IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 9, Issue 6 Ver. II (Jun. 2016), PP 52-59
www.iosrjournals.org

Gas Chromatographic Monitoring and Quantification of the Estrus -Specific Pheromone Compounds in Female Murrah Buffalo Urine

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Abstract: *The objective of this work is to quantify the estrus-specific volatiles in the urine of murrah buffalo, Bubalua bubalis. Using the gas chromatographic analysis the estrus-specific volatiles such as 4-methylphenol and 9-octadecenoic acid was quantified. The level of estrus- specific volatiles 4 – methyl phenol was ranged from 2 ppm to 5 ppm and 9 – octadecenoic acid was ranged from 5 ppm to 10 ppm during the estrus phase of female buffalo.*

Keywords: *Buffalo, Estrus, Gas Chromatography, Quantification, Volatiles*

I. Introduction

Mammals exhibit spontaneous estrous cycles and the natural estrous cycle is regulated by hormonal changes. Thus, endocrine changes may cause excretion of different signals that communicate different reproductive states. Urine is a reliable indicator of the physiological status of an animal since it functions as the major means by which metabolic waste is eliminated [1]. Therefore, it is not surprising that urine can convey to the external world much information concerning the internal physiological state of the animal, and so provides a source of chemical signals in many species. The urinary compounds of few mammals have been thoroughly studied, and their relative effects have been established as potential source for pheromones [2,3].

Sex pheromones in female urine of house rat during estrus was identified [4] namely hydroperoxide, 1-nitropentane and 4-azidoheptane. In mouse, the urinary sex pheromones have been assessed in both male and female namely 1-iodo-2-methyl undecane, 3-ethyl-2-methylhexane respectively and the behavioural assay confirmed that the pheromones have specific sex attractant [5,6]. The estrus specific compounds, 1-iodoundecane and di-n-propyl phthalate were detected during estrus in bovine [7]. However, the behavior study revealed that the compound 1-iodoundecane exhibited extraordinary attraction in bull by expressing flehmen behaviour but other volatile di-n-propyl phthalate. It indicates that the 1-iodoundecane in the estrus female urine is an effective estrus-specific chemical signal which may be used as a marker to detect estrus in bovine [8]. Though the sex pheromones have been identified in certain mammals, quantification of the sex pheromones in most of the species is not yet been evaluated.

Nowadays, urine is believed to use as a tool in diagnosing the status of an individuals. Sampling of blood, cerebro-spinal fluid is an invasive method and it disturbs the normal functions of the body. Urine sampling is a very simple and non-invasive. The scientific study of olfaction and chemesthesis (i.e., chemical sensory irritation) inevitably requires some attempts to deliver controlled amounts of chemical vapors. For pheromone study, it is essential for identification of volatile compounds and behaviour assessment then only the involvement of pheromone communication can be confirmed in an individual. Apart from these assessments, quantification of estrus- specific compounds would give reliable information about the required amount of volatiles to evoke the specific behaviour. The estrus specific pheromones 4-methylphenol and 9-octadecenoic acid are reported as the sex pheromones in buffalo[9]. Certain volatile are reported to be present in more than species, such cases the quantity of compounds may vary. The identified sex pheromones in buffalo are not yet quantified. Since, our long term objective is to develop a kit based on the urinary pheromone compounds, the level of the urinary compounds needs to be evaluated. Hence, the present study focused on quantification of estrus specific compounds, 4-methyl phenol (4 MP) and 9-octadecenoic acid (9 ODA), in the urine of estrus phase of buffalo.

II. Materials And Methods

2.1 *Animals*

Six healthy murrah (*Bubalus bubalis*) buffalo were chosen for the present investigation and the study was carried out at Veterinary College and Research Institute, Namakkal district, Tamil Nadu, India.

2.2 ***Determination of estrous cycle***

The phases of the estrous cycle was carefully determined for three naturally occurring consecutive cycle and confirmed by trans-rectal palpation by assessing the morphological changes in the internal reproductive organs and also the reproductive behaviour of bulls like flehmen, mounting, licking and penile erection [10]. As mentioned elsewhere in the thesis the estrus phase was confirmed by fern pattern analysis from cervico-vaginal mucus samples. The cervico-vaginal mucus samples were collected by using a sterile cotton swab insertion to the cervical region and then it was smeared between two glass slides and observed for the presence of fern like crystals[11].

