Animal (2007), **1:9**, pp 1321–1327 © The Animal Consortium 2007
doi: 10.1017/S1751731107000614

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Detection of oestrous-related odour in bovine (*Bos taurus*) saliva: bioassay of identified compounds

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(Received 14 March 2007; Accepted 19 June 2007)

The present study was designed to identify the volatile constituents across the oestrous cycle of bovine in order to detect oestrous-specific chemical signal. The bovine saliva was extracted with diethyl ether (1 : 1 ratio, v/v) and analysed by gas chromatography-linked mass spectrometry. Numerous compounds were identified during oestrous cycle of bovine saliva. Among these, the compounds, namely, trimethylamine, acetic acid, phenol 4-propyl, pentanoic acid and propionic acid were specific to oestrous stage. The behaviour assay revealed that the compound, trimethylamine, is involved in attracting the male animal. The result concludes that the trimethylamine is considered as a putative oestrous-specific salivary chemo-signal in the bovine.

Keywords: cows, oestrus, pheromones, saliva, volatile compounds

Introduction

It is now known that pheromonal communication plays an important role in mammalian behaviour and reproductive processes (Halpin, 1986; Dominic, 1991; Johnston, 1998; Beynon and Hurst, 2004; Brennan and Keverne, 2004). In mammals, signalling and primer pheromones are thought to act either singly or in combination through olfaction, auditory, visual (sight) or tactile stimuli (Rekwot *et al*., 2001). Various attempts have been made on the regulation and control of bovine reproduction through the use of hormones and the application of biological agents. Therefore, harnessing the reproductive potential of livestock species in developing countries may require development of management strategies. It is now accepted that the olfactory signals of oestrous body fluids ultimately help the male to detect oestrus (Lin *et al*., 2005).

In fact, saliva is a remarkably complex biological fluid with an extraordinary natural history. The organisation of termite societies depends predominantly on intra-specific chemical signals (pheromones) produced by saliva which induce and modulate individual behavioural responses (Reinhard *et al*., 2002). For instance, sexually experienced males preferred the saliva of oestrous females to that of non-oestrous females in Mongolian gerbils (Block *et al*., 1981). In boars, pheromones are secreted in saliva to cause oestrous sows to take up the mating stance (Austin *et al*., 2004). The integration of various compounds in specific ratios may contribute to the formation of a specific odour (Albone, 1984). Therefore, a successful artificial insemination (AI) programme must incorporate efficient and accurate detection of oestrus and timely insemination relative to ovulation (Walker, 1984).

Chemical investigations on mammalian pheromones have been relatively less. However, in the last two decades, there has been a considerable study on the chemistry of mammalian pheromone in the mouse (Novotny *et al*., 1984; Achiraman and Archunan, 2005), rat (Archunan and Achiraman, 2006), bobcat (Mattina *et al*., 1991), tiger (Brahmachary, 1996), white-tailed deer (Jemiolo *et al*., 1995), horse (Ma and Klemm, 1997); bovine (Rameshkumar *et al*., 2000); elephant (Rasmussen *et al*., 1997) and human (Stern and McClintock, 1998). These findings indicate that mammalian pheromones may be a single compound or a mixture of compounds and that each of the major fractions are faithfully involved in conveying specific signals related to reproductive and social behaviours.

Volatile odours from the opposite sex conspecifics contribute mate recognition in several mammalian species, including rat (Selvaraj and Archunan, 2002), bovine (Rameshkumar *et al*., 2000) and buffalo (Rajanarayanan and Archunan, 2004). As the cow reaches oestrous, the bull becomes excited and follows her closely, licking and smelling her external genitalia and often exhibiting reproductive behaviours (Steinel, 1977). To date, chemical investigation has not been performed on saliva from different stages of the bovine oestrous cycle and its biological functions.

