Original Research Article

MALE PHEROMONE ISOLATION, CHEMICAL ANALYSIS AND THE PERFUMES DEVELOPMENT FROM GOAT HAIR WASTE FROM SOME LOCAL FARMS IN THAILAND

Abstract

Goat male pheromones are secreted through their hair and play a prominent role in "male effect". In this study, we aimed to isolate some male pheromone from the goat hair, waste from local goat farm from the Southern Thailand. Goat hairs were collected from three farms in three breeds and different parts of goat bodies compared in both males and females. The goat hairs extraction was carried out by maceration with hexane. The isolation of the extract was performed by column chromatography using sephadex LH-20 to give 100 fractions. The further separations yielded the 4-ethyloctanoic acid reported as goat scent and the exert releaser pheromone activity to attract females. Quantitative analyses using GC-MS demonstrated 12.73±0.78% w/w in the extract. The results showed 17 fatty acid compositions, such as palmitic acid, stearic acid, cis-9-octadecenomic acid, lauric acid, cis-vaccenic acid as prominent compounds. The qualitative of the extract by GC-MS illustrated 13 compounds in which the chemical fingerprint was confirmed by using ^{1}H NMR technique. The microbial contamination of goat hair extract was further evaluated by spread-plate technique, showing no microbial contamination with *Staphylococcus aureus*, *Clotridium spp.* and *Salmonella spp.*. The extract was used as a raw material for perfumes blended development and no irritation on human skin was observed.

Keywords: Goat hair; Male Pheromone; 4-ethyloctanoic acid; Goat hair perfumes

1.Introduction

In goat seasonal breeders, the introduction of a mature male into a flock of females eventually induces the ovulation. It is generally accepted the olfactory signal, the pheromone plays a dominant role in the 'male effect', since it is seen when the presence of male is replaced by its fleece/hairs[1] or their extracts [2]. The Gonadotropin-Releasing Hormone (GnRH) pulse generator in the hypothalamus governs pulsatile GnRH secretion and thereby regulates pulsatile Luteinzing Hormone (LH) secretion from pituitary [3]. It has been demonstrated the exposure to male goat hairs promptly stimulates the GnRH pulse generator in female goats [4] which is a striking contrast to other other mammalian pheromones. Therefore, the male effect could serve as a unique model to investigate the neural pathways participating in pheromone signal transduction. Pheromone signals regulate conspecific behavior and physiology, releaser pheromone induce specific behavior , exerting acute effects on the neural response while the primer induce physiological changes with long-lasting effects by changing the neuroendocrine status of the recipients [5]. The olfactory signal molecule that activates from GnRH pulse generator in male goats has been identified using GC-MS reported in several ethyl-branched aldehyde and ketones. The 4-ethyloctanal is stronger suggested to be a primer pheromone for the 'male effect' and found 18 compounds appears to have stronger activity than 4-ethyloctanal, can be oxidized in the atmosphere to become 4-ethyloctanoic acis, the main constituent of the goaty odour character and reported to exert releaser pheromone activity to attract goat females [6]. In this study reported for the first time of natural goat male pheromone separation and analysis could not only is efficiency of livestock for economically important worldwide but also a novel discover of perfume product development from the goat hairs.

2. Materials and Methods

2.1 Materials

Goat hairs were collected from three difference local goat farms including 1) Sri Pong farm, Krabi Noi sub-distric, Muang district, Krabi province, by Mr. Chuan Phukhaoluan. The Saanen, Shami and Shaanen hair, male goat breeder, was cut on the 2nd November 2020 and 11st November 2020, respectively. The Boer, female goat hair was cut on 11st November 2020. 2)The Toggenburg goat hair was collected from, Learning centre of goat raising farm, Krabi Noi sub-distric, Muang district, Krabi province by Mr. Sombat Boonthawon, on 11st November 2020. The goat hair collected differently such as body, head, beard, leg and genitalia parts of goat body. 3)The goat hair was collected from Jindarat farm, U-Di Charearn sub-distric, Kuan Ka Long district, Satun province on 28th Apirl 2021, Shami male breeder by Mr. Weerasak Duksukkeaw. 4. The Anglo-Nubian
male goat hair was collected from Rajamangala University of Technology Isan Nong Rawiang Education Center was cut on 7th Apirl 2021.

Table1 Goat hairs were collected from three difference local goat farms including.

