European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 07, Issue 08, 2020

A Study On Pheromonal Compounds In Sheep (*Ovis Aries*) Faeces And Their Behavioural Analysis With Special Reference To Estrus Detection

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Abstract

Ramnad white is an important indigenous sheep breed in Tamil Nadu and has a decreasing trend in population, which is alarming as the breed is in critically endangered. Artificial insemination is needed for frequent breeding and for the conservation of these animals. Further, the artificial insemination is also difficult in sheep because of few external estrus signs. In mammals, detection of estrus is one of the most critical components for successful breeding. The accurate and consistent detection of estrus is also necessary for successful fertilization and increase the percentage of success in artificial insemination. Normally, reproduction depends upon the odours released from the female during the estrus phase. It is well known that mammals emit chemo-signals to the surroundings via., faeces, saliva, urine and specialized scent glands. Analyzing chemical signals in the faeces of Ramnad white will aid us to develop estrus detection kit for easy detection of estrus, so that we can proceed for the improvement of livestock production. Therefore, the prime objective of the present work is to identify the marker for ovulatory phase in females and to develop a kit, which would enable to easily identify the reproductive stage of the animal and allow it for mating and artificial insemination. On the other hand, chemical signals (pheromones) would be used for provoking sexual behavior in males. The present work has been designed to analyze the volatiles present in faeces across the various estrous phases in order to identify estrus markers in female sheep and a behavioral analysis are also used as supporting information for our present findings. The samples were collected from sheep in different stages of estrous viz. proestrus, estrus and postestrus. They were extracted using dichloro methane for GC-MS analysis. The results showed the presence of nine different compounds with varying molecular weight and retention time. Among the identified compounds, β-caryophyllene has been exclusively present only in estrus stage and absent in all other stages. The present study concluded that the estrus specific compound β-caryophyllene may act as a pheromonal compound involved in ram communication.

Keywords : Ram; Gas Chromatography Mass Spectrum; pheromone; estrus; artificial; Insemination; β-caryophyllene.

1. INTRODUCTION

In India, animal husbandry has been practiced from centuries old, whereas, Tamil Nadu is gifted with some recognized breeds of cattle, buffalo, sheep and goat (1). Among these, sheep is considered as an important livestock in rural areas of Tamil Nadu and it contributes as rich livehood resources for poor farmers (2). Normally, sheep have adapted to all region of the country of natural condition (3) and when compared to other cattle (4). The main advantage in rearing sheep is that they are best suitable to uncultivable lands and semi-arid tropics with marginal and sub-marginal lands (5). Sheep play an important role in agro economy areas for milk, meat and manure production (4). The survey on the sheep population was gradually decreases 1956-61, 1977-82, 1982-89, 1997- 2004 and 2004-07 in Tamil Nadu. Tamil Nadu is known for domestication of farm animals and home for many recognized sheep breeds. Ramnad white is one of the breed, they are hardy and best suitable for the migratory system of rearing. Ramnad white breed have exclusive economic and traditional status with special adaptation characteristic to the drought prone areas of the South India. According to 1972 census, the total Ramnad white sheep population was 7.82 lakhs, which has reduced to 7.50 lakhs as per 1977 census, and in the latest survey, 2008 less than 500 breedable females are enumerated as pure Ramnad white sheep population. Normally, indigenous animals have developed superior heat tolerance and diseases resistance. These indigenous Ramnad white sheep are sturdy and can able to cover long distance during migration. They are relatively better adapted to local temperature during summer season as compared to other crossbreed sheep. The declining population threads the alarm as the pure breed is critically endangered. To retain these indigenous breed, many assisted reproduction technologies are used but most of them failed due inability to detect accurate estrus stage. The detection of estrus is very much difficult in farm animals especially sheep hence, using of instrument like ultrasound, estrus detector were available, but it is often quite expensive, time consuming and it needs some veterinary assistance. Farmers and veterinarians are very much interested in finding out the suitable method to detect accurate estrus stages. Discovery of a pheromone would make possibility to develop a field test method, for detecting estrus stage, which could be economically reliable (6).

Estrus detection in sheep is difficult to identify because it is a seasonal breeder and this animal shows few external symptoms of estrus; hence the pheromonal cues may offer a new approach to detect the accurate estrus stages. Olfactory system and vomeronasal system are the two primary sites of reception for pheromonal chemical signals present in mammals. Normally, the recipients use these specific olfactory pathways in the sensitivity to chemical signals (7). They are excreted in bodily fluids and fecal matter which are detected by the olfactory systems. Therefore, in reproductive and social behavior including sexual attraction, mounting (8,9), territorial marking (10,11), mother-young interactions (12) and individual identification (13) pheromones play key role. During estrus, the females release chemical signals through their excretory matters to attract the male of same species, called as pheromones, which serve as sexual attractants including sexual arousal and mating behavior. The timing of physiological events of ovulation and co-ordination of sexual behavior is important for successful fertilization (14).

It is well accept that the female produces a specific odor during estrus through biological products like urine (15; 16; 17;18), feces (19), vaginal fluid (20) and specialized scent glands (21; 22) which act as a major source of chemical communication in mammals (23; 24) and may be considered an accurate indicator of female receptivity (25;26; 27). The

research on biological implication in relation to bio-communication in saliva, feces and milk involving estrus detection are scanty (28). However, there may be need to detect estrus stage accurately through other body fluids by adapting simple method and it should be economically (29; 21; 22).