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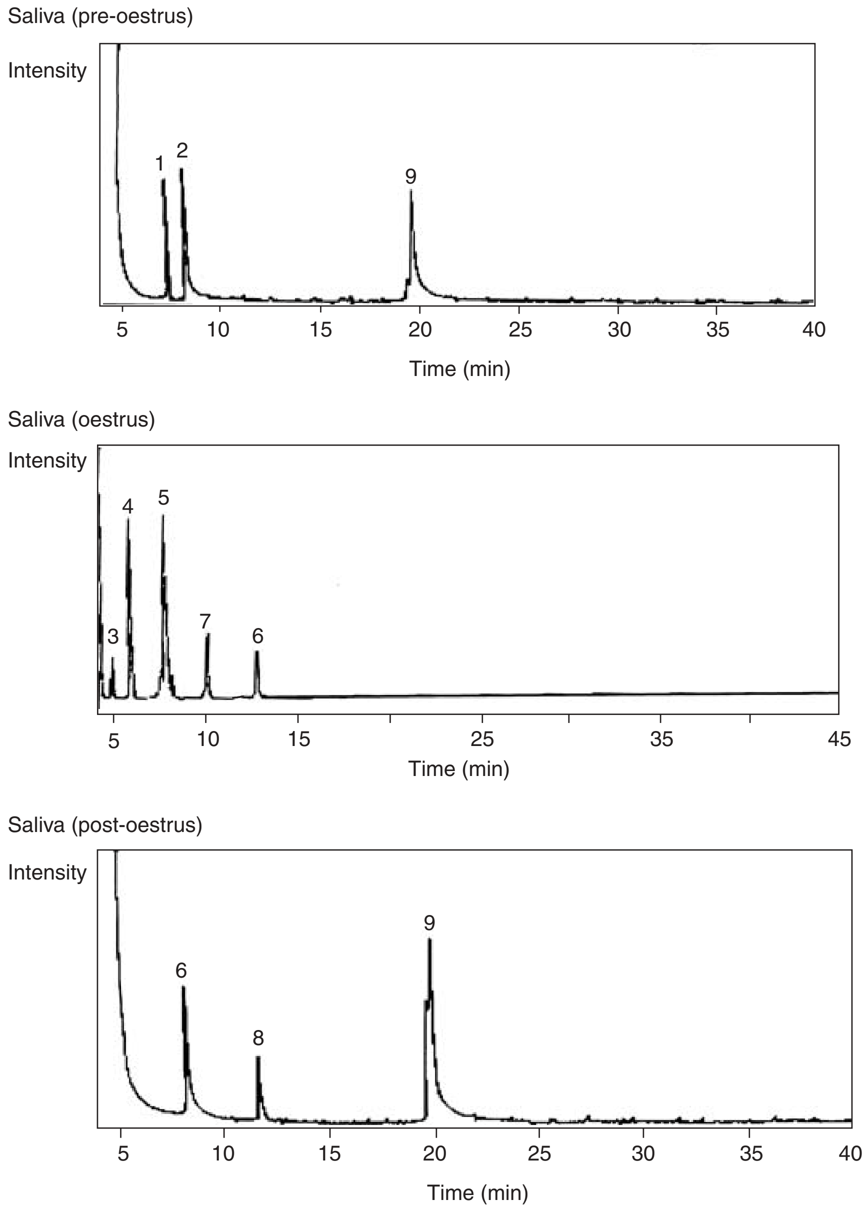

Figure 1 Gas chromatographs of the saliva compounds identified in the three stages of oestrous cycle in bovine, *Bos taurus*.

Hence, the objective of this study was to examine the profiles of bovine volatile compounds across the oestrous cycle so as to clarify whether the bovine saliva contains information potentially useful in chemical communication.

Material and methods

Sample collection

Six cows and six teaser bulls of *Bos taurus* (Jersey) were used in the present study for sample collection and behavioural assay in the exotic cattle-breeding centre, Eachankottai, Tanjore District, Tamil Nadu, India. They were artificially bred, and the females were approximately 20 to 30 months old and the males were 30 to 36 months old. The stages of the oestrous cycle were carefully determined for two to three consecutive cycles and confirmed by transrectal palpation through assessing the morphological changes in the internal reproductive organs. Cows were observed twice daily for 30 min for behaviours. The animals were housed in sheds and paddocks, fed with standard diet and *ad libitum* water. The cows were considered to be in oestrus if they accepted the mounting by a teaser bull and allowed the bulls' behavioural observations for further confirmation. Immediately after screening, the samples were stored in frozen at −20°C and analysed by gas chromatography-linked mass spectrometry (GC-MS).

