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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

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 (%) = $(A_s-A)/A_a$ 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.

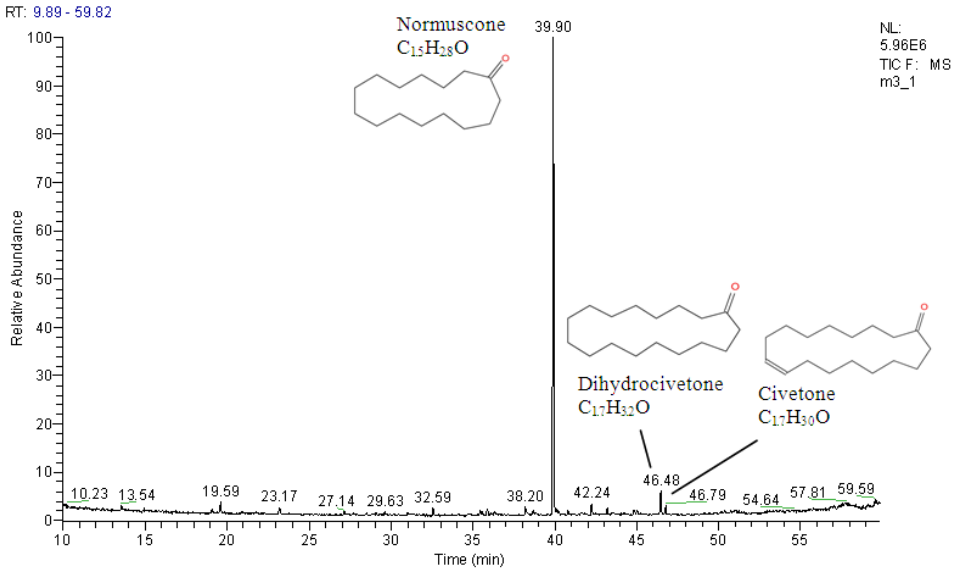

Figure 1 GC chromatogram of male small Indian civet secretion

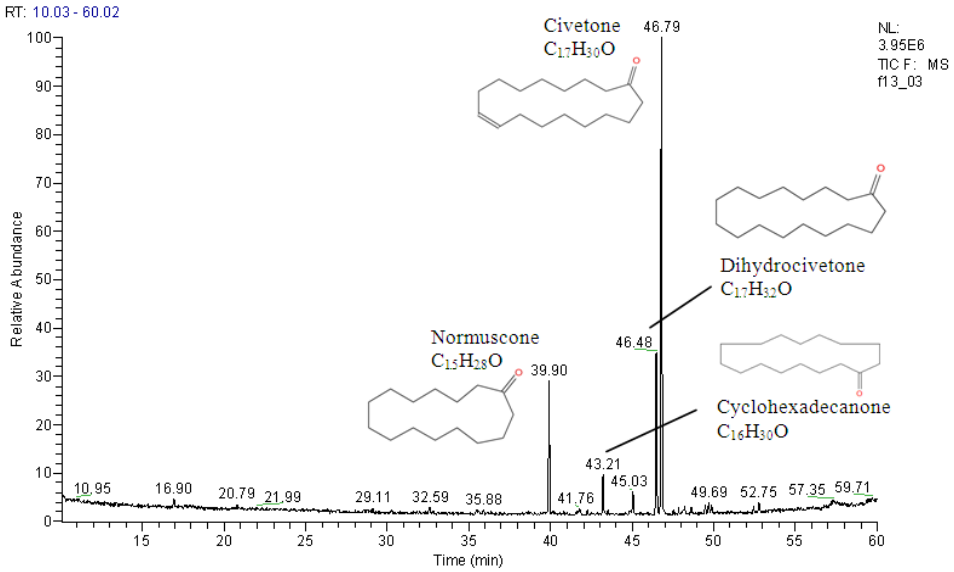