2.3 ***Experimental design***

The urine samples were collected during estrus phase following the method of Rajanarayanan and Archunan (10). Urine was collected from the experimental animal at three hours interval and immediately analysed for monitoring of the estrus specific volatile compounds by extracting with the dichloromethane. Synthetic compounds were procured from Sigma-Aldrich and the standard curve was prepared by dissolving the compound in solvent as parts per million concentration and run in GC-FID. Test samples were run under the same programme and the level of the compound was calculated using the standard graph.

2.3 ***Sample preparation for monitoring the 4-methyl phenol and 9-octadecenoic acid***

Since, the pheromone compounds 4-methyl phenol (4 MP) and 9-octadecenoic acid (9 ODA) are particularly present in buffalo estrus, the present study focused to collect the sample during estrus phase for quantification. Urine samples from different time intervals of the estrus phase i.e., every three hours were collected regularly processed and analyzed in the GC-FID for 4-methyl phenol and 9-octadecenoic acid monitoring. The standards of 4-MP and 9-ODA were run separately under the programme and the retention time was noticed. Likewise, the samples collected during the estrus phase were run and the duration of estrus was calculated by the appearance of 4-MP and 9-ODA peak and its time of disappearance. By plotting the concentration obtained from the GC-FID response the quantity of 4-MP and 9-ODA was calculated.

III. Results

Estrus was confirmed by observing the fern pattern in vaginal mucus smear on the slide. Crystallization of vaginal mucus fern pattern was noticed in the slide. More crystallization with numerous branches was observed in the vaginal mucus of estrus phase (Fig. 1).

After the confirmation of estrus the urine was collected for GC-FID and quantification of estrus - specific compounds such as 4-MP and 9-ODA was carried out. The two estrus-specific compounds such as 4-MP and 9-ODA were quantified, in the urine collected in estrus animals at different time from the onset of estrus, using the standard graph (Figs. 2 and 3).

Authentic standard of 4-MP and the 9-ODA were run and the peak was observed and recorded at the retention time of 7.8 minutes (Fig.4) for 4 MP and 21.0 minutes for 9ODA (Fig.5). Both the compounds were quantified in the samples collected from different time of estrus urine through the analysis in the GC-FID. During the onset of estrus, the 4 - MP was slightly appeared as baseline and gradually increased to a maximum with the frequency of sampling and lowered corresponding to the time (Figs. 6 - 13). The quantification carried out in samples during estrus in buffalo was ranged from 13 to 27 hrs. From the present observation, the quantity of 4-MP was calculated as 2, 5, and 10 ppm in early hours and increased maximum of 4.8 and 5.8 at 15 and 18 hrs respectively(**Table 1**). However, the level was decreased as 2 .75 ppm at late estrus after 24 hours following the onset of estrus. Likewise, the concentration of 9-ODA was remained as 10 ppm between 9-18 hrs after the onset of estrus except the early and late estrus (**Table 1**).

