Lipids of the anal sac secretions of the red fox, *Vulpes vulpes*, and of the lion, *Panthera leo*

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Abstract Lion anal sac secretions were found to be richer in lipids and to contain more complex, less uniform mixtures of lower molecular weight lipids than the anal sac secretions of the red fox. In the lion, homologous series of 1-alkylglycerols and 2-hydroxy-fatty acids were identified. Phenylacetic, 3-phenylpropionic, and related hydroxylated acids were also observed. Gas–liquid chromatography profiles of fox anal sac secretion lower molecular weight lipids were found to be less variable in their major constituents and to be dominated by relatively few large peaks, mainly (derivatized) fatty acids. Indole was also identified. Free cholesterol, and, occasionally, stanols were observed in fox and lion secretions. In the red fox, total cholesterol levels averaged 0.93 mg/g (% free, 56.4), n = 5. Findings are discussed in relation to histological and anatomical similarities and differences between the anal sacs of the lion and the fox and in the context of the role of these secretions in chemical communication.

Supplementary key words glyceryl ethers · 2-hydroxyalkanoic acids · cholesterol · aromatic acids · indole · scent production · chemical communication · gas–liquid chromatography · mass spectrometry

The anal sac is an odor-producing organ common to many carnivores (1, 2). In the red fox, the two anal sacs are approximately spherical reservoirs (approx. capacity, 1 ml) situated laterally to the anus, each sac opening on to the inner cutaneous anal zone through a short duct. The anal sac secretions of such dissimilar carnivores as the lion and the red fox exhibit certain notable similarities. Both are aqueous, odorous liquids that contain variable quantities of cell debris and lipid, and both are rich in the same low molecular weight compounds, which are known to be (or are suspected of being) produced by the action of the restricted resident sac microflora on the sebaceous and apocrine secretions and on the desquamated cells from the cornified surface layers of the sac epidermis, which together constitute the primary input to the sac from the sac wall. These compounds include the volatile fatty acids (C_2 to C_6), putrescine, cadaverine, and ammonia (3).

The precise chemical communicatory function(s) of the scent of anal sac secretion is little understood in any carnivore (3–5). However, compounds of ecochemical significance are likely to be included among the lipids of molecular weight less than about 300 (6) and, for this reason, prime attention was given to this group of substances in the present study.

In this paper, we present preliminary findings concerning the lower molecular weight lipids encountered in crude, unsaponified anal sac secretions. Prefractionation into lipid classes was not employed because it was intended to obtain profiles of the major lower molecular weight lipids as they occurred in the natural secretions, irrespective of compound class, and not to investigate the presence of particular compounds or groups of compounds among the minor constituents of the secretions.

MATERIALS AND METHODS

Secretion samples

All lion anal sac secretion samples (*A,B,C,D*) were obtained from adult, live, male lions (*A,B,C,* Aug/Sep 73; *D*, Aug 74). Each sample was obtained from a different animal. Secretion samples *A, B,* and *C* (300–1000 mg) were acidified with hydrochloric acid and extracted with diethyl ether. For gas–liquid chromatography, the lipids were methylated with diazomethane and further derivatized with bis(trimethylsilyl)acetamide. Sample *D* was extracted at natural pH (ca. pH 7) and similarly derivatized.

Red fox anal sac secretion samples were obtained without sedation from untamed captive animals,

Abbreviations: GLC, gas–liquid chromatography; MS, mass spectrometry; TLS, thin-layer chromatography; TMS, trimethylsilyl.

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raised in captivity, by using external digital pressure and directing jets of secretion from the two sacs into a collecting vessel. For gas–liquid chromatography, secretions were acidified with hydrochloric acid, extracted with diethyl ether, and derivatized as for the lion secretion extracts.

Chromatography

Gas–liquid chromatography was performed using a Pye 104 gas chromatograph fitted with a 4.3 m (or 1.5 m) × 3 mm ID glass column containing 3% OV-17 on Gas Chrom Q (100/120 mesh) or a Varian 2700 gas chromatograph fitted with a 3 m × 1.5 mm ID stainless steel column containing 3% Dexsil 300-GC on Gas Chrom Q (100/120 mesh).