Figure 2 GC chromatogram of female small Indian civet secretion

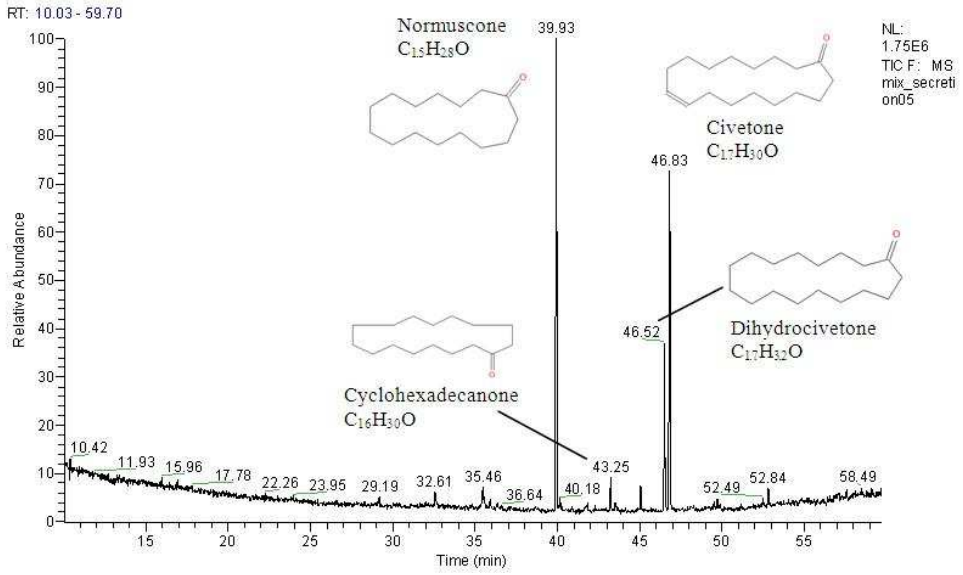

Figure 3 GC chromatogram of pooled small Indian civet secretion

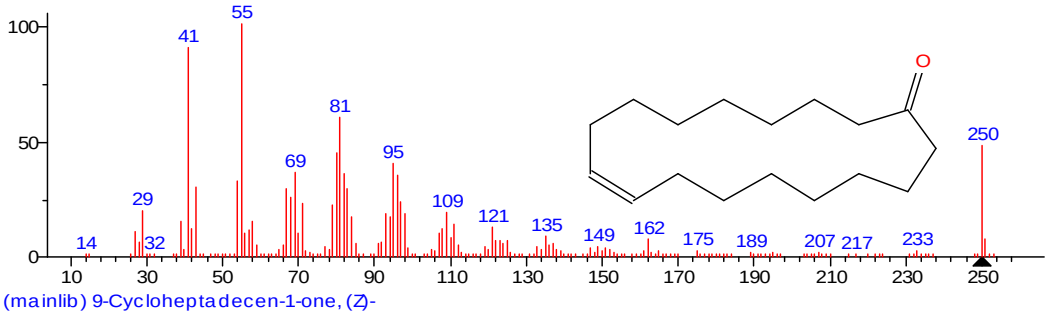

Figure 4 Mass spectrum of civetone

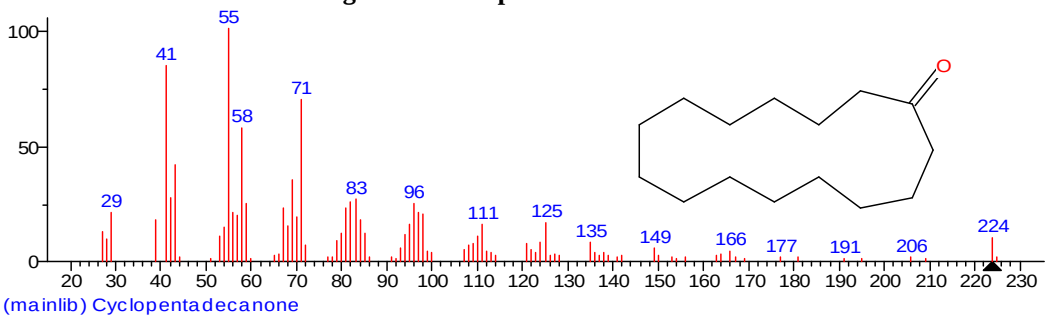

Figure 5 Mass spectrum of normuscone

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).