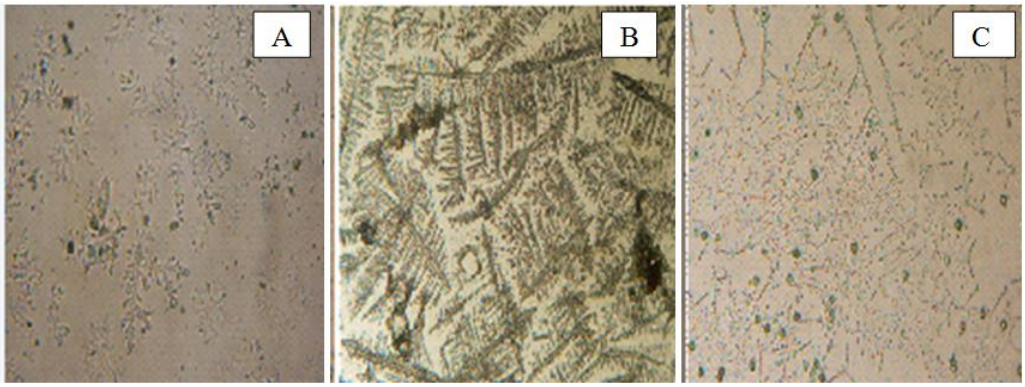

Fig. 1. Fern pattern A) Early estrus B) Mid-estrus C) Late estrus

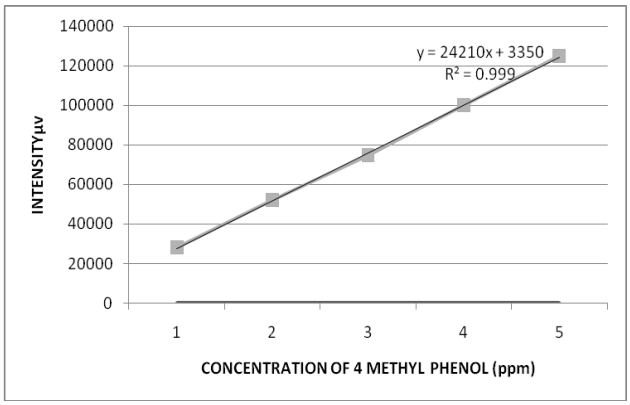

Fig. 2 Standard graph of the authentic synthetic compound, 4- methyl phenol

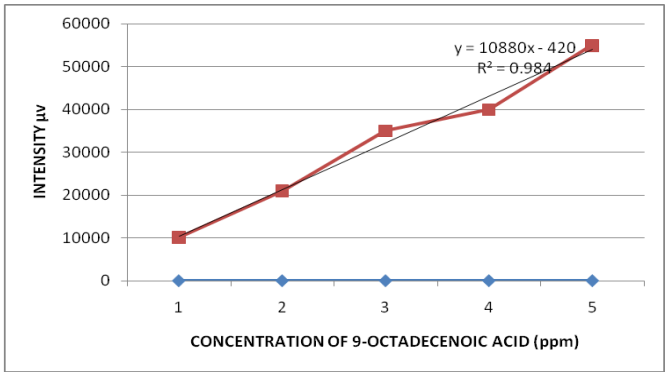

Fig. 3 Standard graph of the authentic synthetic compound, 9-octadcenoic acid

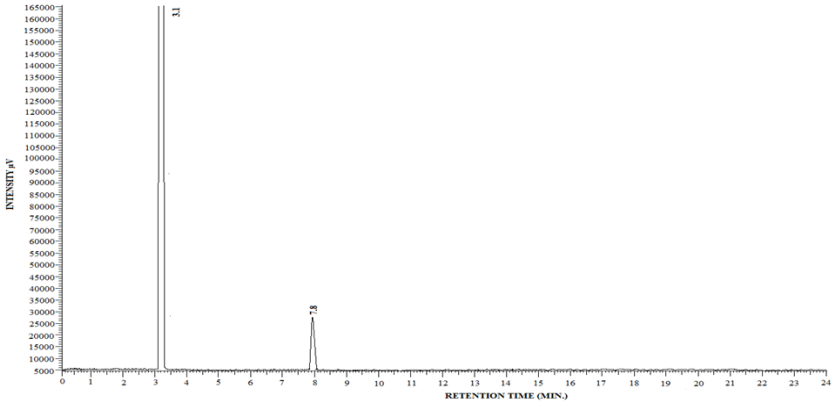

Figure 4 Representative chromatogram of the standard 4 – methyl phenol