Combined gas–liquid chromatography–mass spectrometry was carried out using a Varian 1200 gas chromatograph fitted with the Dexsil column and interfaced with a Varian MAT CH-7 mass spectrometer via a single stage Watson-Biemann separator. The computer-assisted techniques employed with these samples have been described in detail elsewhere (7).

Thin-layer chromatography was performed with petroleum ether (60–80°C)–diethyl ether–acetic acid 70:30:1 (v/v), or with benzene, on silica gel G (0.25 mm) followed by sulfuric acid charring at 220°C.

Cholesterol determination

Anal sac secretions were extracted (×3) with 3 ml of methanol–chloroform 2:1 (v/v) and a two-phase system was formed by addition of water and chloroform. Total cholesterol was determined colorimetrically in the chloroform extract using ferric chloride in orthophosphoric acid (8) (free cholesterol was determined similarly after digitonin precipitation). In some cases, cholesterol was determined in total secretion by the cholesterol oxidase method using a commercially available kit (BDH Ltd., Poole, England, catalog no. 25043).

RESULTS

Results of the computer-aided GLC–MS analysis of derivatized lion anal sac secretion extracts are in **Tables 1** and **2**. Multicomponent peaks were frequently noted, although usually the spectrum of one dominant component characterized a given peak. Occasionally, more than one recognizable component contributed significantly to a given peak, and these are listed in Table 1 as "impure peaks." Such peaks are entered under more than one classification.

The absence of a component from Tables 1 or 2 does not imply its complete absence from the sample, but merely that no definitive spectrum of the compound was obtained. The presence of other components with retention times similar to that of the substance sought rendered detection more difficult, particularly where that substance lacked characteristic high mass fragment ions. Thus, mass fragmentograms indicated the probable presence of small quantities of methyl *n*-octadecenoate in secretions *A* and *C*, although mass spectra of a quality sufficient to justify entry in Table 1 were not obtained. Similar indications of the presence of alkan-1-ol TMS ethers, e.g., octadecan-1-ol TMS ether, were also obtained, but only as minor components of complex peaks. Similarly a mass fragmentogram of the *m/e* 205 ion, a characteristic fragment of 1-alkylglycerol TMS diethers, $(CHOSiMe_3CH_2OSiMe_3)^+$ (9), suggested the presence in sample *C* of low concentrations of *n*-decyl, *n*-undecyl, and branched undecyl, dodecyl, tridecyl and pentadecyl glyceryl ethers in addition to the compounds listed in the table (**Fig. 1**). Identifications of 1-alkylglycerols were confirmed by high resolution mass spectrometry (7) and chemical ionization techniques (10).

Analysis of sample *C* was further complicated by the presence of trimethylsilyl alkanoates (C_{14}, C_{15}, C_{16}, C_{18}), arising from incomplete methylation, in spite of the use of excess diazomethane. Thus trimethylsilyl *n*-hexadecanoate was present in the methyl 2-hydroxy(*n*-pentadecanoate) TMS ether peak.

In all cases a major steroid TMS ether peak was observed. In *A*, *B*, and *C* this was the largest peak in the chromatogram. In secretions *B* and *C* the spectrum corresponded with that of cholesterol TMS ether, while in *A* and *D*, the spectrum was dominated by that of a derivatized stanol. This was probably 5α-cholestan-3β-ol TMS ether (11). Thus, secretion *A* gave *m/e* (relative intensity): 460(6.8), 445(16.7), 403(6.2), 370(8.0), 355(14.6), 306(13.5), 215(46.9), 75(100).

For the fox, GLC analyses were carried out on derivatized extracts of anal sac secretions obtained from three red foxes over 8 weeks (bulking for each fox every two weeks). One fox was an adult male, the second was a barren female, and the third was a pregnant female which gave birth, killed its cubs, and terminated lactation during the sampling period. GLC was conducted using a 4.3 m column containing 3% OV-17, programming from 70° to 300°C at 5°/min. Carbon numbers were determined by coinjection of a mixture of *n*-alkanoic acid methyl esters. Major lower molecular weight lipids, carbon numbers 780–2700, exhibited more constant, simpler GLC profiles than were noted for lion secretion extracts. The largest peak in this range corresponded to $C_{18:0}$, and only 4 to 8 peaks per chromatogram had

TABLE 1. Lower molecular weight lipid composition of lion anal sac secretion (following derivatization[a])

