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The fixative lipid of tiger pheromone

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Abstract

Tigers communicate with one another with the help of Marking Fluid (MF) which is a lipid-rich fluid sprayed upwards and backwards through the urinary channel of both the sexes. The volatile molecules of the MF are made to last longer with the help of lipid 'fixatives' the total amount of which is 1–2 mg/ml. This lipid comprises cholesterol ester, wax ester, triglyceride, free fatty acids, diglyceride, monoglyceride, free sterol and phospholipid as revealed by thin layer chromatography. The gas liquid chromatogram of fatty acid methyl esters of the total lipid of the MF, when compared with the fatty acids of groin and body fat of tiger (analysed by other workers), reveals differences in higher proportions of palmitoleic and myristic acids and of highly unsaturated fatty acids. The palmitoleic acid content of total lipid and triglyceride is high in comparison to the wax esters and cholesterol esters of the MF-fat. The unique feature of the alcohol part of the wax esters is a series of saturated straight chain primary alcohols of C_{14} to C_{20} and these are accompanied by the corresponding monoenoic unsaturates.

Keywords: Tiger (*Panthera tigris tigris*); Pheromone; Marking fluid; Lipid; Fatty acids; Fatty alcohol

1. Introduction

Tigers communicate with one another by spraying Marking Fluid (MF) (Brahmachary and Dutta, 1979; Brahmachary and Dutta, 1981; Brahmachary and Dutta, 1987). MF, which acts as a chemical messenger, is a lipid-rich fluid sprayed upwards and backwards through the urinary channel of both the sexes. MF, which is distinctly different from ordinary urination, contains an array of volatile odorant molecules and a considerable amount of lipid. Matthews (1969) first pointed out that a tiger excretes lipids through urine and MF (the distinction between the two was unknown at that time) to a considerable amount. As tigers demarcate a large area of territory by spraying MF on leaves of trees and shrubs, rocks, etc. it is necessary to fix the volatile pheromonal compounds for a longer duration so as to minimize the frequency of ejection; thus, the message can be conveyed to other tigers even days later. Brahmachary and Dutta (1987) noticed that MF sprayed and dried in a clump of *Mangifera indica* leaves smells even after keeping under running tap water overnight and even after at least ten days on the surface of *Butea* leaves. It was also observed that the odour of MF clings to the leaves of *Heritiera* sp even when these are kept submerged in estuarine water for twenty two hours (Brahmachary, personal communication). In the course of numerous experiments Brahmachary and Dutta (1979, 1981) and Brahmachary et al. (1990) established the fact that aroma substance(s) rapidly vanish on being separated from the lipids by steam distillation. Thus these lipids probably act as fixatives. In the present study a chemical analysis of lipids of MF was undertaken so that their chemical nature and uniqueness, if any, might be revealed.

2. Methods

2.1. Collection of MF

MF was collected from live, untranquilised tigers of Nandan Kanan Biological Park, Orissa, India about 500 km away from Calcutta. This was only possible from four out of forty tigers which were not shy in the presence of human beings. The method of collection in clean trays as the tigers squirt MF on certain points on the fencing has been perfected over the years but it is still far from fool-proof. The tigers were first introduced in a small enclosure adjoining the large open-air space. As the tigers moved beyond the chain-like mesh, trays were kept ready; as the tiger raised its tail, the prelude of squirts, the tray was held up and a part of the spray could be collected at that time. After collection, a known amount of methanol was added to the samples used for lipid studies. In some cases, chloroform was also added at this stage to give the 2:1:0.8 v/v ratio of methanol:chloroform:MF used for extraction process (see Section 2.2). Samples were than brought to Calcutta under ice and stored at $-70°C$.

2.2. Extraction and estimation of total lipid

The lipid of MF was extracted by a slight modification of Bligh and Dyer's method (Bligh and Dyer, 1959). To an aliquot of MF chloroform and methanol were added to give a final v/v ratio of 2:1:0.8 respectively. Chloroform and distilled water each one third the volume of this solution were added. The lower chloroform phase was collected over anhydrous sodium sulphate and stored at −20°C under nitrogen atmosphere. Solvents used for different experiments were obtained from several commercial companies of India including SISCO Research Laboratories, Glaxo Laboratories (India), Alembic India, SD's Fine Chem, etc. as spectroscopy grade. These were redistilled in our laboratory following the methods mentioned in Vogel's Practical Organic chemistry (Vogel, 1956).

An aliquot of the chloroform phase was evaporated to dryness under reduced pressure and finally with a flow of nitrogen. The residue was measured and expressed as mg lipid/ml of MF.