Sample analysis

The samples collected from the particular stage according to the experimental protocol were pooled to minimise the effect of individual variation. Diethyl ether was used to extract the compounds from the saliva samples. Triplicate 5 ml of samples were taken from the pooled samples and separately mixed with 5 ml of diethyl ether. The supernatant was filtered through a silica-gel column (60 to 120 mesh) 30 min at room temperature. The filtered extract was reduced to 1/5 of its original volume by cooling with liquid nitrogen to condense it.

The sample was fractionated and chemical compounds were identified by GC-MS (QP-5050, Shimadzu Corporation, Kyoto, Japan). Two microlitres of extract were injected into the GC-MS system on a 30 m glass capillary column with a film thickness of 0.25 μm (30 $\times$ 0.2 mm i.d. coated with UCON HB 2000; Union Carbide Corporation, Danbury, CT, USA) using the following temperature programme: Initial oven temperature of 40°C for 4 min, increasing to 250°C at 15°C/min and then held at 250°C for 10 min. The GC-MS was run under computer control at 70 eV. The solvent (diethyl ether) peak was seen at 4.0 min. The saliva was analysed repeatedly six times and subjected to cross-checking 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 substances (NIST 6221B).

Behavioural assay

The identified compounds were procured and individually applied manually onto the genital region particularly vulval region of non-oestrous animals (dummy cows). Those cows which did not show any oestrous symptoms were considered as non-oestrous animals. The period within 3 to 5 days before oestrus was considered as pre-oestrus, and that within 2 to 4 days after oestrus as post-oestrus. The onset of oestrus