Component	Sample A[b]	B[b]	C[b]	D[b]
		%[c]		
(a) *Fatty acids*				
Methyl alkanoates (straight chain)				
$C_nH_{2n+1}COOMe$				
n = 10		0.6		
n = 11	0.9		1.4	0.1
n = 12				0.2
n = 13	4.6		3.1	0.4
n = 14	2.4	0.4	1.4	1.1
n = 15	9.3	3.2	7.0[d]	6.9
n = 16	1.3	0.8		1.0
n = 17	4.2	2.6	7.4[d]	5.7
n = 18	1.1	0.8		0.5
n = 19	2.7	0.9	2.0	3.1
n = 20	0.5	0.5		
n = 21	1.6	2.3	2.2	1.0
n = 22	0.6	1.1		0.4
n = 23	2.2	3.8	3.1	1.4
n = 24		0.5		
n = 25			0.9	
Methyl alkanoates (branched chain)				
$C_nH_{2n+1}COOMe$				
n = 10		1.1		
n = 14	2.4	0.6	3.6[d]	1.4
n = 16		0.7		
n = 18		0.6		
Methyl alkenoates (straight chain)				
$C_nH_{2n-1}COOMe$				
n = 15		0.6[d]		4.0
n = 16				5.0
n = 17		5.8[d]		40.5
n = 18				2.4[d]
Total	33.8	26.9	32.1	75.1
(b) *2-Hydroxy-fatty acids*				
2-hydroxyalkanoic acids, methyl esters, TMS ethers (straight chain)				
$C_nH_{2n}(OSiMe_3)COOMe$				
n = 13	8.7	0.6	2.4[d]	
n = 14	4.1		7.6[d]	
n = 15	5.2	5.8[d]		
n = 16	0.9			
n = 17	1.8	1.6		
n = 18	0.6[d]			
n = 20	0.4[d]			
n = 21	0.7			
n = 23	1.5			
2-hydroxyalkanoic acids, methyl esters, TMS ethers (branched chain)				
$C_nH_{2n}(OSiMe_3)COOMe$				
n = 14			0.5	
2-hydroxyalkenoic acids, methyl esters, TMS ethers (straight chain)				
$C_nH_{2n-2}(OSiMe_3)COOMe$				
n = 13	0.6			
n = 14	3.9			
n = 15	9.6		2.7[d]	
n = 16	0.9			
n = 17	1.9			
n = 21	0.7			
Total	41.5	8.0	13.2	
(c) *Aromatic acids*				
Methyl phenylacetate		8.5		
Methyl 3-phenylpropionate		42.3		
Methyl *p*-hydroxyphenylacetate (TMS)		10.3		
Methyl 3-(*p*-hydroxyphenyl) propionate (TMS)	18.8	1.1	1.0	
Total	18.8	62.2	1.0	
(d) *Other peaks* excluding 1-alkylglycerols (Table 2)	5.9	9.3	15.8	24.9
(number of peaks)	(15)	(7)	(20)	(20)

[a] Diethyl ether extract derivatized sequentially with diazomethane and bis(trimethylsilyl)acetamide.
[b] Sample codes, see text.
[c] Percentage contribution to gas chromatogram estimated on peak height basis. All peaks after solvent up to, but not including, cholesterol (TMS), having peak height >0.1% (Sample *A* and *D*), >0.4% (sample *B*), >0.5% (sample *C*) total, included. Column conditions: 3% Dexsil 300-GC on Gas Chrom Q (100/120 mesh), programmed from 100°C (120°C sample *D*) at 8°/min.
[d] Impure peak.

an area that was more than 10% of the $C_{18:0}$ area, while 37 peaks had an area greater than 1%. Not all the minor peaks were observed in each chromatogram. GLC—MS showed $C_{16:0}$, $C_{18:0}$, and $C_{18:1}$ acids (as methyl esters) to be major components. Other components corresponded with other *n*-alkanoic acids and phenylpropionic acid. No major profile differences appeared to be associated with sex or sexual status for the fox, but minor features were suggestive of such differences in the small number of samples examined.