2.3. Fractionation of total lipid into different classes by thin layer chromatography (TLC) and isolation of these respective classes by preparative TLC

An aliquot of the chloroform phase was spotted on TLC plate and developed in a solvent system *n*-hexane:diethyl ether:acetic acid (90:10:1.5) (Stahl, 1965). Authentic compounds were run simultaneously with the sample. After development, plates were sprayed with 0.2% 2,7-dichlorofluorescein (Christie, 1987) in ethanol and viewed under long wave UV-light. Some of such plates were sprayed with Libermann-Bürchard reagent (Huang et al., 1961) and heated at 100°C for 10 min. Molybdenum blue reagent (Kates, 1972) was also used as a developer. Spots visualised by the reagents were marked and compared with authentic compounds.

After detection and identification of the classes, bands were isolated from preparative TLC plates and the contents were eluted separately with chloroform. The eluted material (in solvent) was concentrated and subjected to direct methanolysis (Chalvardjian, 1964).

2.4. Formation of methyl ester from the total lipid of MF and from the fractionated classes

A mixture of methanol, benzene and conc. H_2SO_4 (86:10:4 v/v) were added to the chloroform extract of total secretion (MF; see Section 2.2) and the fractionated classes separately and heated for 3 h at 80–90°C (maintained by placing a beaker filled with water over a steam bath). After cooling, the volume of methanol was reduced under vacuum and water was added. The fatty acid methyl esters (FAME) were extracted thoroughly with *n*-hexane (3 × 1 ml). The solvent extract was washed several times with distilled water, dried over anhydrous sodium sulphate and kept for gas liquid chromatographic analysis (GLC). The nonsaponifiables were recovered from the aqueous layer after addition of 6 M HCl and extraction with diethyl ether. The FAME thus obtained were purified on preparative TLC plates and extracted with *n*-hexane and stored under N_2.

2.5. Formation of acetate derivatives from nonsaponifiables of the fractionated lipid classes

The ether extracts of nonsaponifiables were dissolved in a mixture of acetic anhydride and pyridine (5:1 v/v), heated at 50–60°C for 3 h and left overnight at room temperature. One ml of 0.5 N HCl was added to hydrolyse acetic anhydride to acetic acid and pyridine was removed by a stream of N_2 with gentle warming. The acetylated products were extracted with *n*-hexane and purified on TLC. The purified contents were extracted with hexane.

2.6. Gas liquid chromatographic (GLC) analysis of FAME and acetylated nonsaponifiables

FAME and acetylated nonsaponifiables of each class were subjected to GLC. The column used for FAME and nonsaponifiables were 15% DEGS and 3% SE-30, respectively, on 100–120 mesh WHP chromosorb packed in 3 m × 3 mm (ID) stainless steel tubes. The column temp. and the temp. of FID and injection port for FAME was 200°C, 250°C respectively. In case of nonsaponifiables a higher column temperature FID and injection port 250°C, 300°C, and 300°C were used. Different saturated and unsaturated fatty acid methyl esters and alcohol acetates used as standards were obtained from Sigma, USA. Methyl esters of 22:5 (*n*-6) and 22:5 (*n*-3) were kindly donated by Prof. J. Dutta, Bose Institute, Calcutta. The fatty acids and alcohols of MF were identified from the chromatograms by:

(1) comparing the retention time (R_t) of standard compounds chromatographed under the same condition;
(2) fitting log R_t of the sample peak in the straight lines obtained by plotting the log R_t of standard compounds against the number of carbon atoms in the molecule; and
(3) in some instances cochromatography of a mixture of the standard compounds containing different FAME and the samples and identifying the sample peaks from enhancement of peak areas.

Percentages of compounds present in the chromatogram of MF were calculated with the help of a data processing system of the instrument (Hewlett Packard, 5840A, dual column) by the method of normalization without further correction for the peak areas.

3. Results

3.1. Total lipid content and different classes

The total lipid content in MF expressed as mg/ml does not vary significantly from sample to sample collected throughout the year in all four tigers. The average lipid content is 1.88 ± 0.75 mg/ml. The total lipid of MF was resolved into sterol ester (SE), wax ester (WE), triglyceride (TG), free fatty acids (FFA), free sterol

(FS), diglyceride (DG) and monoglyceride (MG) (Fig. 1). Phospholipid was detected at the point of application on all TLC plates when sprayed with molybdenum blue reagent.