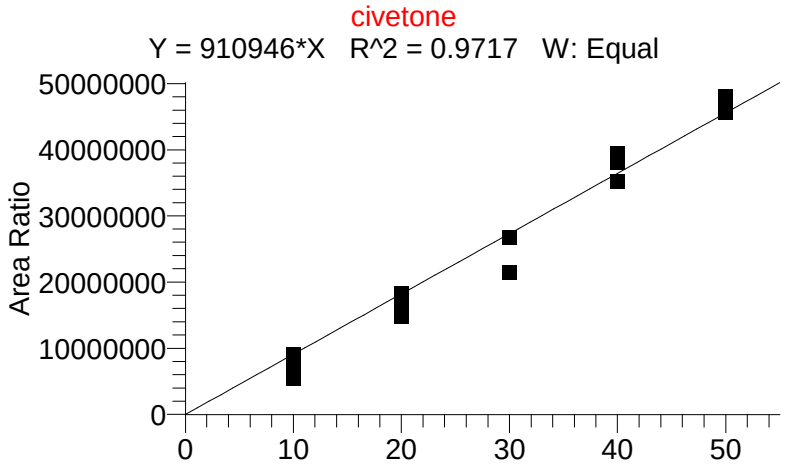

Figure 6 Calibration curve of civetone

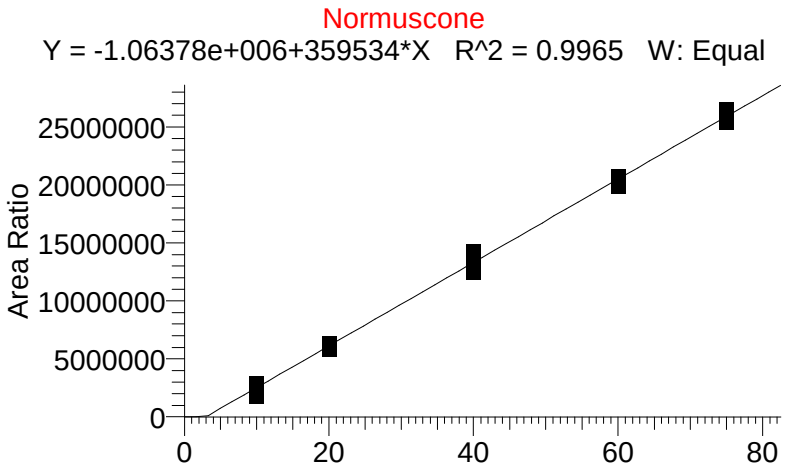

Figure 7 Calibration curve of normuscone

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.

