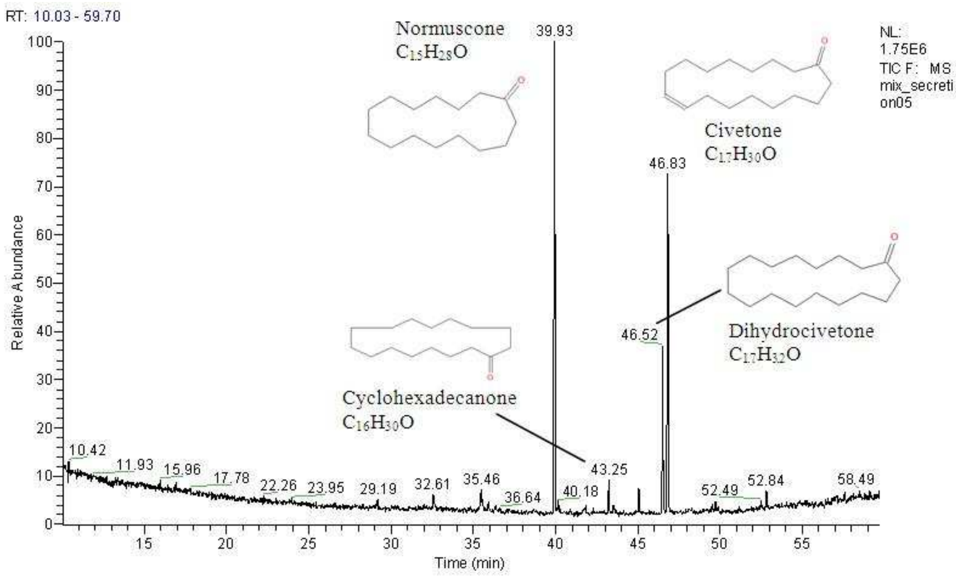

Figure 3 GC chromatogram of pooled small Indian civet secretion

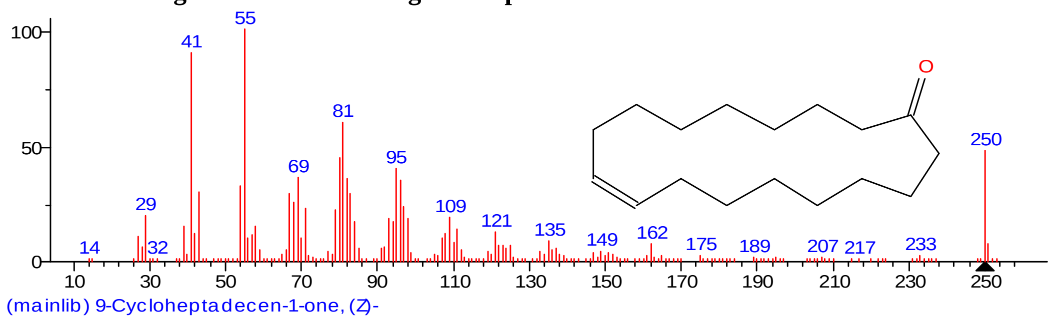

Figure 4 Mass spectrum of civetone

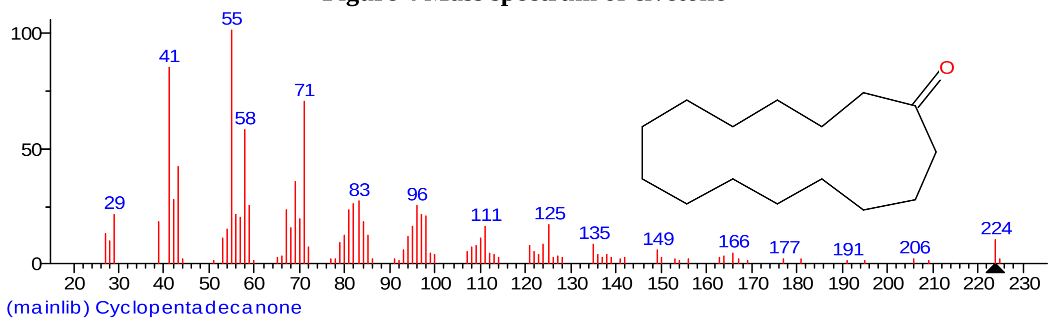

Figure 5 Mass spectrum of normuscone

Commercial civet secretion from civet farms did not divide male and female secretion. The secretion was gathered from all small Indian civets every morning and pooled together. Ten crops of secretion were analyzed and found that the secretion consisted of civetone, dihydrocivetone and normuscone as main components which were related to the previous study [10]. Cyclohexadecanone which found only in the secretion of female civet could be expressed in minor component (Figure 3).