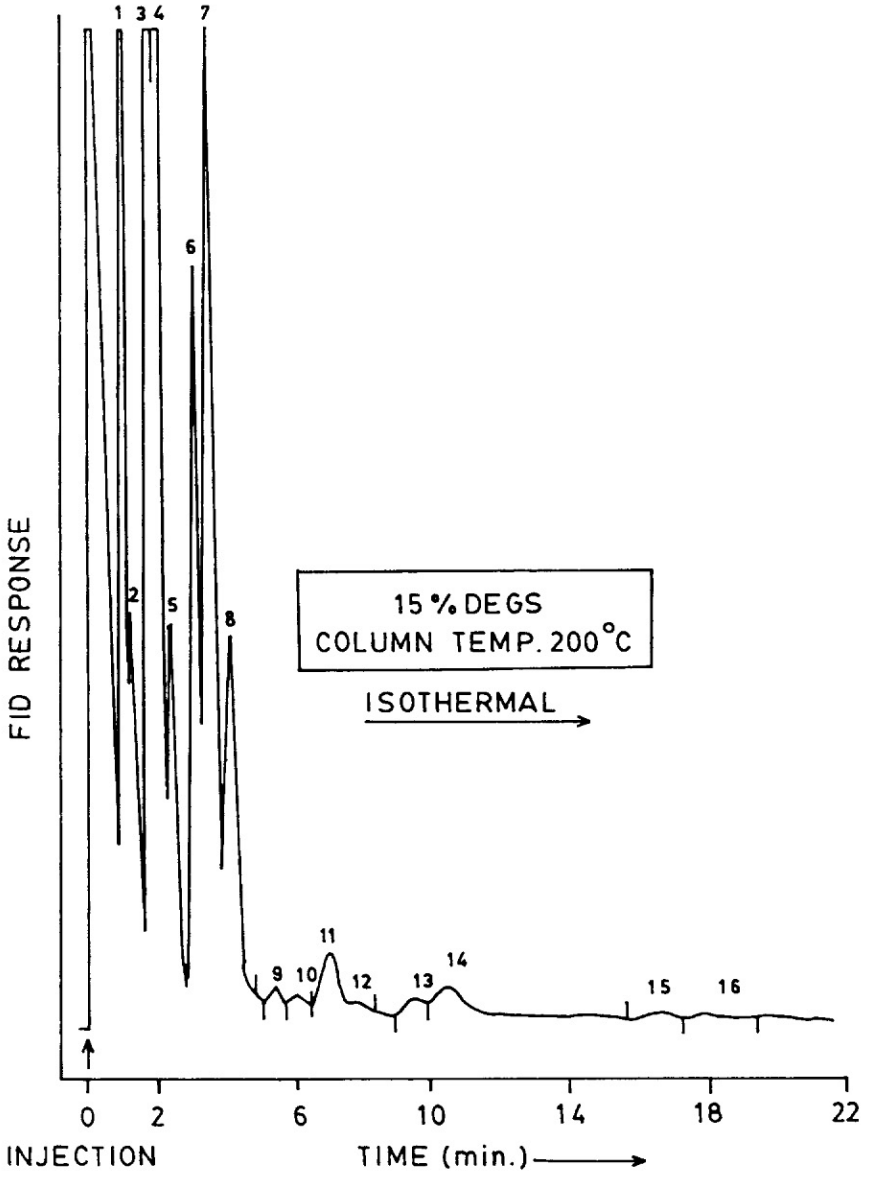

Fig. 1. GLC of FAME of TG isolated from 'fixative' lipid of MF. Peak 1(14:0); 2(14:1); 3(16:0); 4(16:1); 5(16:2 + 16:3); 6(18:0); 7(18:1); 8(18:2); 9(18:3 *n*-6); 10(18:3 *n*-3); 11(18:4 *n*-6); 12(20:3 *n*-6); 13(20:4 *n*-6); 14(20:5 *n*-3); 15(22:5 *n*-6); 16(22:5 *n*-3).

Table 1
Fatty acid composition (w/w %) of total lipid of MF in comparison with the fatty acids (% w) of depot fat of tiger

Fatty acid	w/w % Of fatty acids of total lipid of MF	w % Of fatty acids of depot fat	
		Body[a]	Groin[b]
14:0	18:1	1.0	3.0
14:1	0.4	0.6	0.6
16:0	22.0	22.4	26.8
16:1	20.5	7.1	6.0
16:2+16:3	3.2	—	(Traces of diene and triene C_{16} acid)
18:0	10.5	24.6	10.7
18:1	20.5	39.0	38.1
18:2	7.5	4.1	6.2
18:3 *n*-6+ 18:3 *n*-3	0.1	–	6.8
18:4 *n*-6	1.9		
20:0	Trace	1.2	
20:3	*n*-6	0.1	
20:4 *n*-6	3.5		1.8
20:5 *n*-3	1.2		(Unsaturated C_{20}–C_{22} acid)
22:5 *n*-6	0.3		
22:5 *n*-3	trace		
Total HUFA	5.1		

[a] According to Pathak and Agarwal, 1952.
[b] According to Gunstone, 1955.