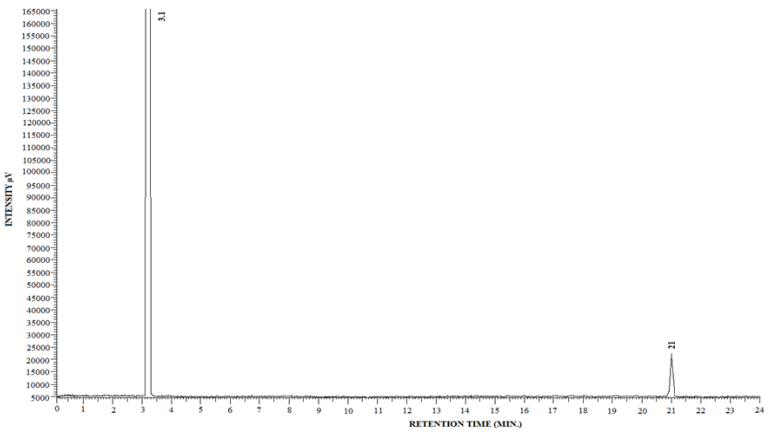

Figure 5 Representative chromatogram of the standard 9-octadecenoic acid

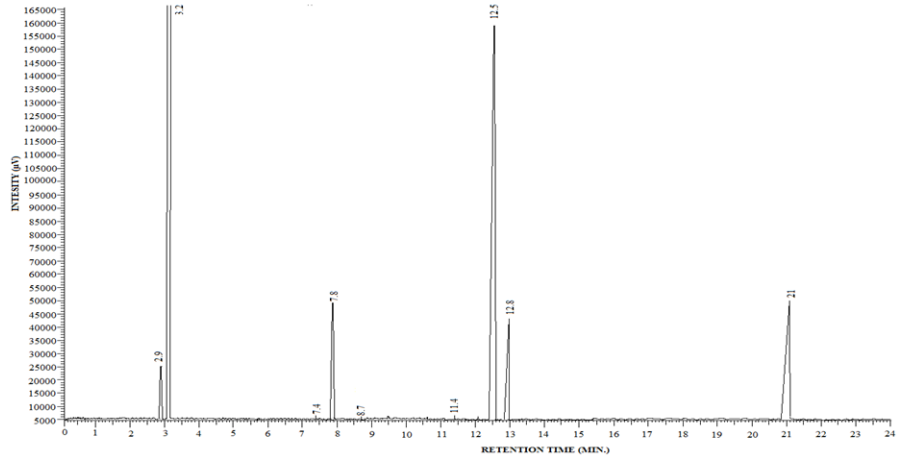

Figure 6 Representative chromatogram of urine (3 hours after the onset of estrus)

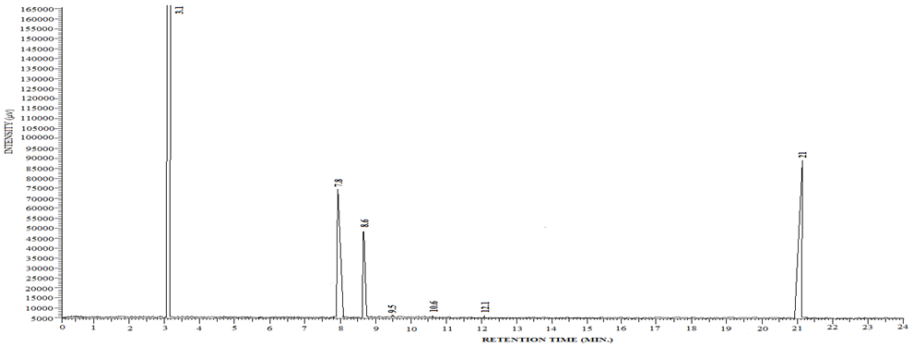

Figure 7 Representative chromatogram of urine (6 hrs after the onset of estrus)

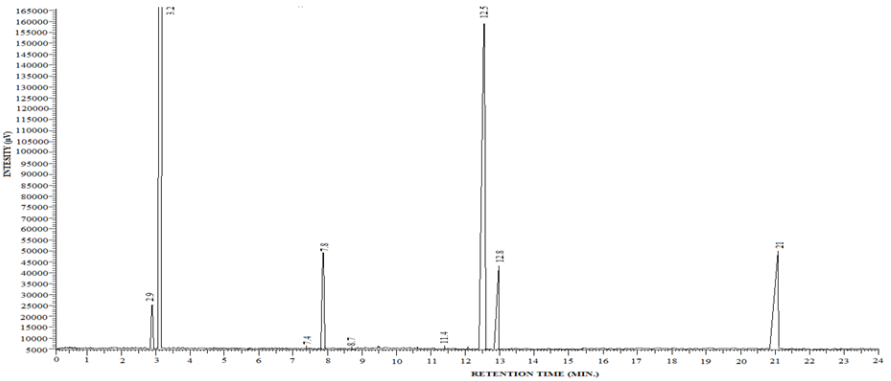

Figure 8 Representative chromatogram of urine (9 hrs after the onset of estrus)

