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## **Detection of Protein Bound Volatile Compounds in Buffalo Urine**

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### **Abstract**

Animal urine contains different non-polar volatile compounds, which are known to stimulate their sexual behavior. These compounds collectively termed as pheromones that remain bound to some urinary proteins, which help in their signaling. The objective of this experiment was to identify the urinary volatile compounds before and after protease treatment in bull and in various reproductive stages of female buffaloes, viz. estrus, diestrus and pregnancy, by chemical extraction followed by thin layer chromatography (TLC). Buffalo bull urine showed different compounds in TLC upon protease treatment, where as no change in retention time values were observed for female buffalo urine upon protease treatment. It was found that buffalo bull urine contains protein bound non-polar compounds, which can be set free upon protease treatment and detected by TLC.

*Keywords:* Buffalo; Urine; Protease; Pheromone; TLC

### **Introduction**

Effective olfactory communication between male and female animals plays a vital role in their high reproductive fitness. Pheromones are secreted or excreted chemical factors that trigger a social response in members of the same species. These are chemicals capable of acting outside the body of the secreting individual to impact the behavior of the receiving individual (Karlson and Luscher, 1959). The urine and specialized scent glands, particularly the preputial gland, have been reported to be major sources of pheromonal communication (Dominic, 1991, Archunan, 2009). Pheromonal molecules integrated into low molecular mass proteins have been found to play a crucial role in pheromonal communication. The biological activity of these low molecular mass proteins, which are called major urinary proteins, is well established in the mouse, rats, buffaloes, etc., and they have been shown to play a significant role in the release of odorant molecules from inside the body into the environment (Rajkumar *et al.*, 2010). Assuming their

primary and tertiary structural homology, these proteins are assigned to the lipocalin super family (Beynon and Hurst, 2004). The pheromone-binding proteins that deliver these chemo-signals and act as transporters in several mammalian species are called by different names, viz.  $\alpha 2\mu$ -globulin in the rat (Saito *et al.*, 2000), pheromaxin in the pig (Rajkumar *et al.*, 2010), aphrodisin in the hamster (Tigoni *et al.*, 2000), and odorant binding protein in bovines (Polverini *et al.*, 2011). In addition to being pheromone transporters, these proteins have been proven to act as slow-releasing agents for pheromonal communication.

Isolation of active fraction and characterization of chemo-signals from urine has been attempted in several mammalian species in recent years. As estrus detection in buffaloes is still a most prevalent managerial constraint, pheromonal cues appear to offer a newer approach that may be developed for accurate estrus detection in these animals.

### **Materials and methods**

Urine samples were collected from five Murrah buffalo bulls and fifteen female Murrah buffaloes in different stages of estrous cycle viz. estrus, diestrus and pregnancy. The stages of the estrous

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cycle were identified by several criteria; including vaginal swelling, frequent urination, bellowing of females, and male Flehmen behavior.

All the samples were divided into two parts. One part was stored at  $-20^{\circ}\text{C}$  as such and other was precipitated with 8% trichloroacetic acid. Protein concentration was estimated in each of the precipitate spectrophotometrically using Nanodrop®, USA. Each of the precipitate was treated with 0.4% trypsin for 30 minutes at  $37^{\circ}\text{C}$  for hydrolysis. All the samples (urine and hydrolyzed precipitates) were subjected to double extraction with dichloromethane solvent in 1:1 ratio. The extracts were vacuum dried and subjected to thin layer chromatography (TLC) as per Patra *et al.* (2010). The distance travelled by solvent front was marked with a pencil. The spots of solute front were identified under UV and marked with a pencil. Retention time (Rf) values for each component were calculated according to the formula: Rf value = Distance travelled by solute front / Distance travelled by solvent front.

## Results

The protein concentration in buffalo bull urine precipitate was found to be  $8.3\pm 0.25$  mg/ml, while that in pregnant, estrus and diestrus buffalo urine

precipitates were  $2.3\pm 0.16$ ,  $1.75\pm 0.09$  and  $1.50\pm 0.12$  mg/ml respectively. Numerous volatile compounds were identified in the urine of female buffaloes at different reproductive phases that differed qualitatively from one stage to another. Several compounds were also detected in bull urine, of which, some were considered as bull-specific. Buffalo bull urine extract gave a single spot, but upon protease treatment it gave 3 spots with different Rf values, which proved that the volatile compounds were protein bound that were liberated upon protease treatment. On the contrary, urine extracts from different phases of cycling buffaloes gave different spots with different Rf values but there was no difference in their Rf values upon protease treatment, which suggests that these compounds in buffalo urine were not protein bound. The results of TLC analysis indicated the presence of six compounds with varying retention time in diestrus buffalo urine extract and two compounds each in estrus and pregnant buffalo urine extract (Table 1), which can be categorized as diestrus-specific, estrus specific and pregnancy-specific urinary compounds respectively. Out of the three compounds in protease treated bull urine extract, one compound had Rf value similar to that of a component of diestrus buffalo urine extract indicating that this compound was present in both buffalo bulls as well as diestrus buffaloes.

Table 1. Retention time values of different volatile compounds in buffalo urine extract

Sl. No.	Buffalo bull urine extract		Female buffalo urine extract					
	Untreated	Protease treated	Pregnant (Untreated)	Pregnant (Protease treated)	Estrus (Untreated)	Estrus (Protease treated)	Diestrus (Untreated)	Diestrus (Protease treated)
1							0.6405	0.6405
2							0.6860	0.6860
3	0.7320							
4							0.7386	0.7386
5					0.7451	0.7451		
6			0.7516	0.7516				
7		0.7710						
8							0.7910	0.7910
9			0.8627	0.8627				
10		0.8690						
11					0.8693	0.8693		
12							0.8760	0.8760
13		0.9477					0.9477	0.9477

## Discussion

Various compounds like 3-cyclohexene-1-methanol, 3-amino-s-triazole, 4-ethyl phenol, 3-ethyl-2-methyl hexane etc. having pheromone like properties have been identified in mouse urine, some of which are male while others are female specific (Archunan, 2009). Several PBPs that deliver these chemo-signals and act as transporters have been identified in several mammalian species, which are known by different names (Tsay, 2010, Rajkumar *et al.*, 2010, Polverini *et al.*, 2011). In addition to being pheromone transporters, these proteins have been proved to act as slow-releasing agents for pheromonal communication (Achiraman and Archunan, 2002). In the present investigation, it is interesting to observe that in buffalo bulls, majority of the volatile compounds do exist in protein bound form but this is not true in the case of their female counterparts. Male animals particularly in wild are territory markers. They do so by giving off secretions containing pheromones and smearing them around on their nearby objects like twigs leaves etc. (Mykytowycz, 1968, Peters and Mech, 1975). This is not so in case of females. Down the evolutionary process, buffaloes might have lost the habit of territory marking, but their urine still contains protein bound pheromones. Protein binding helps in longer retention of pheromones in the environment. In female buffaloes, although none of the volatile compounds were protein bound, but each one of them was specific for different stages of estrous cycle. These compounds may be further identified and probed to detect estrus in buffaloes.

## Conclusion

It could be concluded that qualitative differences in protein bound volatile compounds exist in estrus, diestrus and pregnant buffalo urine and in bull urine, as evidenced by TLC.

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