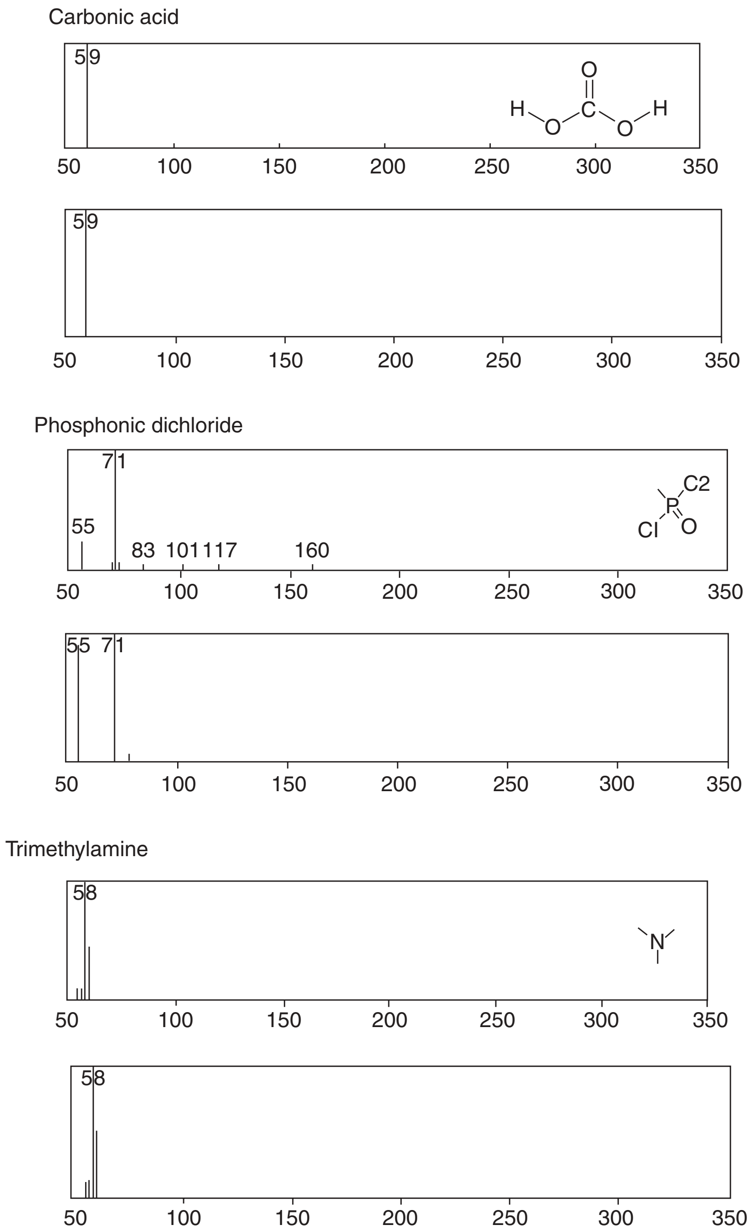

Figure 2 Mass spectrometry of the saliva compounds identified in the three stages of oestrous cycle in bovine, *Bos taurus*. Mass spectra of the main peaks identified (upper) and of standard compounds (lower).

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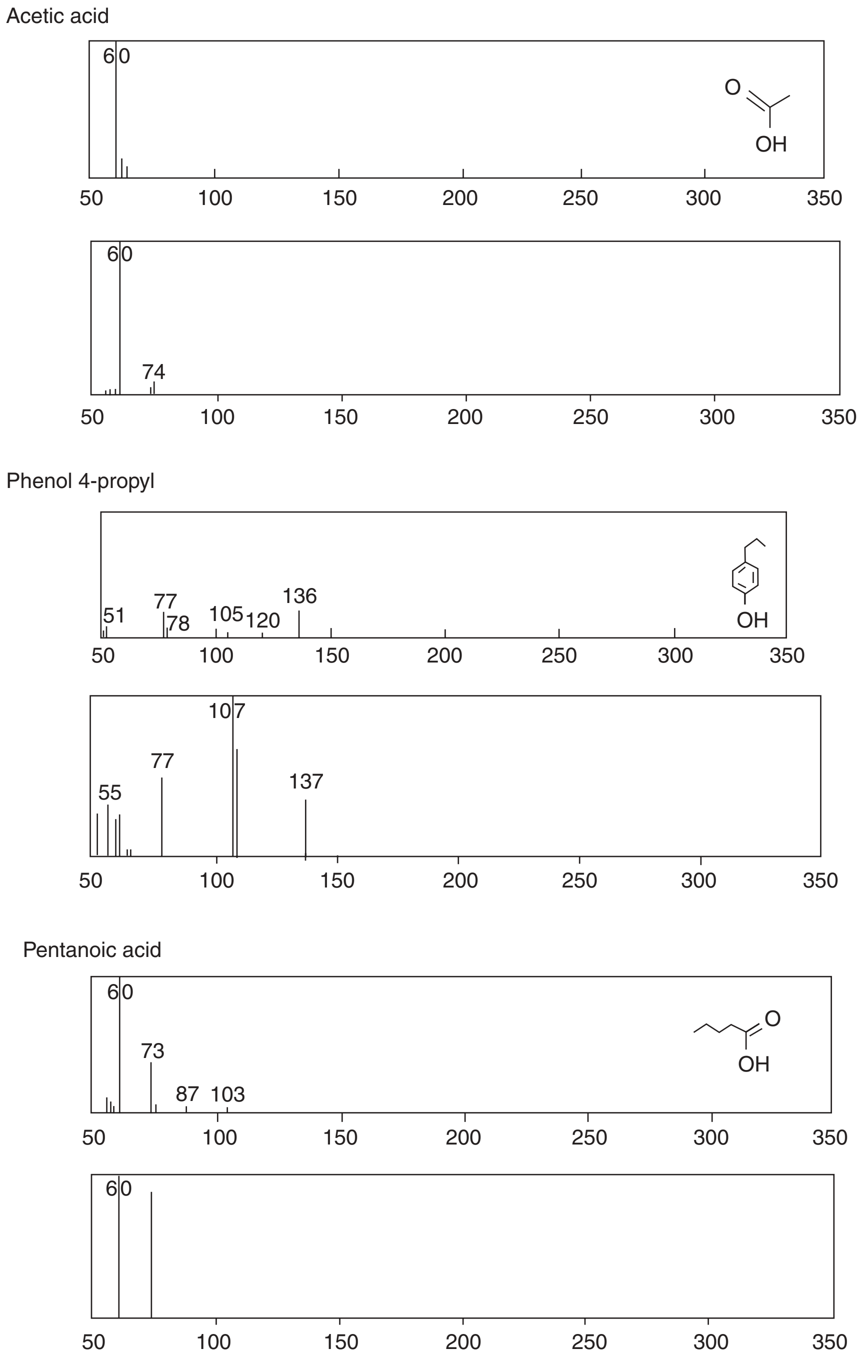