3.2. Fatty acid composition of lipid of MF

The fatty acid composition of total lipid of MF is presented in Table 1. The FAME of the body and groin fat of tiger as revealed by earlier workers (Pathak and Agarwal, 1952; Gunstone, 1955) have also been incorporated in this table. The result shows that the depot fat of tiger and fat of MF are to a large extent similar; but there are also some prominent differences. The major differences lie in the proportion of palmitoleic acid (16:1) fatty acid which is much higher in MF fat (20.5%) in comparison to the body (7.1%) and groin (6.0%). The content of myristic (14:0) in the MF fat is 8.1%—much higher than that of the body (1.0%) and the groin (3.0%). The existence of 5% of highly unsaturated fatty acids (HUFAs) of carbon 20–22 is also a characteristic of MF. These have not been detected in the body fat and are only 1.8% in groin fat.

As TG is the major component of MF, the fatty acid composition of TG (Table 2, Fig. 1) shows close similarity with that of total lipid (Table 1). Like the composition of total lipid the major saturated components in MF-TG are (in descending order of abundance), palmitic acid ($21.9 \pm 4.7\%$); myristic ($10.1 \pm 2.3\%$) and stearic acid ($7.2 \pm 2.3\%$). Both the total fat and TG have 16:0 as the most abundant component. This fat is also very rich in 16:1. Among monoenoics, oleic

Table 2
Fatty acid compositions (w/w %) of TG isolated from the lipid fixative part of MF of tiger

Fatty acid	$x \pm$ SD $n = 8$
14:0	10.1 ± 2.3
14:1	1.3 ± 0.9
16:0	21.9 ± 4.7
16:1	17.2 ± 5.2
16:2+16:3	3.5 ± 1.4
18:0	7.2 ± 2.3
18:1	17.8 ± 4.7
18:2	8.6 ± 2.7
18:3 *n*-6	1.1 ± 1.4
18:3 *n*-3	0.5 ± 0.4
18:4 *n*-6	2.1 ± 0.7
20:3 *n*-6	1.0 ± 0.2
20:4 *n*-6	4.3 ± 3.9
20:5 *n*-3	1.3 ± 1.2
22:5 *n*-6	1.9 ± 2.2
22:5 *n*-3	trace
Total HUFA = 8.5%	

x, Mean; SD, standard deviation; n = no. of samples.

acid (18:1) is $17.8 \pm 4.7\%$, which is about 20.5% in total fat. Appreciable amounts of HUFAs of C_{20} to C_{22} fatty acid (8.5%) are also present in the TG fatty acids.

Table 3
Fatty acid composition (w/w %) of WE isolated from the lipid fixative part of MF of tiger

Fatty acid	$x \pm$ SD $n = 5$
14:0	4.4 ± 0.2
16:0	35.2 ± 1.7
16:1+16:2	1.5 ± 0.1
18:0	15.4 ± 3.4
18:1	10.2 ± 4.1
18:2	5.1 ± 2.1
18:3 *n*-6	2.7 ± 0.7
18:4 *n*-6	trace
20:2 *n*-6	1.3 ± 0.7
20:3 *n*-6	4.4 ± 1.0
20:4 *n*-6	12.4 ± 3.2
20:5 *n*-3	1.4 ± 0.6
22:4 *n*-6	2.2 ± 0.2
22:5 *n*-6	2.8 ± 0.9
22:5 *n*-3	2.6 ± 0.9
Total HUFA = 27.1%; % of total saturates = 55%	

x, Mean; SD, standard deviation; n, no. of samples.