In both captive and wild environment, the determination of fecal chemicals during the reproductive status of many species has been used extensively (30). Fecal determination techniques have a series of advantages that they are non-invasive and allow to analysis of long series of data. Depending upon the sex and stages of reproductive cycle the presence of volatile compounds in the feces may differ. Up to date, only an inadequate amount of information is accessible regarding non-invasive monitoring of pheromones, fecal steroids and behaviors in captive and zoo animals (31, 32). It is documented that, from insects to mammals feces considered as a validated as a source of chemical signals and carries the physiological statuses of an organism either by volatile chemo-communication or non-volatile mode chemo-communication which may various during different phases of the estrous cycle (33). Hence it is reported that in some wild and endangered animals, feces sample used as non-invasive estrus detection, as it could be reflected by the hormonal metabolites (34).

Time of ovulation is very much important for successful fertilization and there are several reports regarding bio-communication involving bovine vaginal fluid and urine. However, reports on other body fluids, such as saliva, feces and milk for their biological significance in relation to estrus detection, are scanty. As it is already known as pheromonal cues may offer a new approach in the detection of estrus. In order to determine their reproductive fitness it is necessary to understand the normal effective olfactory interaction between male and female animals. Therefore pheromonal signals involved in the reproduction of farm animals which was not exploited in sheep. To date, no chemical study was performed in sheep feces with reference to different stages of reproductive cycle in order to define its biological functions. So, the present study is aimed to analyse the fecal volatile compounds excreted in sheep. The identification of compounds gives the information related to their chemical communication. Further, this is a maiden attempt in the field of animal communication.

2. MATERIALS AND METHODS:

Animals

There is a difficulty in tracing the origin of original Ramnad White sheep due to unavailability of documentary proof. However, the elderly sheep keepers briefed that Sethupathhi Maharaja has evolved the sheep during his rule in Ramanathapuram Samasthanam. The original Ramnad White was found to be sporadically distributed in Aruppukottai and Sivakasi block of Virudhunagar district and Vasudevanallur block of Tirunelveli district (35).

Six healthy sheep of Ramnad White breed were used in the present study from District Livestock Farm, Pudukkottai, Tamilnadu, India. The animals were fed cultivated forage, green fodder *ad libitum* throughout the study period.

Estrus determination

The conventional estrus behavior in the sheep is not easy to detect when compared to other cattle. The characteristic behaviors of estrus, such as restless, reddened and swollen vulva, are often difficult to detect because of the wool and small size of the vulva. Some of the secondary behaviors, such as rapid tail wagging and raised tail in the presence of ram are also considered as signs of estrus. The stages of the estrous cycle were carefully determined for two to three consecutive cycles. The sheep were considered to be in estrus if they

accepted the mounting by another sheep. The period within three to five days before estrus was considered as proestrus and that within two to four days after estrus as postestrus.

Sample collection

The fecal samples were collected from sheep during various estrus phases at every morning between 6.00 a. m and 8.00 a.m, the samples were collected within 5 minutes after excretion in vials. The vials were labeled appropriately and were placed in a freezer at - 20 ^{0}C for gas chromatography–mass spectrometry (GC–MS).

Sample analysis

The feces samples collected from were extracted with dichloromethane, the extracted were prepared by taking 15 mg of the pooled samples and mixed with 15 ml of dichloromethane 1:1. The supernatant was filtered through a silica-gel column (60–120 mesh) for 30 min at room temperature. The filtered extract was reduced to 1/5 of its original volume by cooling with liquid nitrogen. Two microliters of dichloromethane extract were injected into the GC–MS system on a 30m glass capillary column with a film thickness of 0.25 m 30mm×0.2 i.d. (coated with UCON HB 2000) using the following temperature programme: initial oven temperature of 40 ◦C for 4 min, increased to 250 ^{0}C at 15 ^{0} C/min; and maintained at 250^0 C for 10 min. The GC–MS was run under computer control at 70 eV. The solvent DCM peak was obtained at 3 min. The feces samples were analyzed repeatedly six times and subjected to crosschecking and confirmation. The identified compounds were then compared with the standard run under the same conditions. These data were already stored in a compact library of chemical substance (NIST 6221B).

Behavioral assay

Procurement of compound

As already characterized, the compound identified in feces of sheep during estrus phase *i.e.,* β-caryophyllene was procured from Aldrich chemical company, USA.

Behavior assay with synthetic compound and estrus feces

The ram behavior in response to synthetic compound were carried out in District Livestock farm, Pudukkottai, Tamil Nadu, India. The identified estrus- specific compound, β-caryophyllene was used for bioassay. Six matured ewe and ram were selected for the present study. The following experiments were carried out in the present investigation. To select the effective concentration for bioactivity of synthetic compound, β-caryophyllene was prepared at several concentrations. Flehmen behaviour and mounting behavior by ram were observed in the field. The solvent, dichloromethane (DCM) has already been used for GC-MS analysis and the synthetic compounds prepare in various concentrations, such as 0.5%, 1.0.%, 2.0% and 5.0%, were used for the present study for behavioral assay. The compound was sprayed in the vagina of non-estrus animal allowed the ram to sniff and lick the synthetic compound, estrous feces and control. There were no estrous females in this study area. In order to condense the conditioning, the tests were conducted at different periods during six months. The ram were experimental for a maximum of 20 min at each presentation.

Flehmen

Animals sniffed the region of female and raise its neck, extended its chin and inhaled with slightly opened mouth, tongue held in a flat position and upper lip curled, so that the nostrils become partly closed. The passive partner does not object to the behavioral activities of ram.

Mounting

An animal mounts from behind or from the side with its forelegs and chest on another animal's back. If an animal mounts a male or a non-estrus female, it then moves away; if it mounts an estrous female, it tries to guard her.

STATISTICAL ANALYSIS

The data were compiled using statistical package for social science program (SPSS 16th version) and subjected to analysis of variance (ANOVA) with post hoc Duncan's test comparison (37) (one–way). $p< 0.05$ was considered as significance.