Goat hairs			
Farm to address	**Courtesy of**	**Species**	**Date cut**
Sri Pong Farm, Krabi Noi sub-distric, Muang district, Krabi province	Mr. Chuan Phukhaoluan	The Saanen, Shami and Shaanen male and female goats hair	2nd November 2020 and 11st November 2020
		The Boer, female goat hair	11st November 2020
Learning Centre of Goat Raising	Mr. Sombat	The Toggenburg male goat	11st November

Farm Krabi Noi subdistric, Muang district, Krabi province	Boonthawon	hair, collected body such as head, beard, leg and genitalia parts of goat body	2020
Jindarat farm, U-Di Charearn sub-distric, Kuan Ka Long district, Satun province	Mr. Weerasak Duksukkeaw	The Shami male goat hair	28th Apirl 2021
Nong-Ra-Wiang, Mueang Nakhon Ratchasima District, Nakhon Ratchasima	Rajamangala University of Technology Isan, Nong Rawiang Education Center	Anglo-Nubian Male goat hair	7th Apirl 2021

2.2 Chemicals

Hexane, dichloromethane, methanol, ethyl acetate, AR grade, Lab-Scan, Thailand.
Sephadex LH-20, GE Healthcare, Sweden
TLC silica gel 60 F254 Aluminium plate, E. Merck, Germany.
UV lamp dual wavelength 254/365 nm., Alltech Ltd., USA

2.3 Chromatography separation

The goat hairs were extracted with hexane and isolated by open column chromatography using sephadex LH-20 as stationary phase, dichloromethane:methanol (3:2) and the TLC plate, silicagel 60 F_{254} aluminium plate (E. Merck) size 20x20 cm.

2.4 Nuclear Magnetic Resonance (NMR) Spectra

The ^{1}H NMR spectra were measured with a Bruker ASCEND 400 FT-NMR spectrometer, operating at 400 MHz (^{1}H). The chemical shifts (δ_H and δ_C) were recorded in ppm. with reference to residual solvent signal $CDCl_3$ (δ_H 7.24), where appropriated and coupling constants (*J*) were given in Hz.

2.5 Gas Chromatography-Mass Spectrometer analysis (GC-MS)

Instrument: GC-MS GC7890A-MS 5975C (Agilent technologies**)**

Analytical conditions:

Gas chromatography

injection volume	1 µl
injection temperature	250 °C
injection mode	Split
Split ratio	10 : 1
Column temperature	40 °C (5min) – (10 °C /min)-250 °C (5min)
Carrier gas	
He (Flow rate 1 ml/min)	
Pressure	7.0699 psi
Total flow	14 ml/min
Average verocity	36.26 cm/sec
Thermal auxiliary temperature	250 °C

Mass spectrometer

Lonization mode	electronic impact at 70 eV
Lon source temperature	230 °C
Quadrupole temperature	150 °C
Scan range (m/z)	35-550

2.6 Determination of fatty acids by GC

Chemicals and Reagents

Standard fatty acid was used, Supelco 37 Component FAME Mix Varied conc. in dichloromethane CRM 47885 from Sigma Aldrich Co. 14% Boron trifluoride (BF_3) (Merck Co., Ltd.) and 0.5 N

Methanolic sodium hydroxide (NaOH) solution. Methanol with purity 99.9% (Analytical reagent grade) Isooctane with purity 99.5% (analytical grade), and Sodium chloride (analytical grade) were obtained from CARLO ERBA Reagent S.A.S

Gas Chromatography (GC)

The separation and detection of the analytes were achieved by GC -2010 Plus and flame ionization detector (FID), respectively. They were purchased from Shimadzu Co., Ltd.

GC Conditions

Column type:	Capillary column: DB-23; 60 m, 0.25 mm ID, 0.15 µm
Split ratio:	30:1
Program rate:	1.0 ^{0}C/min
Injection temp:	250 ^{0}C
Detection temp:	270 ^{0}C
Injection sample:	1 µL

Samples preparation

The 0.2000 g of goat hair extract each sample was balanced by Analytical Balance (Resolution. — 0.0001 g.). In the saponification process, the oil was added 10 mL Methanolic sodium hydroxide (NaOH) solution and stirring for 30 min. at 100 °C in Erlenmeyer flask 25 mL then added with 10 mL of 14% Boron trifluoride (BF_3). Continue stirring for 30 min and added Iso-octane into sample solution. After that added sodium chloride. Finally, the sample solution was injected to GC-FID.