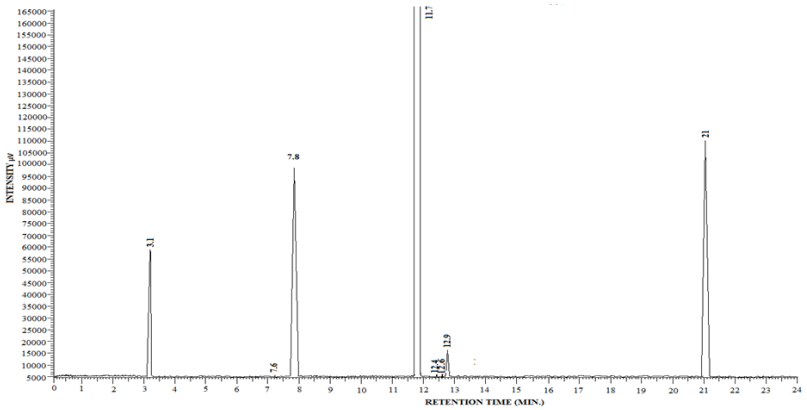

Figure 9 Representative chromatogram of urine (12 hrs after the onset of estrus)

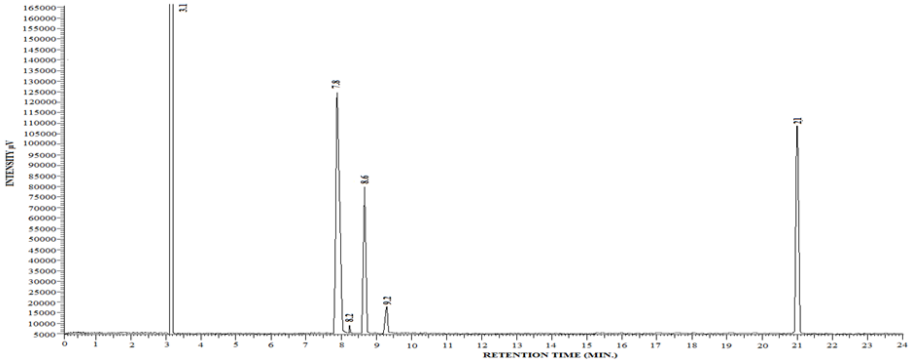

Figure 10 Representative chromatogram of urine (15 hrs after the onset of estrus)

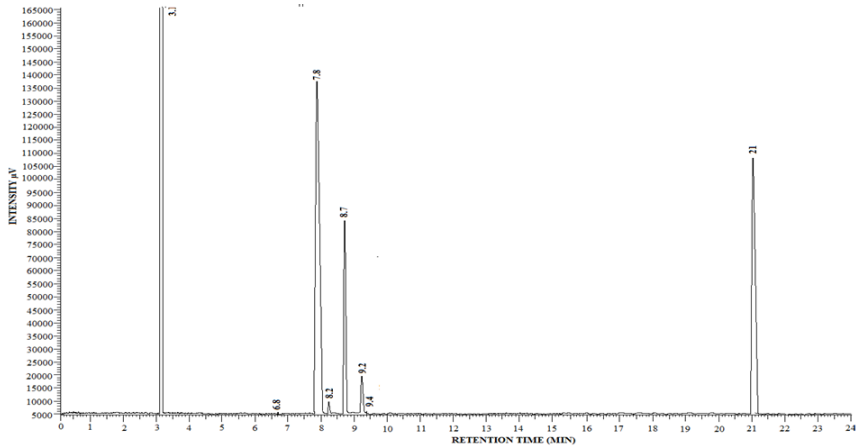

Figure 11 Representative chromatogram of urine (18 hrs after the onset of estrus)