At slightly higher temperatures, gas–liquid chromatograms of fox anal sac secretion extracts invariably exhibited a peak corresponding to choles-

TABLE 2. 1-Alkylglycerol composition of lion anal sac secretion (following derivatization[a]) as TMS diethers, $C_nH_{2n+1}OCH_2(CHOSiMe_3)_2H$

Straight Chain	Sample C[b]	Branched Chain	Sample C[b]
	%[c]		%[c]
n = 12	2.3	n = 14	5.9[d]
n = 13	7.4[d]	n = 16	0.5
n = 14	1.5	n = 17	3.5
n = 15	0.6	n = 18	0.7
n = 16	2.6	n = 19	1.0
n = 17	1.0		
n = 18	5.7		
n = 19	2.3		
n = 20	5.9		
n = 21	0.9		
n = 22	2.7[d]		
Total	32.9	Total	11.6

1-Alkylglycerols were not observed in samples *A*,*B*,*D*. For other footnotes, see Table 1.

terol (TMS ether). This was confirmed by GLC–MS. In one sample, coprostanol (5β-cholestan-3β-ol) was also noted; *m/e* (relative intensity) 403(3), 371(12), 370(42), 355(28), 257(21), 230(10), 217(13), 216(26), 215(54), although a careful GLC analysis (1.5 m column containing 3% OV-17; isothermal at 270°C) of six further red fox anal sac secretion derivatized extracts in all cases demonstrated a large cholesterol TMS ether peak but no peak (>1% cholesterol peak area) corresponding to coprostanol TMS ether (retention time relative to cholesterol TMS ether, 0.76). Prior to derivatization, these secretion extracts all revealed one small peak at lower temperature on 3% OV-17 (eluting at 124°C, programming from 110°C at 2°/min). This was identified as indole by GLC–MS and by retention time comparison with authentic material. Thus, one red fox secretion extract yielded the mass spectrum *m/e* (relative intensity) 118(10.2), 117(100), 116(10), 90(45.7), 89(31.4), 63(13.7).

Determinations of cholesterol in five samples of red fox anal sac secretion using the ferric chloride method yielded a mean total cholesterol level of 0.93 mg/g (σ = 0.39 mg/g) and a mean percentage free cholesterol of 56.4% (σ = 9.2%). Individual determinations were 0.77 mg/g (40.6%), 1.58 mg/g (59.3%), 0.61 mg/g (62.0%), 1.03 mg/g (56.7%), 0.68 mg/g (63.4%). In comparison, determinations of cholesterol in eight samples of domestic dog anal sac secretion using the cholesterol oxidase method yielded a mean total cholesterol level of 2.49 mg/g (σ = 0.60 mg/g), a mean free cholesterol level of 1.60 mg/g (σ = 0.24 mg/g) and a mean percentage free cholesterol of 65.8% (σ = 10.4%). Values ranged from 1.71 mg/g to 3.29 mg/g total cholesterol, from 1.31 mg/g to 1.90 mg/g free cholesterol, and from 50.5 to 77.2% free cholesterol.

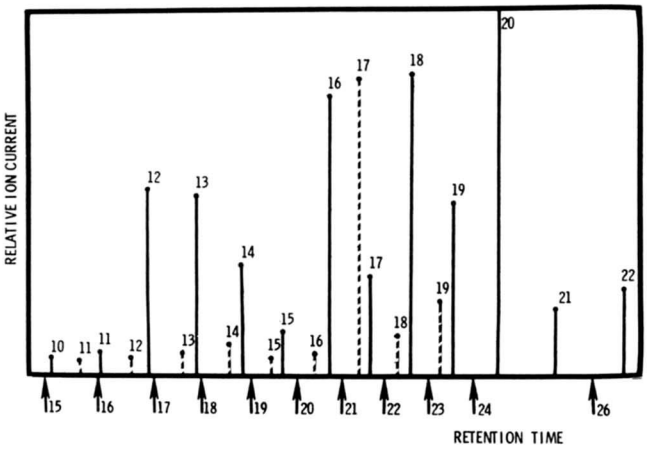

Fig. 1. The distribution of 1-alkylglycerols (as TMS diethers) in a lion anal sac secretion extract (Sample *C*), derived from a mass fragmentogram of the characteristic *m/e* 205 ion. Carbon numbers of the alkyl chains are indicated. The shorter retention time homologous series (dashed line) was tentatively assigned a branched chain structure. The numbered arrows specify the carbon number of the methyl *n*-alkanoate eluting at that retention time on 3% Dexsil 300-GC.