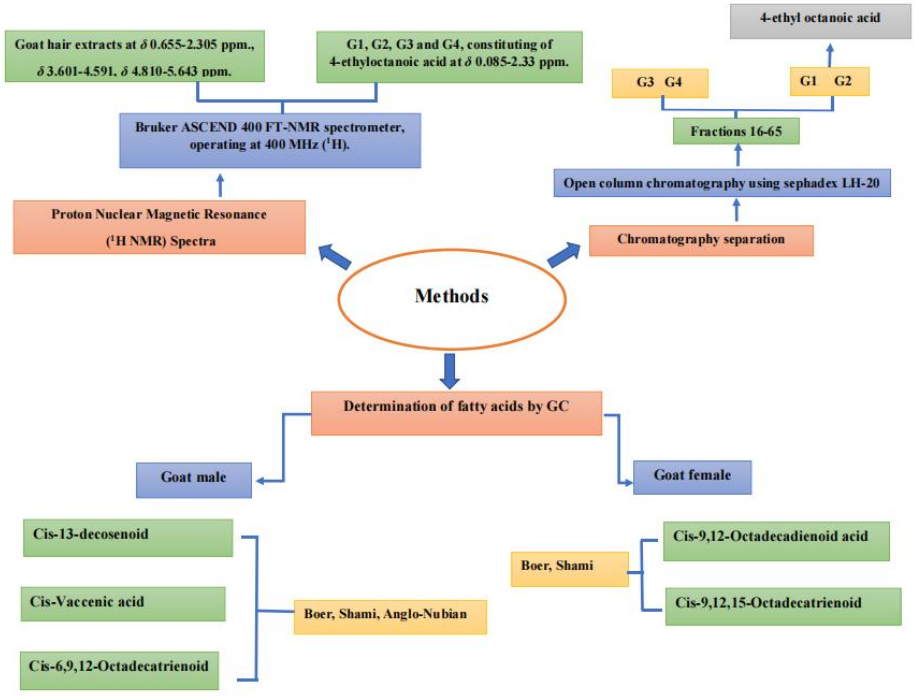


Diagram 2.3-2.6 The preparation and characterization in this experiment

2.7 Study of bacterial contamination by Spread-Plate test

Made a dilution of sample under sterile condition, pipetted 0.1 mL in 1 mL of sterile water mixed well by Vortex mixer. The sample 0.1 mL was spreaded from appropriate desired dilution series onto the centre of the surface of an agar plate by triangle shape glass spreader using sterile DI water as the control. The plate was incubated, containing, *S. aureus*, *Clostridium spp. And Salmonella spp.* on the mannitol salt agar, egg yolk agar and eosin methylene blue (EMB) agar, respectively. The incubation was at 37^0 C for 24-48 hrs. excepted for the *clostridium spp.* was under no oxygen condition using Anaerocult^{R}A. Calculated the CFU value of samples, counted the colonies, multiplied by the appropriate dilution factor to determine the number of CFU/mL in the original samples.

2.8 Perfumes development from goat hair extracts

Perfume ingredients and bottles were purchased from JJ Mall, Chatuchak, Bangkok, Thailand.The ingredients were divided to three notes such as base notes, middle notes and top notes. The goat hair extract was blended in to the base notes ingredients, following by the middle notes and the top notes, respectively. The perfume base oil was added to after all notes combination were blended them mixed the combination well, transferring the perfume to its bottle put the lid on and kept it in room at 25^0C temperature for at least 29 days before used.

2.9 Skin Irritation test in volunteers

Volunteers of 10 people including 3 males and 7 female, their age were in between 21-55 years old. Determination the skin of the volunteers with erythema index by using mexameter probe. Detection the skin at 0 hr after applying the goat hair perfume sample 200 μL, size 2x2 cm^2, flowing by wool attached in the target area for 4 hrs. Detected erythema index of their skin at 4, 24, 48 and 72 hrs.after removed the wool (Jirova *et al*, 2010), compared to the skin applied on positive control (20% w/v SLS) and the blank skin, then calculated the red skin of erythema that has changed.