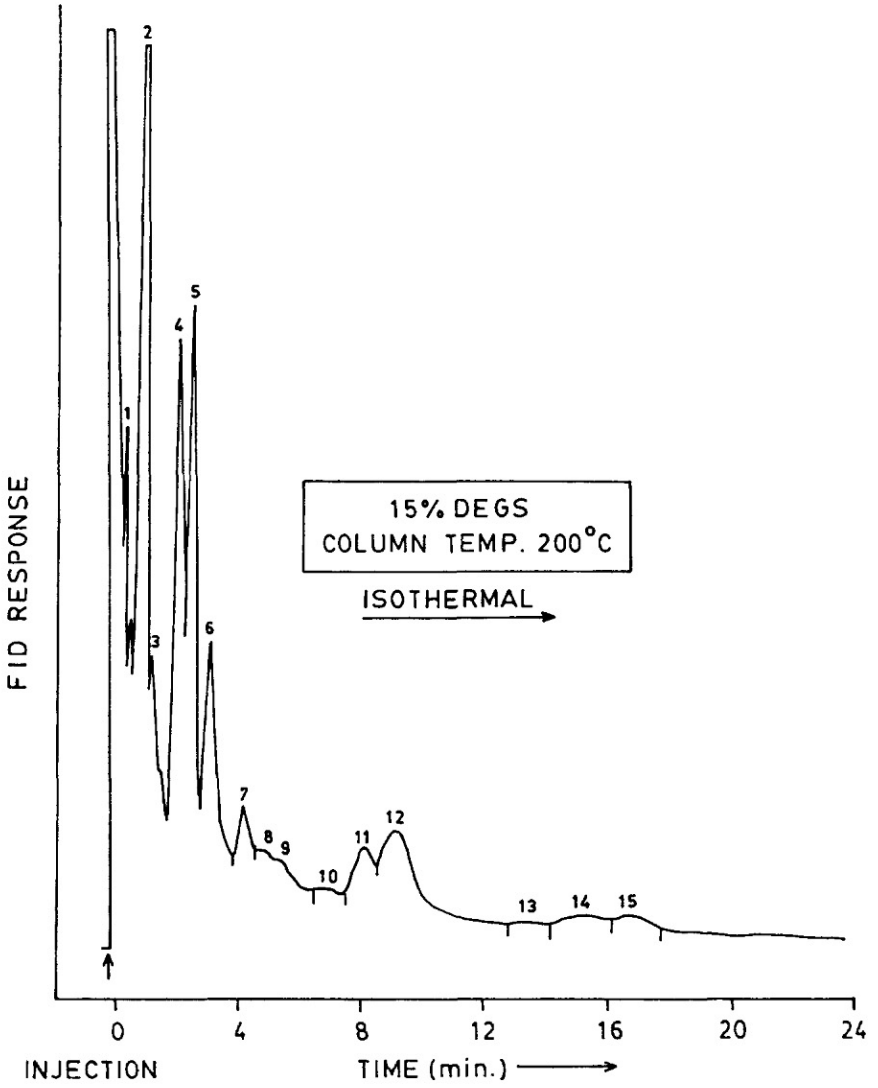

Fig. 2. GLC of FAME of WE isolated from 'fixative' lipid of MF. Peak 1(14:0); 2(16:0); 3(16:1 + 16:2); 4(18:0); 5(18:1); 6(18:2); 7(18:3 *n*-6); 8(18:4 *n*-6); 9(20:2 *n*-6); 10(20:3 *n*-6); 11(20:4 *n*-6); 12(20:5 *n*-3); 13(22:4 *n*-6); 14(22:5 *n*-6); 15(22:5 *n*-3).

The wax esters are more saturated (55%) than the other lipid classes examined (Table 3, Fig. 2). The wax fraction is very rich in the C_{20} to C_{22} HUFAs. Not only is the total percentage 27.1 but this fraction is comprised of different types of HUFAs like 20:3 *n*-6, 20:4 *n*-6, 20:5 *n*-3, 22:5 *n*-6, 22:5 *n*-3 etc.

The composition of fatty acid of the sterol (SE) (Table 4, Fig. 3) is to a large extent similar to those of total fat and TG but it contains a lower amount of 16:1 ($4.2 \pm 1.7\%$) than in total fat and TG. The SE is, in contrast, almost devoid of myristic acid (14:0). Among the HUFAs 20:4 *n*-6 is prominent ($9.8 \pm 4.1\%$). The saturation to unsaturation ratio in the TG and SE fatty acids are similar, i.e. about 2:3.