3. RESULTS:

Female behavior during estrus gives a promising non-invasive approach in identifying the reproductive status of the animal. The chained behaviors was recorded in estrus sheep, initial responses reflected on attraction, that the ram oriented the head towards the estrus sample. The female exhibited like hypersalivation and frequent urination. The male smelled towards the vaginal discharge and exhibited behaviors like licking cheasing etc. The second series of action is a detecting phase, because male wanted odour sexually like Flehmen response. These behaviors were follow-up by vocalization, penis protrusion, mounting and copulation. After copulation all sexual activities were ceased (Table.1, Fig. 1).

3.1. GC–MS analysis

In mammals, olfaction is an important modality affecting behavior and odor can carry messages with in species such as sexual receptiveness. These compounds were supposed to be present in estrus female, but absent in other stages of estrous cycle. In the present study, we are looking for estrus specific molecules in the feces sample of sheep during different stages of estrus. The gas chromatography analysis clearly showed 9 major peaks in the three stages like pro estrus, estrus and post estrus that fell between 4 to 27 min retention time (Fig. 2,3 Table 2).

The compounds identified were belonging to alkane, alcohol and alkene groups, among this alkane are predominant. Among the ten volatile compounds identified, 2-pentanone, 4-hydroxy-4-methyl and 2-hexadecene, 3, 7,11,15-tetramethyl were present in all the three stages. The compound tetracosamethyl-cyclododecasiloxane is absent in proestrus and present in estrus and post estrus stages. Benzenemathol, n-pentadecanol, 1,1,2,2-tetramethyl-1,2-diphenyldisilane and dotriacontane were the compounds present both in proestrus or postestrus, but the compound β-caryophyllene (C15H24) with 204 molecular weight was found exclusively in estrus stage at retention time 14.441 which belongs to alkane groups was absent in proestrus and postestrus stages. The compound β-caryophyllene is a unique compound present only in estrus stage.

Behavior assay with synthetic compound and estrus feces

Ram behaviours like Flehmen and mounting was observed towards estrus feces, estrus specific synthetic compound *β*-caryophyllene along with water at different concentration like 0.5%, 1.0%, 2.0% and 5.0%. Among the concentration, 2.0% is effective to elicit the ram behaviour. However, the other concentrations 0.5%, 1.0% and 5.0% were shown to be ineffective in eliciting the ram flehmen behavior significantly. However, the duration of Flehmen was higher in response to the estrus feces (20.54±0.15) than that of synthetic compounds. The suspected estrus feces compound, *β*-caryophyllene showed higher response (18.05±0.14) of flehmen behaviour. The number of mounting were highly

significant in estrus feces (8.58 ±0.87) when compared to synthetic compound (6.47±0.18) and their mixture at different concentrations (Table 3).

4. DISCUSSION :

The role of sexual pheromones in the reproductive behavior of lab mammals is very well studied, but there was limited evidence found in species-specific chemosignal of farm animals. In livestock species, vaginal mucus, saliva and urine and as well as feces have been investigated (38; 16; 17; 28) for the identification of estrus specific volatile molecules. Thus, it is accepted that for chemo-communication and to identify the physiological status of animals, feces may considerably important source. Reports also gave an additional supports that in honey bees, African wild dogs and farmed goat release pheromones, esters as scent marking (39) in feces play potential role in communication (40, 32).

Pheromones play a key role in anima l communication (41), it is reported that it plays a major role in male to female communication in sheep (42; 43). Pheromones are belongs to group, specifically primer pheromones and releaser pheromones which provoke a physiological response and change the animal behavior respectively (8; 44). The pheromones associated with elicitation of LH response in anoestrous ewes is released from sebaceous glands of the skin and wax collected from the ante orbital region of the eye (45). Ram urine is not effective in stimulating an endocrine response in anoestrous ewes (46) in contrast to evidence in rodents (41). The capacity for ewes respond to rams wool alone indicated that the endocrine response observed when rams are introduced to anoestrous females was due to a specific primer pheromone similar to that in rodents (47).

The physiological effect of exposure to socio-sexual cues is not restricted to an effect of the ram on the ewe; indeed exposure of ewes to rams stimulates an increase in LH pulse frequency in the rams (48, 49). This endocrine response is not dependent on the ewe being sexually receptive; however it is enhanced if the ewe is in oestrus (50). All potential sources of a 'female pheromone' have been investigated by exposure of rams to masks containing female fleece, urine and vaginal secretions. However in contrast to the investigation into the source of the 'ram pheromone (45) there was no endocrine response within rams exposed to the female odours. Hence the present study was designed to identify the estrus specific volatile compounds which are involved in the attraction of males and lead to active mating.

Animals eliminate metabolic products through feces is one of the main path, thus, it is understood that feces convey the internal physiology of the animal to the external world. Thus, the feces give information beyond the source of chemosignals in many species, like rats (51), pigs (52), lizards (53) and blackbucks (27. 54) and goat (32). It is identified that feces act as potent source of chemical signals , which all supports the present opinion. In the present study, numerous volatile compounds were identified at different phases of the estrus stages which are differed from one phase to the other and there were nine volatile compounds identified in the sheep feces, of these β-caryophyllene exclusive in estrus stage and is absent in all other stages. In fact, Achiraman and Archunan (17) reported that the 3-ethyl – 2,7 dimethyl octane, a single volatile compound present in the male mice urine can act exclusively as attractant. Our findings further correlated with the results of Rasmussen et al., (55,56), who identified 7- dodecenyl acetate in Asian elephant, which is in high concentration during ovulation and this compound induces sexual behavior in male. Therefore, the present finding made an additional evidence to the earlier reports that single compound can act as a pheromone in rat and in bovine urine. Normally pheromones are defined as it may be single or a mixture of compounds but every fraction of it will