% relative difference = (value after – value before / value before) x 100

3. Results & Discussion

3.1 Fatty acids composition

The goat hair extract was determined their fatty acids composition using GC-MS yielded most abundant repectively, Palmetic acid, stearic acid and cis-9- octadecenoic acid in every breedings and they were varied between lignoceric acid, Arachidic acid, myristic acid and lauaric acid, respectively. The goat male and female composition were compared found the female was lack of cis-13-docosenoid, cis-Vaccenic acid and Cis-6,9,12-Octadecatrienoid. The male in Boer, Shami, Anglo-Nubian have found no cis-Vaccenic acid while the male Boer and Anglo-Nubian were absent of Cis-9,12,15-Octadecatrienoid when they were compared to The Saanen Males.**(Table2)**

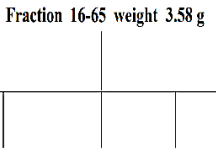
Table2 Fatty acids analysis of goat hair from difference resources.

3.2 Proton NMR analysis

The proton NMR illustrated chemical shifts of goat hair extracts at ***δ*** 0.655-2.305 ppm., ***δ*** 3.601-4.591, ***δ*** 4.810-5.643 ppm. Goat hair extracts with different parts of goat bodies were similar chemical shifts pattern, but difference intensity, especially the only Saanen male breeder containing high intensity at ***δ*** 7.00-8.00 ppm. As can be seen in the Table1 showed the chemicals shifts of compounds G1, G2, G3 and G4 that constituting of 4-ethyloctanoic acid at ***δ*** 0.085-2.33 ppm.

3.3 Separation of goat hair extracts

The Shami male breeder goat hair weigh 4.19 g has been separated by open column chromatography using sephadex-LH20 to give 100 fractions. The fractions 16-65 (3.58 g) were combined and further separated gave 54 fractions (**Scheme1**) The fractions 10-15 were selected and combined to purify for the goat male pheromone. The fraction was developed as a band on to the TLC plate, silicagel 60 F_{254} aluminium plate (E. Merck) size 20x20 cm. using hexane: ethyl acetate (4:1) as the mobile phase yielded G1 (0.01g), G2(0.02g), G4 (0.01g) were appeared as single spot and G3 (0.01g) showed as two spots separately. The G1, G2, G3,and G4 were submitted for ^{1}H NMR compared to 4-ethyl octanoic acid spectrum as a standard showed in **Table3**



Fatty acids		Sources					
		Krabi	Nakhonratcha	Satun	oer, Male Satun	Shami Female Satun	Boer Female Satun
Capric acid	C10:0	0.6	0.1	0.1	0.1	0.1	0.2
Lauric acid	C12:0	3.1	0.3	1.1	0.4	0.5	1.0
Myristic acid	C14:0	2.3	1.5	1.2	1.3	1.1	1.7
Pentadecanoid acid	C15:0	0.6	0.3	0.3	0.3	0.3	0.4
Plamitic acid	**C16:1 n-7**	**10.5**	**9.0**	**25.2**	**18.1**	**22.1**	**17.5**
Palmitoleic acid	C16:1 n-7	1.8	0.3	1.3	0.9	1.1	1.1
Heptadecanoid acid	C17:0	1.1	0.6	0.7	0.7	0.4	0.4
Stearic acid	**C18:0**	**7**	**2.7**	**15.7**	**12.6**	**15.6**	**11.4**
Cis-9-Octadecenoid acid	**C18:1 n-9**	**5**	**2.2**	**13.7**	**7.3**	**15.5**	**11.1**
Cis-Vaccenic acid	C18:1 n-7	2.6	-	-	-	-	-
Cis-9,12-Octadecadienoid acid	C18:2 n-6	0.7	0.3	0.9	0.7	1.0	1.9
Cis-9,12,15-Octadecatrienoid	C18:3 n-3	0.1	-	0.1	-	0.1	0.1
Cis-6,9,12-Octadecatrienoid	C18:3 n-6	0.2	-	-	-	-	-
Arachidic acid	C20:0	1.2	0.7	1.6	1.7	2.2	2.1
Cis-11-Eiccosenoid acid	C20:1 n-9	0.5	0.1	0.8	0.5	1.4	0.9
Heneicosanoid acid	C21:0	0.1	-	0.1	0.1	0.1	0.1
Behenic acid	C22:0	0.7	0.6	0.7	0.8	0.8	1.4
Cis-13-Docosenoid	C22:1 n-9	0.1	0.1	0.3	0.2	-	-
Tricosanoid acid	C23:0	0.1	0.1	0.1	0.1	0.1	0.2
Lignoceric acid	**C24:0**	**1.4**	**1.5**	**1.6**	**2.3**	**2.7**	**6.2**