Linearity

The calibration curves were constructed by plotting the peak area of the standards against their concentration. The regression equations for the linear portion of the standard curves of civetone and normuscone were y = 910946x and y = -735543 + 354744x respectively. Linear calibration curves were obtained with good correlation (r^2 = 0.9717 and 0.9965) for civetone and normuscone respectively. Linearity range of civetone was 0-50 µl/ml and of normuscone was 0-80 µl/ml (Figure 6, 7).

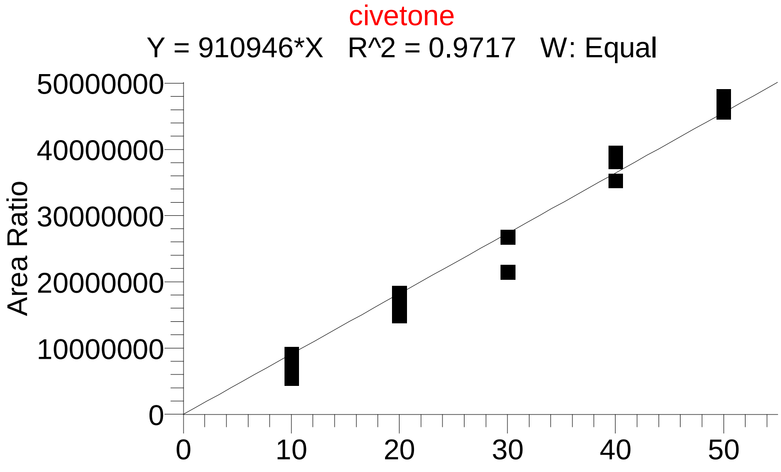

Figure 6 Calibration curve of civetone

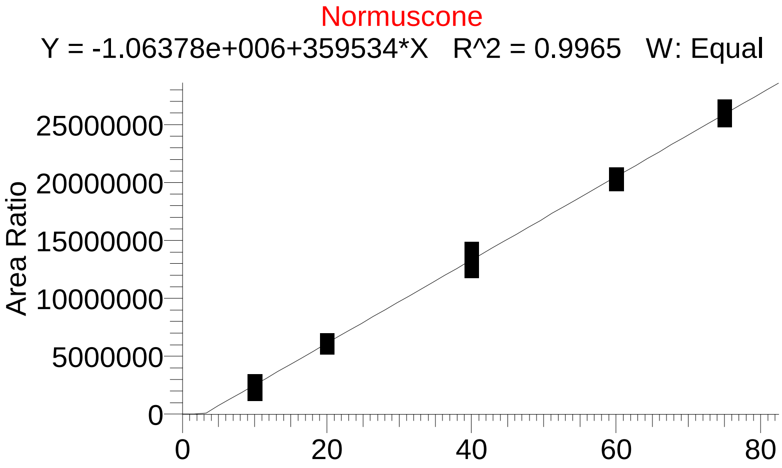

Figure 7 Calibration curve of normuscone

LOD and LOQ

LOD and LOQ for civetone were 0.0087 and 0.0165 µg/mg of secretion and for normuscone were 0.0596 and 0.1154 µg/mg of secretion respectively.

Recovery

The triplicates of each concentration group were analyzed for the recovery. This recovery was shown in percentage amount of civetone and normuscone which extracted from the sample to validate the method. The average recoveries were 97.3-98.0% in secretion and 91.4-105.7% in aromatic remedy for civetone. For normuscone, average recoveries in secretion and aromatic remedy were 98.5% and 90.0-103.0% respectively. The results demonstrated that the method was sufficiently accurate for determination.

Precision

The intra-day and inter-day precision of civetone and normuscone quantitation were determined. The results were presented in Table 1. The intra-day and inter-day RSDs were less than 8% which shown that the method was precise.