responsible for reproductive and social behavior. In our current investigation, a single unique compound has been identified and it may act as a chemo-signal for sexual attraction. Hence, the present study develops an idea that for chemical communication a feces compounds may involve (51; 57; 1995, 58,59). Signoret (60) proposed an alternate origin of the female pheromone to those tested or that the proximity to the stimulus permitted by the masks was insufficient to elicit an endocrine response. Breed, et al., (39) stated that estrus feces of honey bee contain pheromones able involved in scent marking. Further, in African wild dog a pheromone has been identified in feces which would play a potential role in communication (40). Hence, it is adequate that, as the results of metabolism in animals feces is one of the major media and therefore it is not surprising that feces can potentially convey much information about the internal physiology of the animal to the external world and thus provide a source of chemosignals in many species. In rats (51), pigs (52), lizards (53) and blackbucks (54) researchers may point out that feces is a potent source of chemical signals and hence, the present study supports to the concept that feces contain an array of compounds that are involved in chemical communication (61). It is already reported by Sasso et al., 2009, that E-β-caryophyllene was demonstrated to be attractive towards aphid parasitoids, *Aphidius ervi*. It is an sesquiterpene compound having biological properties like aggregation pheromone of the Asian ladybeetle, *Harmonia axyridis*, this gave an additional support to the present findings.

The alcohols, saturated, aldehydes, unsaturated acids, aromatic compounds aliphatic, and non-polar molecules such as alkanes and alkenes to highly polar compounds acidic (acids, phenols) or basic (amines) has been reopted as reported as pheromones (52). In the present study, the majority of compound belongs to alkane groups. Further, in the present study, β-caryophyllenes give an supporting evidence that alkane compounds probably play a significant role in sexual attraction in mammals. For instance, 1-iodoundecane an estrus specific compound only present in estrus bovine urine and is also an alkane which play a significant role in the attraction of bulls (16) and 1,5-diemethy-6,8- dioxodicyclo octane found to act as pheromone in elephants (62). Likewise, alkane present in bitrophic herbivore plant interfaces as well as attractants for oviposition or attraction for feeding in insects, (63).

The compound β-caryophyllene an estrus specific compound has molecular weight of 204, with 15 carbon, β-caryophyllene this result correlated with the properties of pheromones as reported by Archunan (2009), he reported pheromone must have lower molecular weight which is less than 300 molecular weight, 20 carbon, which is may reach the receiver as it is volatile the major properties of pheromones (63; 64). 1-iodo undecane, an urinary estrus-specific compound in bovine, has low molecular weight of less than 300. Similarly, compound 7-(Z) dodecene-1-yl acetate with 13 carbons and a molecular weight of 300 was identified in the urine of female Asian elephants during preovulatory which attracts the males elephant . These compounds act as primer pheromones, that they may normally pass through the vomeronasal organ and reach the accessory olfactory bulb via the vomeronasal nerve. Pheromone may arouse sexual behaviours like erection, mounting, and copulation in male. Hence, the unique compound β-caryophyllene observed during estrus period has physical properties necessary for consideration as chemo-signal and be act as estrus specific volatile compound in sheep feces. Kimura (64) reported that, the reproductive status of animals was noticed by the fecal sample in females. The fecal components indicate the estrus information in bovine by the bull behavior assay (17). Further, it is also useful, to evaluate the reproductive status of the animal as non-invasive method. Dehnhard, et al., (65), proved the reproductive status of the animal through the urine of bear. Sankar and Archunan (17),

noticed that during estrous cycle there was an apparent variation in volatile compounds present in bovine feces. It is very interesting to note that, the compounds 4, -methyl phenol and trans-verbenol are uniquely present only in estrus phase. Mozuraitis, et al., (66) identified m-cresols in the urine of estrus mare, also supports the present findings . Comparisons of the fecal profiles in all samples indicate that there is specific volatile compounds observed in estrus, which are not found in other feces. Since a natural estrous cycle is caused by hormonal changes, the above qualitative variations in the urine may also be due to endocrine changes.

The present investigation clearly indicated that 2.0% concentration of β-caryophyllene compound is significant in flehmen and mounting behaviours. It is interestingly to note that the increases in the concentration of compounds did not enhance the ram behaviour. It indicates that the optimum concentration required to influence the ram behaviour appears to be 2% regardless of the volatiles involved in attractant and mounting activity, this result was also supported by correlated to the results of Prabu and Rameshkumar (67,68) and Sunthanithirakanan and Rameshkumar (69) similarly, Rasmussen, et al., (70) reported 0.5mm concentration of (Z) 7-dodecene -l- yl acetate consistently elicited high levels of flehmen responses equivalent to bio responses elicited by pre – ovulatory urine.

The results further revealed that bulls paying attention and showed frequent flehmen behaviour significantly against the 1-iodo- 2-methylundecane in contrast other compound tetetratetracontane did not elicit significantly. Identification of (2) – 7- Dodecen-1-yl-acetate in female Asian elephant that is released in the pre- ovulatory urine was found to influence the specific behavior include flehmen in male elephants (55). In addition, Archunan and Rameshkumar, (71) identified two estrus specific compounds in cow urine and conducted the bull bioassay using synthetic compounds and found that the compound 1-iodoundecane elicits flehmen behaviour in bull. Likewise, sex specific pheromones have been characterized structurally identified and linked to sexual mammals (65, 72, 73, 74). In addition, the compound 1- iodo- 2-methylundecane is known to putative estrus specific urinary chemo signals of female mouse (Achiraman and Archunan, 2006).