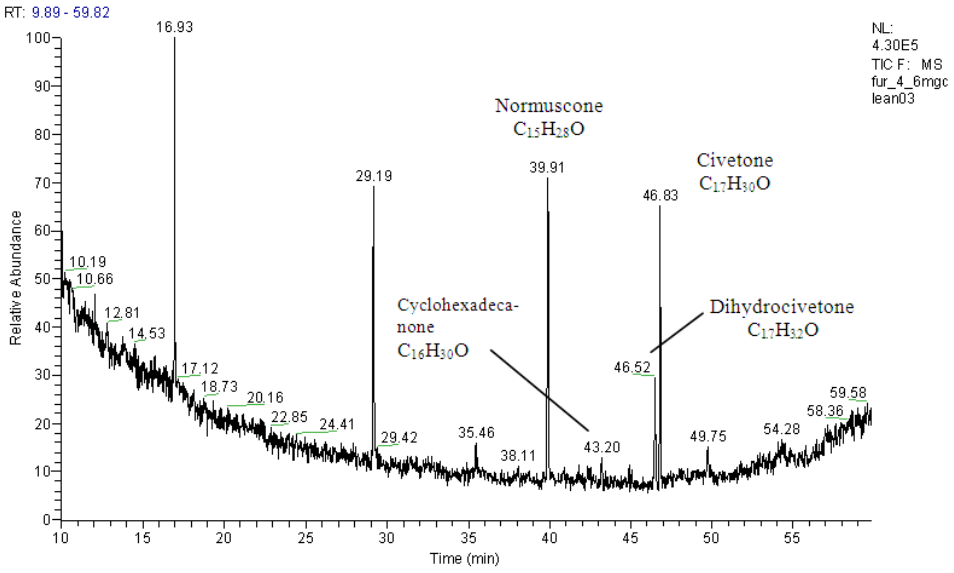

Figure 8 GC chromatogram of small Indian civet fur

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

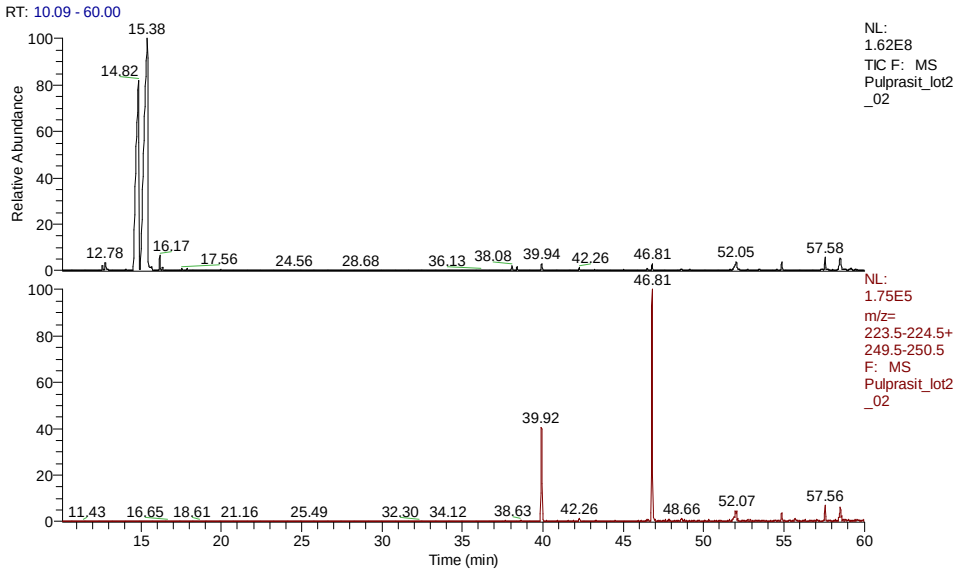

Figure 9 Full scan (upper) and SIM (lower) chromatogram of aromatic remedy

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.

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REFERENCES

[1] CM Francis. Field guide to the mammals of South-East Asia, Princeton University Press, New Jersey, **2008**; 289.
[2] JW Wheeler; DM Lay; MS Bulm; PJ Weldon. Biochemical systematics and ecology, **1998**, 26, 457-458.
[3] H Surburg; J Panten. Fragrance and flavor materials, 5th Edition, Wiley – VHC, Germany, **2006**, 185.
[4] RK Jananie; V Priya; K Vijiyalakshmi. *Journal of Chemical and Pharmaceutical Research*, **2011**, 3(4), 460-466.
[5] S Gopalakrishnan; K Saroja; JD Elizabeth. *Journal of Chemical and Pharmaceutical Research*, **2011**, 3(3), 477-480.
[6] N Rajeswari; S Lakshmi; K Muthuchelian. *Journal of Chemical and Pharmaceutical Research*, **2011**, 3(3), 792-798.
[7] FS Sharopov; IS Gulmurodov; WN Setzer. *Journal of Chemical and Pharmaceutical Research*, **2010**, 2(6), 284-290.
[8] http://www.ich.org/products/guideline/quality/article/quality-guideline.html
[9] http://www.aoac.org/Official_Methods/slv_guidelines.pdf
[10] O Yukio; T Shigeyuki. *Japan Analyst*, **1977**, 26(4), 232-236.
[11] H Lianghua; L Hauchong. *Fenxi Huaxue*, **1983**, 10(11), 781-783.