3.3. Composition of nonsaponifiables of lipid of MF

The wax of tiger MF fat contains basically the long chain monohydric alcohols ranging from 14 to 20 carbon atom (Table 5, Fig. 4) usually found in animal waxes. Each saturated straight chain alcohol was found to be accompanied by the corresponding monoenoic component. The major components are the eicosanol (20:0, $40.2 \pm 13.0\%$), gadoleyl alcohol (20:1, $16.1 \pm 5.5\%$) next to which are Myristyl (14:0, $15.1 \pm 3.2\%$) and myristoleyl (14:1, $4.7 \pm 0.1\%$). The percentage of cetyl (16:0) and palmitoleyl alcohol are 12.1 ± 4.1 and 5.5 ± 0.3 respectively. The percentages of 18 carbon pairs (steryl and oleyl) are very low, $3.8 + 0.1$ and 2.6 ± 1.2 respectively. The nonsaponifiable component of SE shows the presence of cholesterol ester.

4. Discussion

It is evident that MF contains a considerable amount (1–2 mg/ml) of lipid and through the process of ejection of MF, the tiger loses a sizable portion of its metabolic energy. It is a generally valid principle that highly uneconomic expenditure of metabolic energy is not likely to be permitted in the course of aeons of

Table 4
Fatty acid composition (w/w %) of SE isolated from the lipid fixative part of MF of tiger

Fatty acids	$\bar{x} \pm SD$ $n = 9$
16:0	32.2 ± 7.1
16:1	4.2 ± 1.7
16:2 + 16:3	1.5 ± 1.5
18:0	12.3 ± 2.6
18:1	24.4 ± 8.9
18:2	9.8 ± 3.0
18:3 *n*-6	2.2 ± 1.2
18:4 *n*-6	Trace
20:2 *n*-6	1.1 ± 0.8
20:3 *n*-6	2.8 ± 1.8
20:4 *n*-6	9.8 ± 4.1
20:5 *n*-3	Trace
Total saturate = 44.5%	

$\bar{x}$, Mean; SD, standard deviation; *n*, no. of sample.