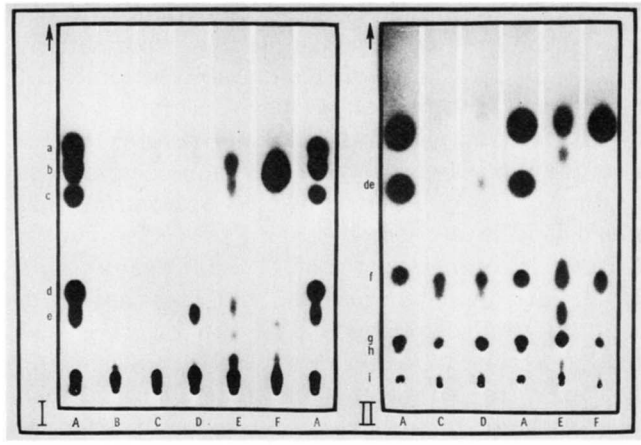

Fig. 2. Thin-layer chromatograms of anal sac secretion (chloroform) extracts on silica gel G (sulfuric acid charring); I, developed with benzene; II, developed with petroleum ether (60–80°C)–diethyl ether–acetic acid 70:30:1. *A*, standard mixture (*a*, squalene; *b*, cholesteryl palmitate; *c*, palmityl palmitate; *d*, muscone; *e*, tripalmitin; *f*, stearic acid; *g*, cholesterol; *h*, dipalmitin; *i*, monopalmitin); *B*, *C*, red fox (♀,♀); *D*, maned wolf (♂); and *E*, *F*, lion (♂,♂) anal sac secretion extracts.

Thin-layer chromatography results are in **Fig. 2** and **Table 3**.

DISCUSSION

Lion anal sac secretions were browner, thicker liquids and contained more suspended material than

TABLE 3. Red fox anal sac secretion lipids.[a]
TLC analysis on silica gel G

Secretion Extract		Standard Mixture[b]	
Zone	R_f	Compound	R_f
Solvent, benzene			
1	0.78	Squalene	0.76
2	0.67	Cholesteryl palmitate	0.69
3	0.57	Palmityl palmitate	0.61
4	0.41	Muscone	0.30
5	0.20	Tripalmitin	0.20
Solvent, petroleum ether (60–80°C)–diethyl ether–acetic acid 70:30:1			
1–5	0.77	Tripalmitin	0.60
	0.61	Muscone	
6	0.50	Stearic acid	0.34
7	0.34	Cholesterol	0.31
8	0.31	Dipalmitin	0.10
9	0.19		
10	0.13		

[a] Seven samples of anal sac secretion were individually acidified (pH 1), extracted with chloroform, and chromatographed.
[b] Chromatographed on the same plate as the secretion extracts.

the straw-colored, watery secretions commonly obtained from the fox. Lion sample *D* was particularly notable in consisting of comparable volumes of aqueous and lipid phases. We have observed no such oil-rich anal sac secretion from any red fox in this or in any previous study. This lion sample was caught as it was expelled as two jets by an unsedated animal held in a crush cage, and proved to consist of a mobile, orange-brown oil (1.6 ml) floating on a turbid, buff, aqueous liquid (2.5 ml). This chance observation confirms that the lion can direct jets of anal sac secretion a meter or so. Reports of marking behavior of the lion in the wild generally have been unclear whether urine, anal sac secretion, or both are involved. In addition, the behavioral implications of the two-phase nature of the secretion merit further study. The fate of the lipid phase in the environment will differ from that of the aqueous phase, so are the messages of the two phases different?

The results presented are illuminated by a consideration of the histology of the anal sac. The anal sac of the domestic dog differs histologically from that of the cat in that the sac wall of the cat contains both sebaceous gland complexes and coiled tubular apocrine glands (12), while that of the dog contains predominantly coiled tubular apocrine glands alone, sebaceous glands being confined to the wall of the duct (13). Gross morphological observations on anal sac tissue taken from one adult male lion in this study revealed a distribution of sebaceous glands in several discrete plaques in the sac wall, similar to those reported in the cat and in the tiger (14). Our histological observations on the anal sacs of two male red foxes indicate a distribution of apocrine and sebaceous glands similar to that reported for the dog. Spannhof (15) described the presence only of apocrine tubular glands in the wall of anal sac of the red fox. These observations suggest that the anal sac secretion of the lion would be rich in lipid relative to the secretion of the fox. Indeed, the very limited histological data presently available suggest that this might be a general difference between the anal sac secretions of felids and canids.