The feces of bovine showed the difference of volatile compounds throughout the estrous cycle (17,28,76). In their assessment, the compounds 4-methyl phenol and trans-verbenol exists only during the estrus phase. Mozuraitis, et al., (66), reported that the urinary compound, m-cresols identified in estrus mare can be used as marker. In *Drosophila melanogaster* a compound 4-methyl phenol present in olfactory receptors contact as pheromone (77). The study in bark beetles, suggested that the compound, trans-verbenol is involved in pheromonal communication (78; 79).

In ungulates, male reproductive behavior like flehmen and mounting are noticed by (10). Ghosal, et al., (33) reported that the during the follicular and ovulatory phases female elephant attract the males to female feces. In our present study, ram shows various attraction levels at different phases towards ewe feces. The behaviors like chasing, Flehmen, penile protrusion and mounting were observed more in estrus sheep. This result coincidence with the report of Price, et al., (80) that estrus male goat shows self-enurination towards estrus female. Pugh, (81); Sukumar, (82); Archunan and Ramesh Kumar, (83), Karthikeyan et al., (84), suggested that flehmen and mounting were significantly different towards estrus specific compounds. Hence, we stated that the female feces may communicate the status of the female to male. Furthermore, we noticed the difference in the male reproductive behaviors towards the estrus female.

The compound β-caryophyllene appeared only in estrus period and not found in any other phases. This is an important finding in the present study and confirms the role of identified compound with ram behavioural studies. The response of ram to the synthetic compound is very much satisfactorily and this finding would give a real impact in farm animal reproduction with real economic importance. It is also possible to use this compound as a marker to develop a biosensor kit for effective estrus detection.

5. CONCLUSION :

The female specific volatiles express its receptivity to attract the male for its pre-mating behavior and coitus and this is the first study on the reproductive behaviors and identification of volatile profiles in correlation with fecal steroids of Ramnad white sheep. Feces may act as chemo-signal prompting behavioral response during reproductive stage. In this regard, the present study highlights that the compound β-caryophyllene exclusively present in estrus stage may be considered as a putative pheromone to attract the males. In addition, it is proved and confirmed that the compound may play a role as chemical signals by the behavioral assay. Hence, the β-caryophyllene may act as an estrus indicator in sheep, which would be act as a marker of estrus detection by non-invasive method.

Acknowledgment

The authors gratefully acknowledged to UGC-RGNF, for providing finical support to carry out this work very successfully, Ref. No. F1 17.1/2011-12/RGNF-SC-TAM- 1690/ SA-III/Website.

Table 1 Behaviors observed in male and female sheep during estrus phase in field condition

Behaviors	**Female**	**Male**
Body rubbing	✔	**X**
Restlessness	✔	**X**
Reduced food intake	✔	**X**
Raising of tail	✔	**X**
Frequent urination	✔	**X**
Vaginal swelling	✔	**X**
Vaginal mucus discharge	✔	**X**
Bellowing (deep loud roar)	✔	**X**
Teasing of male goat	✔	**X**
Homosexual behavior (chasing other females in the absence of males)	✔	**X**

Repeated flehmen	**X**	✔
Mounting	**X**	✔
Penile protrusion	**X**	✔
Copulation	**X**	✔