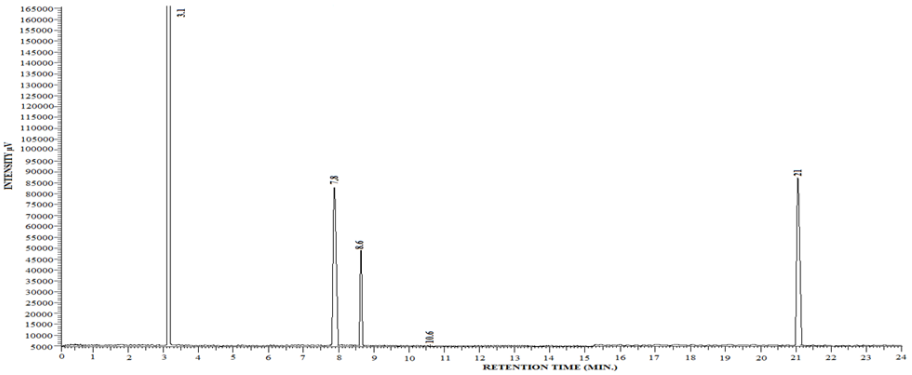

Figure 12 Representative chromatogram of urine (21 hrs after the onset of estrus)

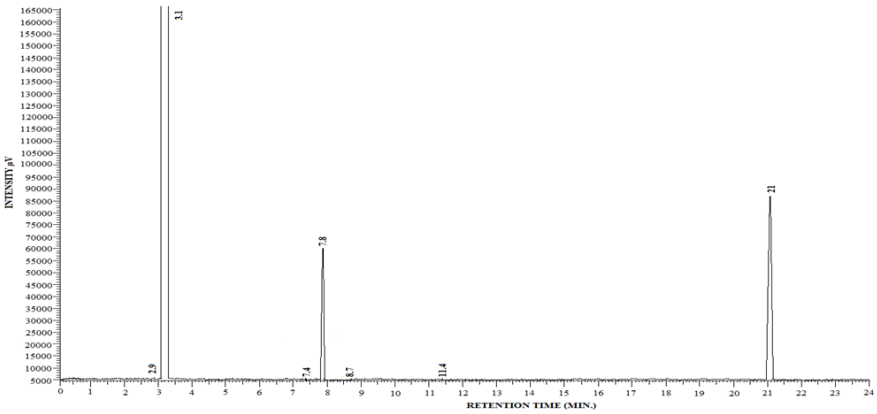

Figure 13 Representative chromatogram of urine (24 hrs after the onset of estrus)

Table 1. Concentration of 4-methyl phenol and 9-octadecenoic acid (Mean ± SE) in the estrus urine sample at different time intervals

S.No.	Time after the onset of estrus (hrs)	4-methyl phenol (ppm)	9-octadecenoic acid (ppm)
1	3	2.10 ± 0.03	5.27 ± 0.03
2	6	2.27 ± 0.06	8.20 ± 0.05
3	9	3.56 ± 0.04	10.06 ± 0.01
4	12	4.17 ± 0.01	10.25 ± 0.21
5	15	5.48 ± 0.07	10.10 ± 0.018
6	18	5.80 ± 0.03	10.03 ± 0.13
7	21	3.53 ± 0.01	7.97 ± 0.01
8	24	2.75 ± 0.01	8.22 ± 0.07

IV. Discussion

The presence of estrus -specific compounds in buffalo urine is confirmed further in the present study. The results are strongly support with our previous investigation [9]. In the present study, the quantification of two estrus specific compounds, 4-MP and 9-ODA, revealed the level of those compounds varied dramatically during estrus. During the estrus phase, the 4-methylphenol was monitored with time intervals and found that the volatile compound was appeared during the onset of estrus, reached maximum with the progression of time particularly during 15-18 hrs after the onset of estrus and decreased in concentration of volatile after 18 hrs. Quantification study was performed by comparison with two authentic synthetic standard compounds. It further provides valuable support for the presence of these two sex pheromones in buffalo. There are many approaches are attempted for detecting estrus in buffalo. For example, tail painting [12,13] pressure activated heat mount detectors [14,15] vasectomized bulls [16], electronic Bovinose [17], trained dog [18]. None of the tool found effective to detect estrus in buffalo.