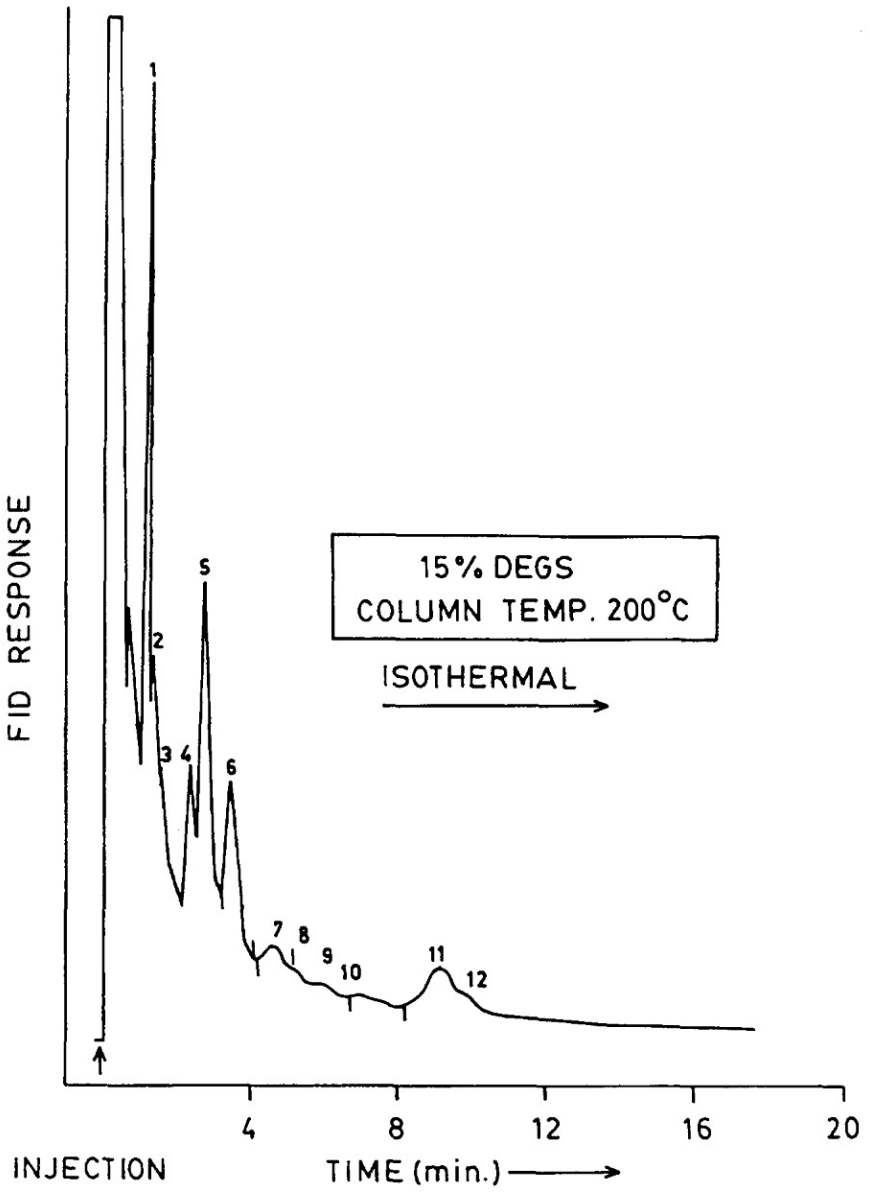

Fig. 3. GLC of FAME of SE isolated from 'fixative' lipid of MF. Peak 1(16:0); 2(16:1); 3(16:2 + 16:3); 4(18:0); 5(18:1); 6(18:2); 7(18:3 *n*-6); 8(18:4 *n*-6); 9(20:2 *n*-6); 10(20:3 *n*-6); 11(20:4 *n*-6); 12(20:5 *n*-3).

evolution. The loss of lipid (1 gm = 9 cal) in the MF and urine of tigers seems, at first sight, to be a wasteful process but the role of lipids as fixatives helps us to resolve this apparent contradiction. The mechanism of fixing volatile molecules by

'lipid' has been observed in *Tupaia belangeri*, one of the most primitive mammals (Stralendorff, 1987). The presence of a diversified class of lipid in green iguana femoral gland secretion also permits initial detection of secretion deposits in the environment through the release of volatile compounds (Alberts et al., 1992). Lipid thrown out in the MF is certainly not a wastage, rather, it serves a useful purpose, namely, to protect the volatile semio-chemicals from quick dispersal by evaporation and washing out by rain. It is also to be noted that a tiger may wander about a range of several square miles (or even 400 sq km in the case of the Siberian tiger; Spitsin et al., 1987). The lingering semio-chemicals, fixed to the lipids, would however discourage or at least alert rivals so as to minimize direct confrontation or to attract the opposite sex. There is however an intriguing point, namely due to weathering and a certain amount of bacterial attack, the constituents of the MF sprayed on a particular site are likely to slowly change. As such, how exactly the signals are altered or decoded, becomes a tricky problem. This is a general problem for all scent markings of different animals especially in the tropics and a satisfactory explanation is not available at the moment.

In spite of certain similarities with the body fat of tiger, the overall composition of MF lipid differ to some extent. Grønneberg (1978) identified wax esters from Castoreum, the secretion of castor sac of the beaver (*Castor canadensis*). This comprises C5–C22 carboxylic acid and C14–C19 alcohol but he did not explain its specific biological function. The interesting feature of wax ester of lipid of MF is that each saturated fatty alcohol from 14–20 carbon atom is accompanied by its unsaturated monoene form.

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Table 5
Long chain alcohol composition (w/w %) of WE isolated from the lipid fixative part of MF of tiger

Primary alcohol	$\bar{x} \pm SD$ $n = 5$
14:0	15.1 ± 3.2
14:1	4.7 ± 0.1
16:0	12.1 ± 4.1
16:1	5.5 ± 0.3
18:0	3.8 ± 0.1
18:1	2.6 ± 1.2
20:0	40.2 ± 13.0
20:1	16.1 ± 5.5

$\bar{x}$, Mean; SD, standard deviation; n, no. of sample.