Fig. 1 Behaviors exhibited by male sheep and estrus female field condition

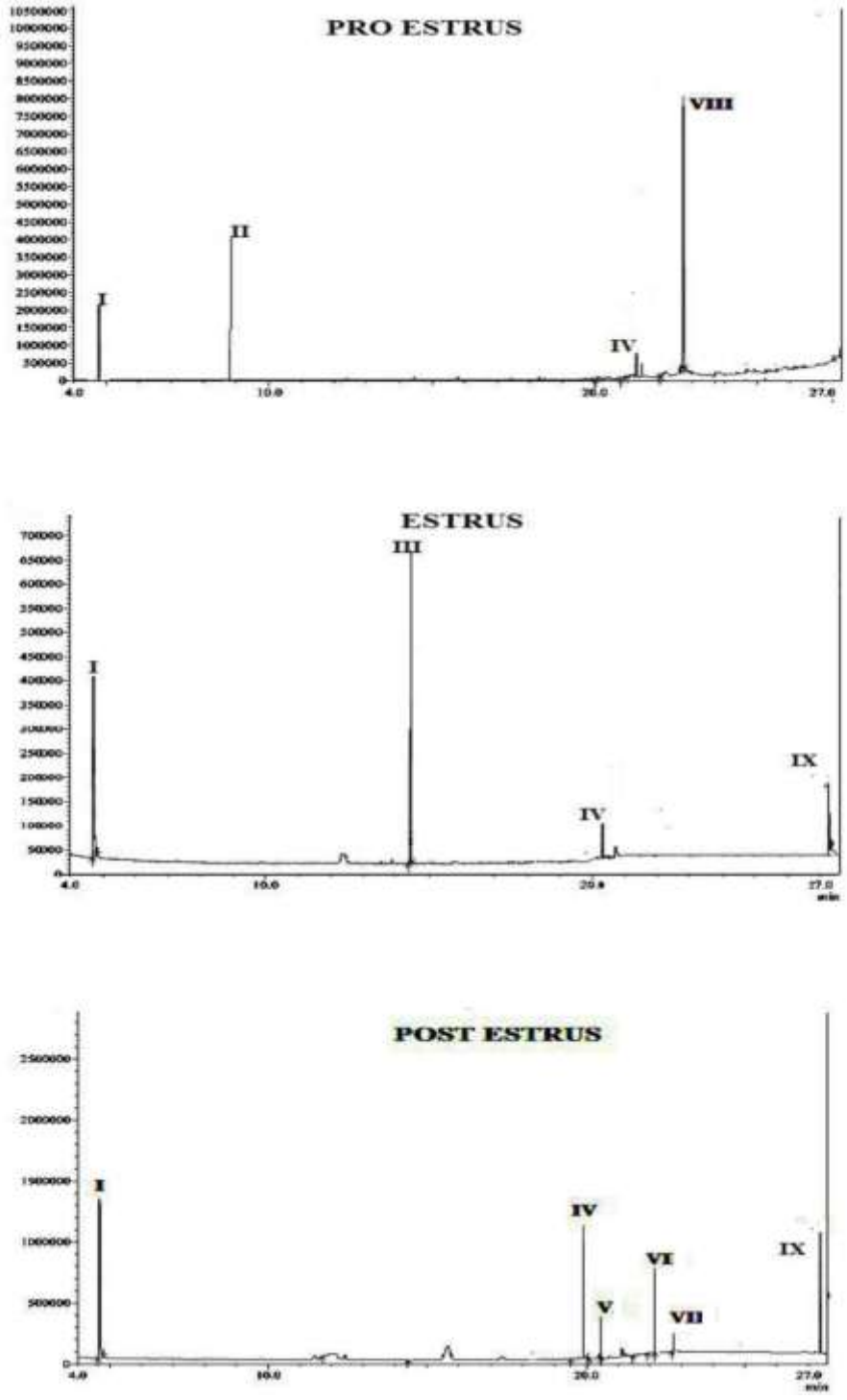
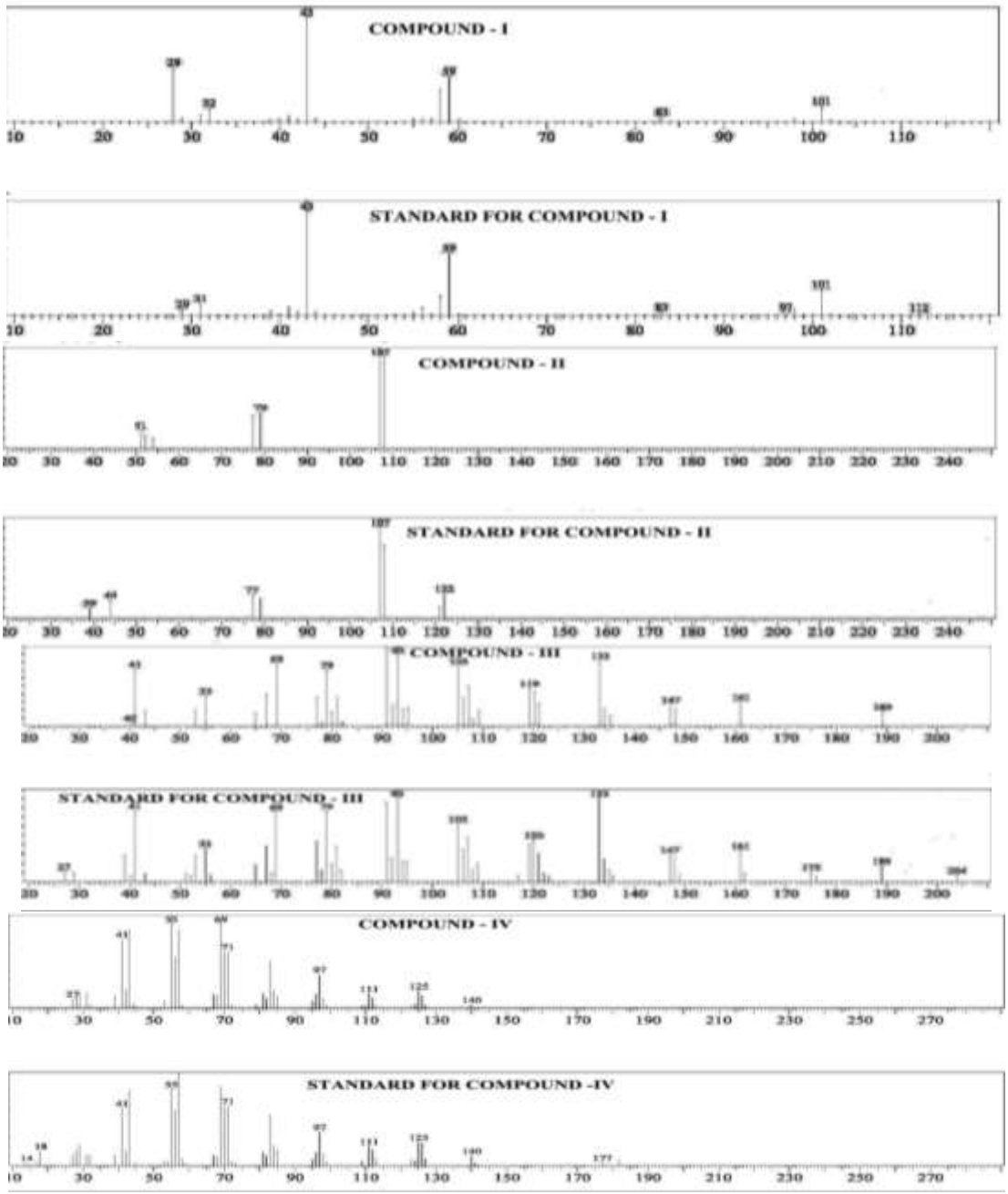
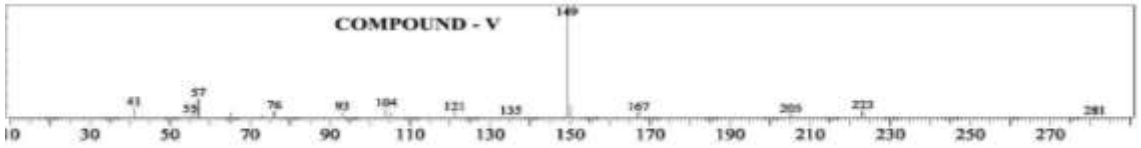


Fig. 2 Gas chromatography profile of volatile compounds identified in sheep

(*Ovis aries*) feces during various estrus stages .
Peak No. referes to Table. 2.