Table 1 Percentage of relative standard deviation of intra-day and inter-day analysis

	Concentration (mg/ml)	Intra-day RSD%			Inter-day RSD% (n=3)
		Day 1 (n=3)	Day 2 (n=3)	Day 3 (n=3)	
Civetone	0.025	3.212	0.821	1.276	0.472
	0.5	1.008	3.530	5.507	0.139
	1	1.660	4.693	3.185	0.180
normuscone	0.025	3.446	4.739	1.889	0.531
	0.5	1.117	4.856	7.649	0.929
	1	1.114	3.920	0.872	0.464

Civetone and normuscone contents in small Indian civet secretion

Table 2 demonstrated the concentration of chemical constituents which was different between secretion of male and female small Indian civet. Civetone was dominated in female whereas normuscone was dominated in male small Indian civet secretion. This finding was in accordance with the civet in China [11]. Analysis of secretion crops which containing both male and female secretion showed higher concentration of normuscone than civetone. This was in accordance with higher male small Indian civets in the farm.

Table 2 The concentration of civetone and normuscone in small Indian civet secretion obtained by GC-MS

	Male	Female	Crop
Civetone	0.788±0.138	23.614±1.469	5.931 ± 1.728
Normuscone	52.121±5.931	19.218±1.584	22.304 ± 5.162

Determination of civetone and normuscone in small Indian civet feces

There was no civetone and normuscone in the chemical constituents of small Indian civet feces.

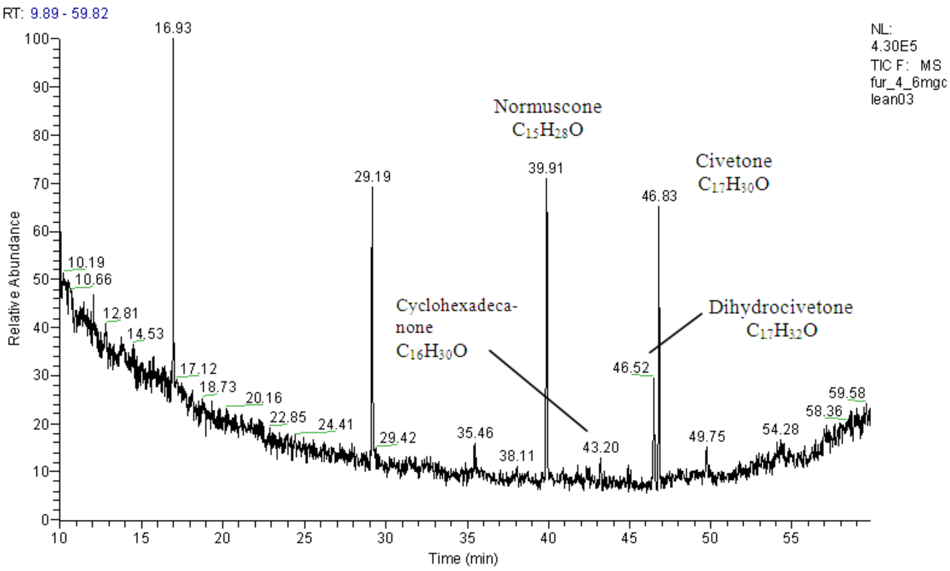

Figure 8 GC chromatogram of small Indian civet fur

Determination of civetone and normuscone in small Indian civet fur

The collected civet secretion was frequently mixed up with civet fur. The fur sticked with civet was one of commercial products form civet farms. However after exhausted washing, it was still found that the small Indian civet fur presented four chemical constituents (Figure 8) including civetone, dihydrocivetone, normuscone, and cyclohexadecanone that related to the small Indian civet secretion. The concentration of civetone and normuscone in small Indian civet fur were

0.23 ± 0.09 and 1.27 ± 0.17 µg/mg of washed fur respectively. Besides full scan analysis of mass spectrum, selected ion monitoring (SIM) of civetone and normuscone were analyzed for confirmation.