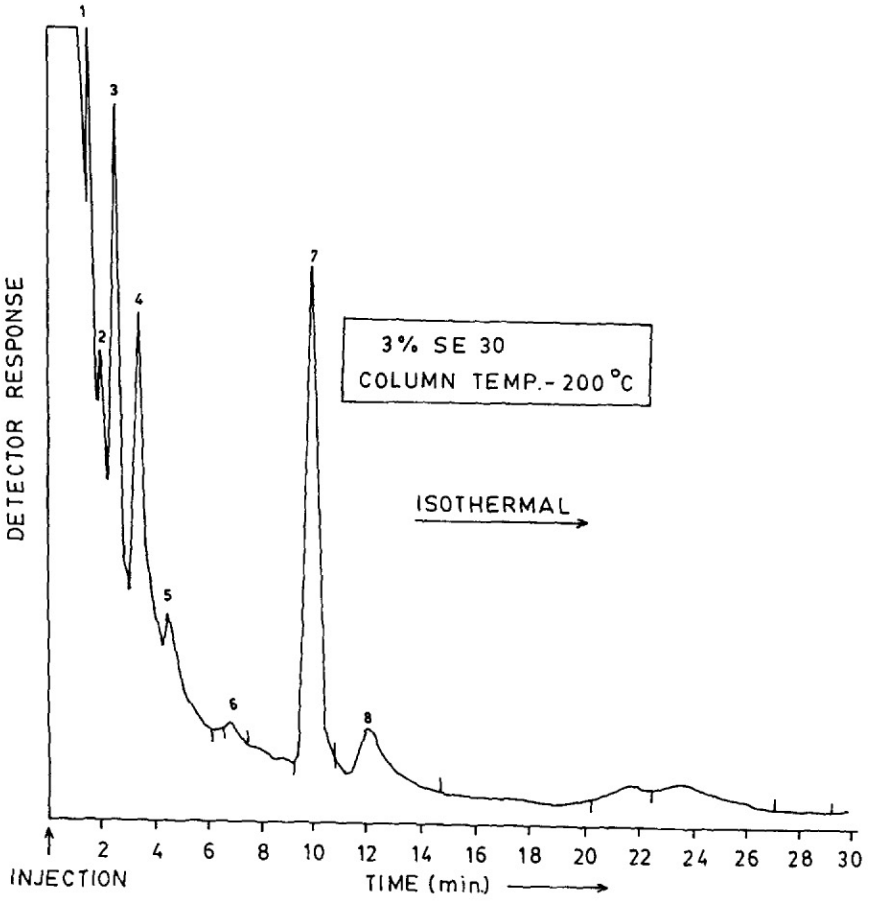

Fig. 4. GLC of long chain alcohol acetate of WE isolated from 'fixative' lipid of MF. Peak 1(14:0); 2(14:1); 3(16:0); 4(16:1); 5(18:0); 6(18:1); 7(20:0); 8(20:1).

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References

Alberts, A.C., Sharp, T.R., Werner, D.I. and Weldon, P.J. (1992) Seasonal variation of lipids in femoral gland secretions of male green iguanas (*Iguana iguana*). J. Chem. Ecol. 18(5), 703–712.

Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Brahmachary, R.L. and Dutta, J. (1979) Phenylethylamine as a biochemical marker of tiger. Z. Naturforsch. 34C, 632–633.

Brahmachary, R.L. and Dutta, J. (1981) On the pheromones of tigers—experiments and theory. Am. Nat. 118, 561–567.

Brahmachary, R.L. and Dutta, J. (1987) Chemical communication in the tiger and leopard. In: Tilson, R.L. and Seal U.S. (Eds.), Tigers of the World, Noyes Publication, USA, pp. 296–302.

Brahmachary, R.L., Poddar-Sarkar, Mousumi and Dutta, J. (1990) The aroma of rice... and tiger. Nature 344, 26.

Chalvardjian, A.M. (1964) Fatty acids of brown and yellow fat in rats. Biochem. J. 90, 518–521.

Christie, W.W. (1987) Lipid Analysis, 2nd ed., Pergamon Press, Great Britain.

Grønneberg, T. (1978–1979) Analysis of wax ester fraction from the anal gland secretion of beaver (*Castor fiber*). Chemica Scripta 13, 56–58.

Gunstone, F.D. (1955) Animal fat—the component acids of tiger fat and of puma fat. Biochem. J. 59, 455–458.

Huang, T.C., Chen, C.P., Wefler, V. and Raltery, A. (1961) A stable reagent for the Libermann-Bürchard reaction—application to rapid serum cholesterol determination. Anal. Chem. 33, 1405–1407.

Kates, M. (1972) In: Work, T.S and Work, E. (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, 3 part-II, North-Holland Publishing Company, Amsterdam.

Matthews, L.H. (1969) The Life of Mammals, Weidenfeld and Nicolson, London.

Pathak, S.P. and Agarwal, C.V. (1952) J. Sci. Food Agric. 3, 136.

Spitsin, V.V., Romanov, P.N., Popov, S.V. and Smirnov, E.N. (1987) The Siberian tiger *Panthera tigris altaica*-Temminck 1884 in the USSR: status in the wild and captivity. In: Tilson R.L. and Seal, U.S. (Eds.), Tigers of the World. Noyes Publication, USA, pp. 64–70.

Stahl, E. (1965) Thin Layer Chromatography—A laboratory hand book, Academic Press.

Stralendorff, F.V. (1987) Partial chemical characteristic of urinary signalling pheromone in tree shrews, *Tupaia belangeri*. J. Chem. Ecol. 13, 655–697.

Vogel, A.I. (1956) Practical Organic Chemistry, 3rd ed., Longman-Green, London.