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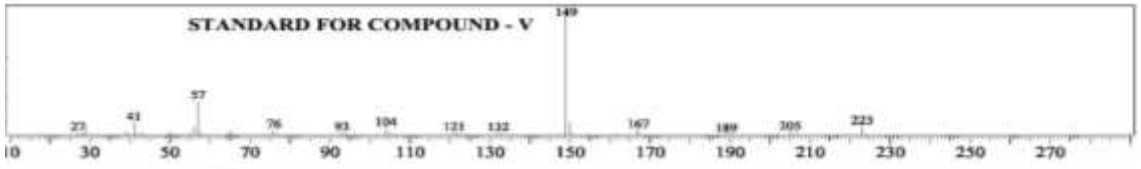
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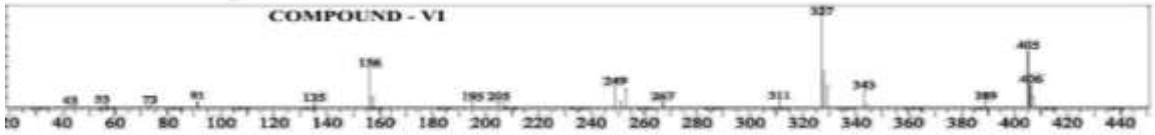
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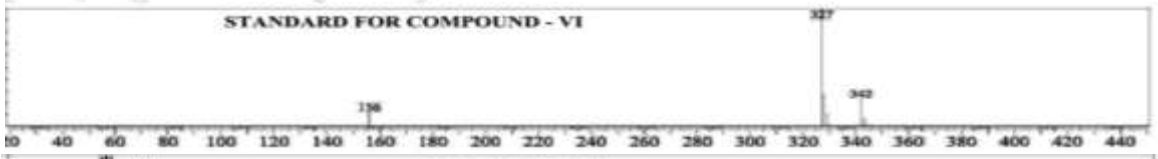
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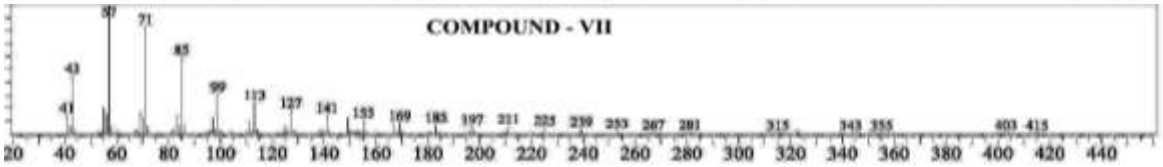
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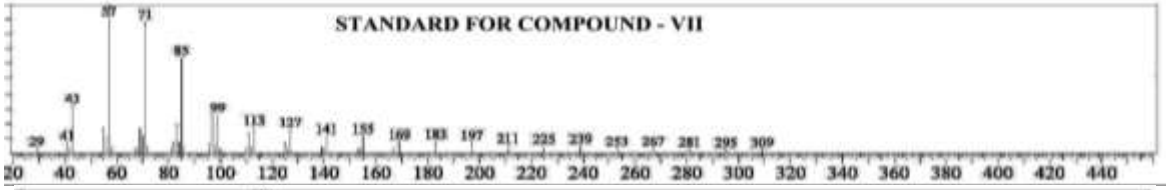
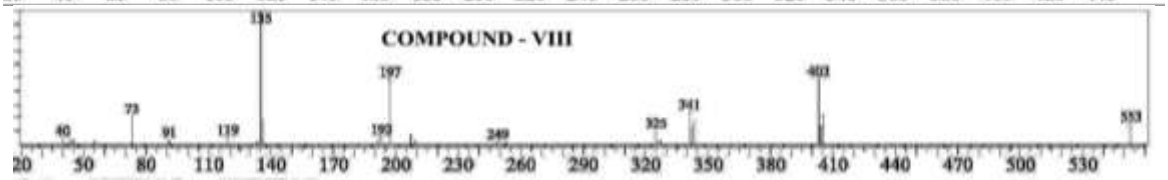
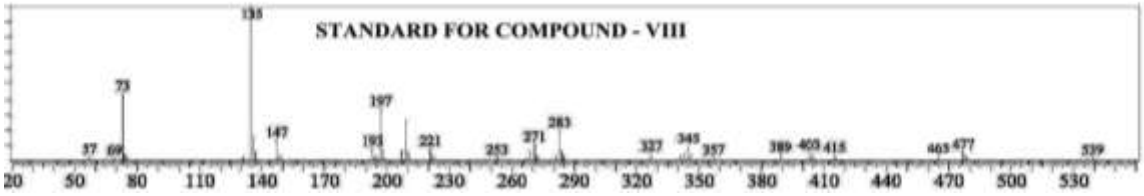