Table 3 The concentration of civetone (µg/mg of sample) in aromatic remedies obtained by GC-MS

No. of aromatic remedies	Lot number			Mean	SD
	1	2	3		
A	3.679 ± 0.32	2.785 ± 0.169	3.727 ± 0.773	3.397	0.531
B	2.913 ± 0.263	3.772 ± 0.341	6.876 ± 0.355	4.520	2.085
C	4.259 ± 0.323	4.540 ± 0.141	5.523 ± 0.521	4.774	0.663
D	10.011 ± 1.471	5.045 ± 0.459	9.686 ± 1.502	8.247	2.778
E	15.696 ± 1.601	10.386 ± 0.713	11.158 ± 1.152	12.413	2.869
F	1.233 ± 0.084	0.830 ± 0.520	1.155 ± 0.250	1.073	0.213
G	< LOQ	3.108 ± 0.213	5.978 ± 0.321	3.029	2.990

Table 4 The concentration of normuscone (µg/mg of sample) in aromatic remedies obtained by GC-MS

No. of aromatic remedies	Lot number			Mean	SD
	1	2	3		
A	8.225 ± 0.429	6.441 ± 0.552	8.593 ± 0.782	7.753	1.151
B	6.754 ± 0.727	9.219 ± 1.54	14.739 ± 0.799	10.238	4.087
C	10.447 ± 0.979	10.767 ± 1.257	13.846 ± 1.069	11.687	1.877
D	23.696 ± 2.701	10.792 ± 0.167	13.106 ± 2.467	15.865	6.880
E	41.040 ± 0.585	27.792 ± 2.162	24.251 ± 2.140	31.028	8.849
F	2.874 ± 0.884	2.089 ± 0.474	3.313 ± 0.186	2.759	0.620
G	< LOQ	6.644 ± 0.520	12.159 ± 0.675	6.268	6.088

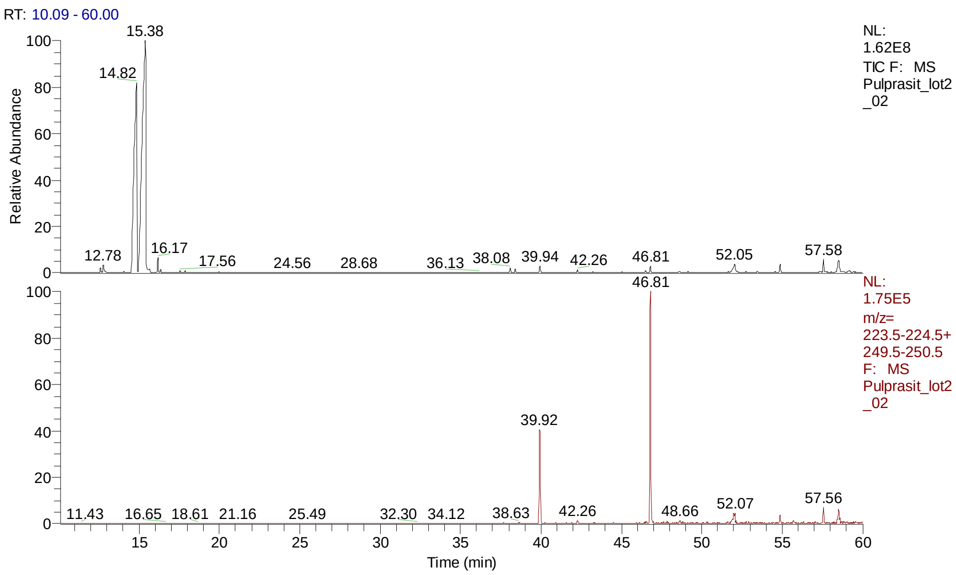

Figure 9 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy

Determination of civetone and normuscone in aromatic remedies

Civetone and normuscone were found in all aromatic remedies which claimed to use the small Indian civet secretion as an ingredient. The concentration of normuscone was higher than civetone in all remedies. The different in content of civetone and normuscone among each aromatic remedy might be due to specific formulary. The difference content of civetone and normuscone among each lot of same remedy might be due to the variety of each crop of civet secretion.

CONCLUSION

The chemical constituents of Thai *V. indica* secretion were different between male and female. Either civetone or normuscone could be used as marker for civet secretion ingredient in Thai traditional medicine products including aromatic remedies. The quality control of civet secretion on crude drug should be concern for sex dependent chemical compositions. The GC-MS method is precise and accurate for civetone and normuscone determination in small Indian civet secretion as well as in aromatic remedies.

Acknowledgements
The authors are thankful to the Herbal Remedies and Alternative Medicine Task Force of STAR: Special Task Force for Activating Research under 100 Years Chulalongkorn University Fund. The authors are also grateful to College of Public Health Sciences, Chulalongkorn University and all the staff members for necessary assistance and instrumental support.

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