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was determined as the first of two mounts received within 4 h. The end of oestrus was confirmed while the last mount was received, taking the account of a mount 4 h before, and no mount during the next 12 h. The number of mounts was calculated through the number of times a cow was mounted by herd mates. The duration of oestrus and the number of mounts received per hour of oestrus were recorded. The bulls were allowed to sniff the genital region of experimental cows for a period of 30 min in the AI centre of the farm. The compounds were soaked with diethyl ether on cotton wool and used for behavioural assay. The solvent diethyl ether was used for GC-MS analysis as well as dissolving the compounds in various concentrations e.g. 0.5%, 1.0%, 2.0% and 5.0%. Since 0.5% concentration of oestrous-specific compounds were found to be effective in eliciting the flehmen and other behaviours of penile erection and mounting, 5 ml of the same concentration (5.0%) was used throughout the experiment to assess the bioactivity. The duration of flehmen behaviour exhibited by the bulls in response to dummy cows that received (oestrous and non-oestrous) compounds was recorded. Subsequently, other behaviours like penile erection, mounting and act of copulation were also observed. Flehmen was recognised as follows: when the male sniffs the genital region of female and raises its neck, extends its chin and

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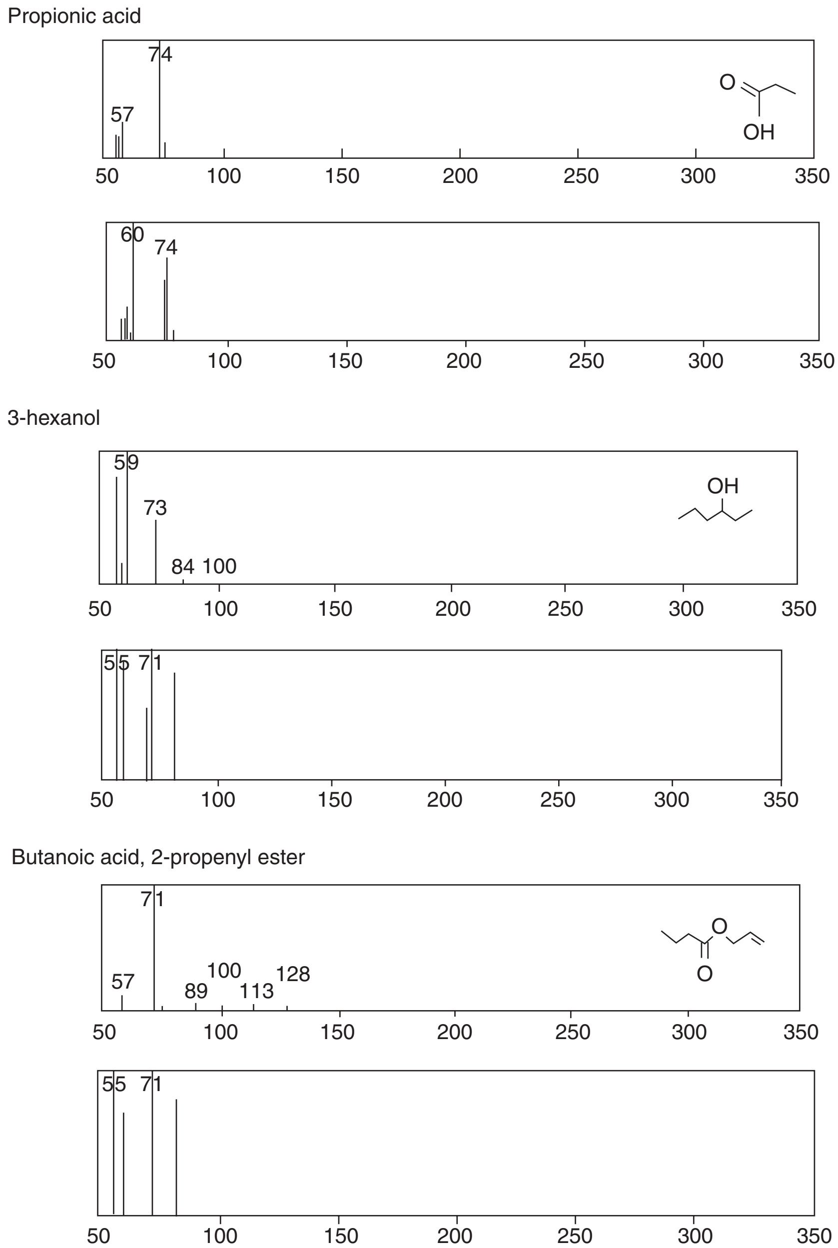