STANDARD FOR COMPOUND - VII


COMPOUND - VIII


STANDARD FOR COMPOUND - VIII

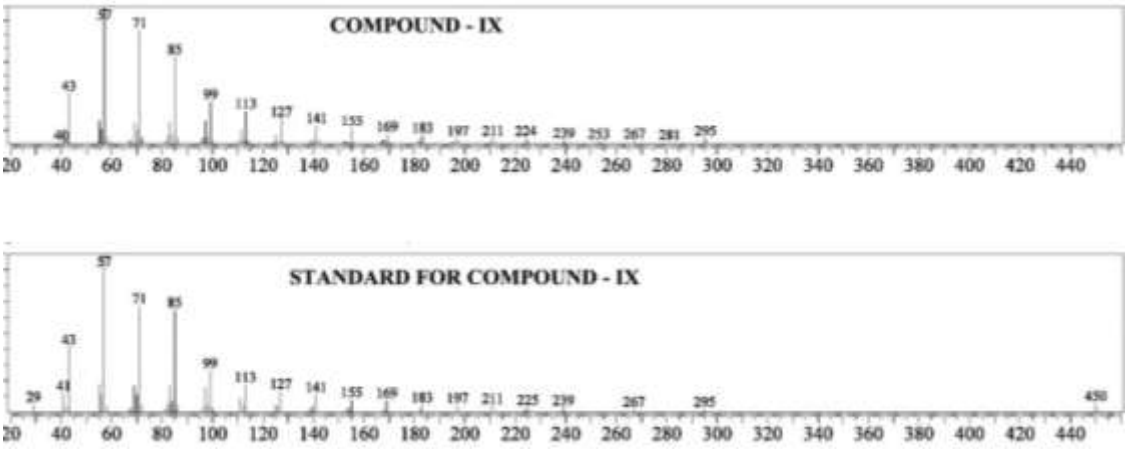
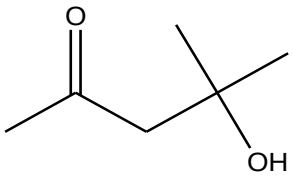
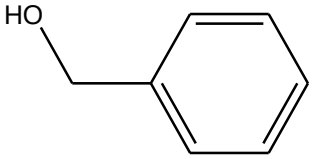


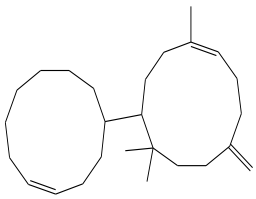
Fig. 3 Mass spectrum of sheep fecal compounds identified in various stages of estrus



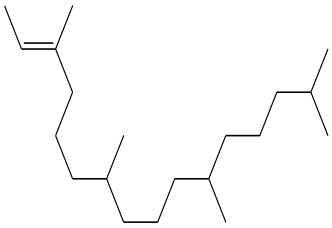
2-Pentanone, 4-Hydroxy-4-methyl



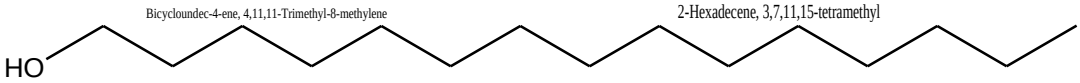
Benzenemethanol



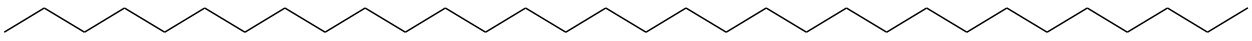
Bicycloundec-4-ene, 4,11,11-Trimethyl-8-methylene



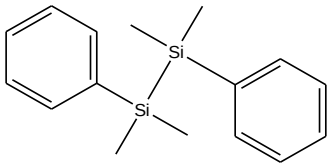
2-Hexadecene, 3,7,11,15-tetramethyl



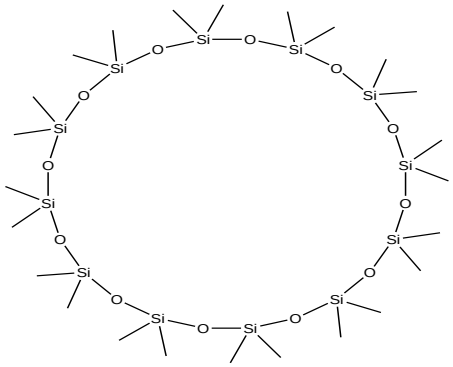
n-Pentadecanol



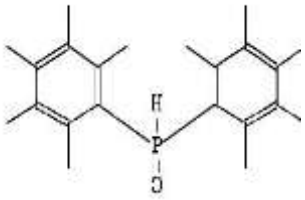
Dotriacontane



1,1,2,2-Tetramethyl-1,2-diphenyldisilane



Tetracosamethyl-cyclododecasiloxane



1,4-Bis tetrafluorobenzene

Fig. 4 Chemical structures identified in sheep fecal in various stages of estrus

Table. 2. Fecal compounds identified in sheep during various estrus stages

Peak No.	Compounds	R.Time	Molecular Formula	Molecular Weight	Nature	Pro	Estrus	Post
I	2-Pentanone, 4-Hydroxy-4-methyl	4.707	$C_6H_{12}O_2$	116	Diacetone alcohol	✔	✔	✔
II	Benzenemethanol	8.733	$C_{10}H_{10}F_{2O}$	184	Alcohol	✔	**X**	**X**
III	***β*-caryophyllene**	**14.441**	**$C_{15}H_{24}$**	**204**	**Alkane**	**X**	✔	**X**
IV	3,7,11,15-tetramethyl 2-hexadecene	20.100	$C_{20}H_{40}$	280	Alkene	✔	✔	✔
V	1,4-Bis tetrafluorobenzene	20.478	$C_{16}H_{18}F_4Si_2$	342	Alkene	**X**	**X**	✔
VI	n-Pentadecanol	21.958	$C_{15}H_{32}O$	228	Alcohol	**X**	**X**	✔
VII	1,1,2,2-Tetramethyl-1,2-diphenyldisilane	22.767	$C_{16}H_{22}Si_2$	270	Alkane	**X**	**X**	✔
VIII	Dotriacontane	22.733	$C_{32}H_{66}$	450	Alkane	✔	**X**	**X**
IX	Tetracosamethyl-cyclododecasiloxane	27.392	$C_{24}H_{72}O_{12}Si_{12}$	888	Alkane	**X**	✔	✔

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