Relatively little is known of the chemical compositions of apocrine secretions (16) compared with present knowledge of the lipid compositions of the seba of many species (17). The lower molecular weight lipids reported in lion anal sac secretion in this study are hydrolysis products of compounds identified in the sebaceous secretions of other species. Thus 2-hydroxyalkanoic acids are present in cat sebum as type I diester waxes (66% skin surface lipid) (18), while 1-alkylglycerols have been encountered as diesters in mouse (sebaceous) preputial gland (19), although it has been stated that these compounds have not been reported in the skin lipids of any species (17).

The anal sac is a microenvironment of high microbial activity. All secretions studied here were rich in volatile fatty acids (C_2–C_6), known microbial breakdown products of amino acids, and other compounds (3). It is suggested that the lower molecular weight lipids identified in lion anal sac secretion may be largely microbial hydrolysis products, formed within the sac from components likely to be present in lion sebum, just as skin surface lipid free fatty acids in man are derived by hydrolysis of excreted sebum triglyceride (20).

Macrocyclic ketones, such as have been identified in the preputial scent glands of the muskrat and in the scent gland of the civet (21), were not observed among the major constituents of these anal sac secretions.

Thin-layer chromatography of the oil phase of sample *D* revealed a major zone moving in the approximate position of a sterol ester (cholesteryl palmitate standard) with other zones moving with stearic acid and cholesterol standards (Fig. 2, F). In comparison with the TLC of this and of the more complex extract of a second lion secretion (Fig. 2,E), those of the fox (and, for comparison, of a second canid, an adult male maned wolf (*Chrysocyon brachyurus*) were poor in nonpolar lipids (Fig. 2,B,C,D). A careful TLC analysis of extracts of seven further red fox anal sac secretion samples revealed, on H_2SO_4 charring, nine separate zones corresponding to compounds less polar than cholesterol (Table 3). Only zone 3 was not detected in every extract, although in most cases only a weak charring reaction was observed. Zone 10 gave a strong charring reaction in every case, zones 7 and 8 in all but one case, and zone 2 in only two cases. By comparison with the TLC behavior of standard compounds, it was inferred that hydrocarbons, sterol esters, triglycerides, free fatty acids, and free sterols were present. 2-Hydroxyalkanoic acids and 1-alkylglycerols do not move under the chromatographic conditions used (batyl alcohol and 2-hydroxypalmitic acid, $R_f < 0.03$ in petroleum ether–diethyl ether–acetic acid 70:30:1).

Free cholesterol levels determined in five samples of red fox anal sac secretion by the ferric chloride method ranged from 0.31 mg/g to 0.94 mg/g (mean, 0.53 mg/g). For comparison, free cholesterol levels in three further red fox anal sac secretion samples were 0.24, 0.24, and 0.8 mg/g, and in eight domestic dog

anal sac secretions, ranged from 1.31 to 1.86 mg/g (mean, 1.60 mg/g), all being determined using the cholesterol oxidase method.

Of the lower molecular weight lipids (Tables 1 and 2) in lion anal sac secretion, 2-hydroxy-fatty acids were present in significant quantities only in *A* (42%) and *C* (13%) and 1-alkylglycerols were important only in *C* (45%), while aromatic acids dominated *B* (62%) and fatty acids contributed substantially to *D* (75%). The presence of aromatic acids has been reported previously in lion anal sac secretion (22).

In contrast, the GLC profiles of fox anal sac secretion extracts revealed a greater uniformity and simplicity in their major lower molecular weight lipid composition. While not implying that the peaks of the GLC profiles are necessarily "pure", an examination of the patterns provides a valuable starting point for further ecochemical studies. Although no major profile differences appeared to be associated with sex or sexual status, minor features were suggestive of such possible correlations and these will be the subject of future study.

The occurrence of stanols in some anal sac secretion samples is not unexpected in view of the anaerobic microbial conditions and the abundance of cholesterol in the anal sac; however, its occurrence as a significant constituent of anal sac secretion, at least in the fox, appears not to be common.

Indole was identified for the first time in fox anal sac secretion.

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