Results on chemical analysis of goat hair fractions

Samples	TLC	Proton NMR chemical shift (***δ*** ppm.), in $CDCl_3$, 400 MH$_Z$
Goat hair extract	G1	0.021-0.066,0.665-2.017, 0.859-0.891,1.178-1.312, 1.480-1.549, 2.269-2.307, 4.669 (*s*)
	G2	0.020-0.050, 0.610-1.967,0.865-0.894 (few), 1.186-1.292 (few)2.254-2.260 (few), 1.868-1.967, 3.418-3.495, 4.620 (*s*), 5.276-5.289
	G3	0.021-0.048, 0.668-2.037, 0.860-0.876, 1.233-1.320, 1.546-1.6022.256-2.331, 2.256-2.356, 3.603-3.637, 4.463-5.582,
	G3,1	0.021-0.057, 0.658-2.036, 0.859-0.885, 1.233-1.294, 1.558-1.6112.286-2.353, 3.606-3.639, 2.256-2.353, 3.606-3.639
	G3.2	0.021-0.048, 0.662-2.036, 0.853-0.882, 1.232-1.319, 1.545-1.6022.256-2.322, 2.256-2.376, 3.603-3.636, 4.463-5.582
	G4	0.021-0.048, 0.656-2.027, 0.860-0.885, 1.205-1.603, 1.551-1.6032.251-2.314, 2.221-2.374, 4.033-5.356,
4-ethyl octanoic acid (STD)	G1, G2	0.866-0.883, 1.197-1.311, 1.555-1.608, 2.283-2.323, 10.927.9 (*s*)

Scheme1 Separation of goat male pheromone

Table3 Chemical analysis of goat hair fraction

3.4 Chemicals analysis by GC-MS

The chemicals analysis of the goat hair extract by GC-MS resulted in ethyloctanoic acid, germacranen-decyldocosane, 5-octadecane, 2-hexyl-1-octanol, nonadecane, 1-exadecene, ethyl palmitate, propionaldehyde allyldrazone, 6-methyltridecanea-hexadecene, 2-ethyl-1-dodecanol, cholasta-4,6-diene-3-ol as goat hair main component while it comprised of 12.73±0.78% w/w of 4-ethyloctanoic acid.

3.5 Microorganism contamination by Spread-Plate test

The results found no contamination of microorganism *Staphylococcus aureus* , *Clotridium* spp. , *Salmonella* spp. in goat hair extracts by Spread-Plate technique.

3.6 Skin Irritation test in volunteers

The irritation test of the perfume development from goat hair extract found no caused of irritation from the red skin of volunteers were appeared after applied the perfume at 4, 24, 48 and 72 hrs found no difference from 0 hr while found the significant difference ($p<0.05$) of the red skin applied of 20% W/V SLS in the same experiments after 4 hrs compared to 0 hr. As shoen in the **Table4**.

Table4 The erythema index, EI in 10 volunteers after applied of goat hair perfume at 4, 24, 48 and 72 hrs compared to 0 hr.

	Erythema index (EI)				
Examples	Before test	After test (hrs.)			
	0 hr	4	24	48	72
Goat hair perfume	172.33±54.10	172.85±49.16	170.33±50.89	172.70±53.08	171.81±54.91
20% w/s SLS	170.11±32.43	238.63±23.97	178.93±48.40	184.41±42.56	177.70±30.06
No application	171.37±39.40	172.93±31.46	170.81±42.71	189.07±49.74	170.22±49.97

4. Conclusion

The goat hair extracts possess thirteen chemicals constituents as well as the similar pattern of chemical shift in their proton NMR except the Saanen male breeder containing high intensity of aromatic compounds at δ 7.00-8.00 ppm. could provide the strong goat smell which promotes as

good perfumes smell to be more secreted and mysterious. The main chemical composition of goat hair extract was 4-ethyl octanoic acid, reported as goatty smell and a releaser goat male pheromone. The separation found G1-G4 comprised of the releaser male pheromone. The goat hair extract gave no contamination of prohibited microorganism and skin irritation on volunteers when the perfume was blended.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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