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inhales with slightly opened mouth, tongue held in flat position and upper lip curled. The time taken by the bull from sniffing to the curling of the upper lip was recorded as duration of flehmen. When the dummy animal was mounted fully by a bull, then it was considered as mounting.

Statistical analyses

The data for the behavioural assays were compiled using statistical software (Sokal and Rohlf, 1995) and subjected to the analysis of Duncan's multiple-range test (DMRT).

Results

The GC-MS profiles shown in Figures 1 and 2 are the representative of saliva obtained in the pre-oestrous, oestrous and post-oestrous periods. The saliva of oestrus showed five peaks, pre-oestrus exhibited three peaks and post-oestrus exhibited three peaks. Eleven different peaks were noted in the saliva of three different phases (Figure 1). Of these, trimethylamine, acetic acid, propionic acid and phenol 4-propyl were unique in the oestrous phase but were absent in the other phases. However, the compounds, carbonic acid, phosphonic dichloride, butanoic acid and 2-propenyl ester were found only during pre-oestrus (Table 1). The compounds 3-hexanol, butanoic acid, 2-propenyl ester and pentanoic acid were found during post-oestrus. However, pentanoic acid was present in oestrus and also in post-oestrus.

The volatile compounds identified in the saliva have the molecular weight range between 60 and 174 kDa (Table 1). The statistical analyses (DMRT) showed that the flehmen

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Table 1 *Volatile compounds identified in bovine saliva with their nature, molecular formula and weight*

Sample no.	Compound name	Pre-oestrous	Oestrous	Post-oestrous	Nature of compound	Molecular formula	Molecular weight
1	Carbonic acid	+	−	−	Carboxylic acid	H_2CO_3	62.02
2	Phosphonic dichloride	+	−	−	Phosphoric compounds	$C_5H_{11}Cl_2OP$	188
3	Trimethylamine	−	+	−	Amines	C_3H_9N	59
4	Acetic acid	−	+	−	Carboxylic acid	$C_2H_4O_2$	60
5	Phenol 4-prophyl	−	+	−	Phenols	$C_9H_{12}O$	136
6	Pentanoic acid	−	+	+	Carboxylic acid	$C_5H_{10}O_2$	102
7	Propionic acid	−	+	+	Carboxylic acid	$C_3H_6O_2$	130
8	3-Hexanol	−	−	+	Alcohol	$C_6H_{14}O$	102
9	Butanoic acid, 2-propenyl ester	+	−	+	Ester	$C_7H_{12}O_2$	164

Table 2 *Bioactivity of oestrous-specific compounds of bovine saliva (values are expressed as mean $\pm$ s.e. (n = 6)*

Sample no.	Oestrous-specific compounds	Duration of flehmen (s)	No. of mounts
1	Acetic acid	4.60 ± 0.13	4.94 ± 0.28
2	Propionic acid	4.54 ± 0.27	4.99 ± 0.23
3	Trimethylamine	5.55 ± 0.13	7.07 ± 0.12
4	Phenol 4-propyl	4.16 ± 0.13	4.74 ± 0.20
5	Pentanoic acid	3.80 ± 0.21	3.49 ± 0.12
6	Mixture of acetic acid and propionic acid	4.74 ± 0.07	5.24 ± 0.25
7	Mixture of acetic acid, propionic acid and phenol 4-propyl	4.88 ± 0.23	5.74 ± 0.09
8	Butanoic acid, 2-propenyl ester (negative control)	2.46 ± 0.08	2.47 ± 0.26

behaviour of bulls was greatly influenced by trimethylamine ($P < 0.001$) than that of individual compounds and combination of components (Table 2). In fact, the number of mounting events and flehmen (Table 2) was higher in response to trimethylamine than that of an individual and a combination of two compounds.