Generally, the expectancy towards the level of 4-MP was higher during early estrus time or pre-estrus. But in the present observation the maximum concentration of 4-MP (5.80 ± 0.03 ppm) was observed at mid estrus in female that shows apt time for insemination and invites the bull to copulate. The mid estrus may provide threaten signal to the bull that the period of receptivity of the female comes to an end soon. This is coincide with the reports of Rasmussen *et al*. [19,20,21] who identified and characterized the 7-dodecen-1-yl-acetate as pre-ovulatory indicating pheromone found in high concentration. In the present study, it shows that the presence of estrus specific compounds in buffaloes may vary from estrus urine in concentration wise.

Fern pattern study was performed for confirming the estrus. The branched like fern was observed in the vaginal mucus of estrus but there was no fern in non-estrus animals. Cervical mucus is a visco-elastic fluid constantly secreted from the mucus producing cells of endo-cervix [22].Visual appearance of cervical mucus is a good indicator of reproductive health status of animal. Appearance of estrual cervical mucus changes during different stages of estrous cycle in cattle ranging from transparent to translucent. Crystalization of vaginal mucus was more at mid estrus and during the initial and late time of estrus the branches of crystallization is very low. [23] Reported that the appearance of more intensive venation in cervical mucus at estrus due to the increase in peripheral estrogen concentration. Reproductive behaviours like sniffing, licking chin resting, flehmen and mounting of the bull was maximum during early, mid time of estrus rather than late time of estrus (data is not included). Among which, the bull exhibited intermittent and thurstful mounting activity towards the early or mid time estrus female than the late time of estrus and non-estrus female. From the present investigation it is evident that the early and mid time of estrus phase appear to be pronounced effect on males to proceed copulation.

In the present investigation, a raise fall pattern of 4-MP in the urine of estrus phase corresponds with the study of Mozuraitis *et al*., [24] which also irregular quantitative changes of 4-MP until 3–4 days before ovulation and afterwards concentration began to increase reaching a peak a day before ovulation. They further reported that on the day of ovulation amounts of the metabolite decreased sharply almost to basal concentrations and remained low for 6 days when sampling was completed. Monitoring the amounts of m- and p-cresols during and a few days before and after estrus revealed the presence of clear peaks for both compounds one day before ovulation [25].

The estrus- specific compounds quantified in the present study may helpful in making estrus detection kit as well as it may prescribe the dosage for exhibiting the bull behaviour. It is also suggested that the optimum dosage of estrus - specific compounds may require to increase the sperm quantity and quality in bull. In the present study, the 4-MP was available about 5 ppm and 9-ODA was available about 10 ppm concentration during mid estrus in which the female probably exhibits maximum sex desireness towards bull. This may be considered as the optimum period for insemination. Interestingly, the quantity of 4-MP was lower in buffaloes when compared to the estrus mares [25]. Hence, it presumes that, if the similar pheromone compound is present in more than one species to act as a sex attractant in different species then it may differ in concentration to attract the conspecifics. The 4-MP was also reported in the insect tsetse fly [26]. It is also suggested that, the 4-MP having germicidal properties [27] and this compound may act as antiseptics, preventing the genitals of both

sexes during sexually active periods. It is further noticed that the presence of p-cresol in the urine and feces of both mares and stallions was reported [25]. Likewise, the sex pheromones i.e., 4 MP and 9 ODA are identified in urine [9] and feces [28] of buffalo during estrus. The present study in regard to quantification of estrus-specific pheromone compounds would definitely help in making biochemical kit or even biosensor for detection of estrus in buffalo.

V. Conclusion

The quantification of estrus – specific volatiles such as 4 – MP and 9 – ODA was monitored across the estrous cycle. The level of estrus – specific volatiles 4 – MP was ranged from 2 ppm to 5 ppm of and 9 – ODA was ranged from 5 ppm to 10 ppm during the estrus phase of female buffalo. This will be used to develop a marker based economically low cost effective kit for the detection of estrus in buffalo at farms/ herds and provide the information for the time of insemination which leads to success in conception rate.

Acknowledgement

We thank DBT and ICAR national fund, New Delhi for the financial support. The facility availes from UGC-SAP II phase as well as DST-FIST II phase is acknowledged.

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