Discussion

In the present study, numerous volatile compounds were identified in the bovine saliva at different phases of the oestrous cycle, which qualitatively differed from one phase to the other. The present results revealed that trimethylamine, acetic acid, propionic acid and phenol 4-propyl appeared during the oestrous phase but were not found in the other phases. Among the compounds identified in oestrous saliva, the acetic and propionic acids belong to fatty acids, the trimethylamine is in the amine class and phenol 4-propyl is in the phenol class.

Oestrous saliva is known to contain chemo-signals and the compound identified specifically in this period may be considered as behaviourally important chemical signals that might attract males (Archunan, 2003). Smith and Block (1991) reported that adult female Mongolian gerbils were preferentially attracted to saliva from adult non-sibling males when paired with saliva from their male siblings. The present study indicated that the compound, trimethylamine, may be the putative chemical signal excreted during oestrous (Table 2). The study also provided additional support to the previous finding (Lunden *et al.*, 2002) that trimethylamine can be considered as one of the volatile component in indicating the physiological state of the animal. Fish flavour is derived from volatile compounds like trimethylamine, which may stimulate the odour receptors (Methven *et al.*, 2007). Recently, Mitchell *et al.* (2007) identified a unique constituent, trimethylamine, in human urine and reported that it may act as a pheromone.

Among the volatiles identified (acid, amine and phenol) in the oestrous phase, the DMRT test clearly showed that the trimethylamine was found to be effective ($P < 0.001$) compared with those of individual and combinations of components (Table 3). On the other hand, the present study also indicates that the male animal showed good preference towards the mixture of oestrous-specific compounds when compared with trimethylamine. This suggests that combinations of components have also more pheromonal effect. Fatty acids have been reported to act as sex attractants (Mattina *et al.*, 1991) as well as individual identification (Poddar-Sarkar and Brahmachary, 1999) in certain mammalian species.

The bulls revealed a high frequency of flehmen response when exposed to trimethylamine as well as the mixture of synthetic compounds tested. The appearance of these chemical compounds during a specific period in the female reproductive cycle and the male response to these compounds confirmed their bioactivity. It indicates that the oestrous-specific salivary compounds are able to activate the bull to many behavioural activities, which usually occur prior to mating. It is consistent with our previous findings that body fluids like saliva, faeces and milk have oestrous-related odours that are probably involved in bovine bio-communication (Sankar and Archunan, 2004).

The chemosensory responses and pre-mating behaviours of the adult male to the female urinary cues have been well documented in *Bos taurus* (Klemm *et al.*, 1987). The behavioural activities observed in the bull in response to

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Table 3 *Analysis of variance with* post hoc *comparison (one-way)*†

Behaviours		Sum of squares	d.f.	Mean squares	F
Duration of flehmen	Between squares	36.261	7	5.180	
	Within groups	6.844	40	0.171	
	Total	43.105	47		30.275
Mounting behaviour	Between squares	80.432	7	11.490	
	Within groups	7.541	40	0.189	
	Total	87.973	47		60.944

†The means were compared using Duncan's multiple-range test (DMRT). **Level of significance at 0.001. Means for groups in homogenous subsets are displayed comparison of means using DMRT.

oestrous synthetic compounds show striking similarities to that seen in the bull in responses to natural oestrous samples. The results are consistent with the reports of Reinhardt (1983) that the bull frequently mounts the cows that are in oestrous. Our findings confirmed that the compounds identified during oestrous are particularly involved in activation of the bull behaviours, it would therefore be considered as sex attractants. These salivary compounds may be used as non-invasive biomarker for oestrous detection.

Acknowledgement

The investigation was partially supported by DST and UGC-SAP, New Delhi.

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