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# Comparison of volatile compounds of anal sac secretions between the sexes of domestic dog (*Canis lupus familiaris*)

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## Abstract

Volatile compounds of anal sac secretions are odorant chemicals used across the carnivores for social communication such as identifying individuals and group membership. Odor profiles taken from expressed anal sac secretions of some species of carnivores have been detected in previous studies. In this study, the volatile compounds of anal sac secretions between five male and five female domestic dogs (*Canis lupus familiaris*) were compared. Volatile chemicals were extracted, separated, and analyzed by gas chromatography-mass spectrometry with solid-phase micro-extraction and identified from their electron ionization mass spectra and Kovats retention indices. The results showed the presence of various types of compounds including organic fatty acids, ketones, aldehydes, esters, and alcohols in the anal sac secretions of dogs. Greater amounts of diversity and esters, and lower amounts of alcohols were detected in the anal sac secretions of females compared to males. This was accompanied by finding citrate and acetic acid ester only in the females. Furthermore, presence of some sex-specific organic compounds like dimethylcyclopentyl ethanone indicates that the volatile profiles of anal sac secretions in 10 domestic dogs are differentiated by host sex.



## Introduction

Most carnivores have a pair of anal sacs that contain the secretions of the anal glands opening to each side of the anus.[1] Anal sac secretions contain mostly volatile compounds emitting strong pungent odors used for scent communication in mammals. Qualitative observations of domestic dog (*Canis lupus familiaris*) behavior suggest that these anal sac secretions may also function in sexual attraction.[2] Anal sac secretions are used for defense by the skunk (*Mephitis macroura*)[3] and the honey badger (*Mellivora capensis*),[4] as well as territory marking by the hyena (*Crocuta crocuts*)[5] and the wolf (*Canis lupus*).[6] These chemicals are used for individual identification by the ferret (*Mustela furo Linnaeus*),[7] the mongoose (*Herpestes auropunctatus*),[8] the giant panda (*Ailuropoda melanoleuca*),[9] and the spotted hyena (*Crocuta crocuta*),[10] and sex recognition by the brown bear (*Ursus arctos*),[11] the giant panda (*Ailuropoda melanoleuca*),[12] and some mustelids (*Mustela spp.*).[13,14] Chemical analyses of anal sac secretions of the red fox (*Vulpes vulpes*), coyote (*Canis latrans*), wolf (*Canis lupus*), and mongoose (*Herpestes auropunctatus*) have identified volatile short-chain free fatty acids, such as acetic acid, propanoic acid, and butanoic acid as being key constituents of anal sac odor profiles.[15-19] The domestic dog has a pair of anal sacs lined with glands of two types, apocrine and sebaceous, within the ventrolateral perianal region.[20] Reportedly, anal sacs release a pungent liquid secretion into a dog's feces for territorial marking, and such secretions may have information about sex, reproductive state, and recognition of individuals.[21] Surprisingly, however, there has been little research on the chemical composition of anal sac secretions in domestic dogs and their contributions to chemical communication. Most studies of anal sacs have focused on disorders, including carcinoma[20,22,23] and gross and cytological characteristics for diagnostic purposes.[24] Although millions of dogs coexist with humans worldwide, the scientific community is only beginning to understand the cognition and behavior of dogs.[25] Considering that chemical signals emitted from the body are important for scent communication in dogs,[26] it is important to examine the function and chemical composition of anal sac secretions. Such studies will improve our understanding of behavior

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and scent communication in domestic dogs, and also may help to address their behavior issues accordingly.[27]

The purpose of this study was to determine whether the chemical volatile profiles of anal sac secretions varied by sex in ten domestic dogs. In this study, the headspace gas of the anal sac secretions was analyzed for chemical profiling of the volatile compounds emitted from the secretions using a thermal desorption gas chromatography–mass spectrometry (GC-MS) with solid-phase micro-extraction (SPME).

## Materials and Methods

**Animals and sample collection.** Anal sac secretions were collected from 10 mixed breed clinically healthy dogs (2 - 4 years old) including five intact males (identification numbers (ID): M1-M5) and five females (ID: F1-F5). Anal sac secretions of five male dogs (M1-M5) and five female dogs (F1-F5) were obtained only once as described below. The dogs were housed individually in cages being kept at 22.00 ˚C under 12-hr light: 12-hr dark conditions. The Animal Research Committee of the Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, approved the protocols for sample collection and all behavioral experiments using laboratory dogs (No 5800, 20-12-2021). Anal sac secretions were obtained (after disinfecting the external area of perinea by 70.00% alcohol) by squeezing each pair by gloved hands (lavender nitrile powder-free exam gloves; Kukatpally, Hyderabad, India). The secretions were collected into 20.00-mL glass vials (headspace screw top clear glass 20.00 mL SPME vial Sigma Aldrich, St. Louis, USA) using a spatula, and then the caps were sealed (magnetic screw cap, septum: silicone blue/polytetrafluoroethylene white, 45.00° Shore A, 1.30 mm; Sigma Aldrich) using vial crimper, and kept in the refrigerator at a temperature of 4.00 ˚C for later analyses. Before preparing the sample, the container was kept at room temperature for one hr in order to reach the equilibrium with the ambient temperature.

**Gas chromatography–mass spectrometry analysis of headspace gas from anal sac secretions.** Twenty milligrams aliquot fresh samples of anal sac secretions from the 10 dogs were analyzed within 1 hr after being kept at room temperature. The headspace gas above the sample was concentrated into an adsorption polyvinyl chloride nanotube containing multi wall carbon at 40.00 ˚C by purging with pure nitrogen gas at 50.00 mL per min for 30 min. Volatile compounds trapped in the SPME fiber were desorbed at 200 ˚C for 5 min using a thermal desorption system (Trace 2000; Thermo Finnigan, Warrington, UK), and then injected directly into a Trace GC-MS Plus equipped with a DB_5 capillary column (length: 30.00 m; internal diameter: 0.25 mm; layer thickness: 0.20 µm; Agilent, Santa Clara, USA). The GC was performed in splitless mode. The oven temperature was held at 50.00 ˚C for 1 min, increased to 260 ˚C at 0.50 ˚C per min, and held at 260 ˚C for 4 min. The mass spectrometer was operated in electron impact mode at the electron energy of 70.00 eV and ion source temperature of 200 ˚C. Mass spectra were obtained in full-scan mode from 35.00 to 300 m $z^{-1}$. The molecular species of the volatile compounds were identified by comparing the mass spectra and GC retention time indices with National Institute of Standards and Technology library data and synthetic standards. The amounts of compounds emitted from 20.00 mg anal sac secretions by purging with pure nitrogen gas at 50.00 mL per min for 30 min at 40.00 ˚C were quantified by the area obtained from the reprocessed chromatogram using the characteristic m $z^{-1}$ fragments.

**Overview of analyses.** The initial analyses used GC-MS to identify volatile compounds in the samples and the raw instrument data, including selecting peaks from the total ion chromatogram were processed by GC-MS Xcalibur Software (version 3.0; Thermo Fisher Scientific Inc., Waltham, USA). The relative abundance of each compound was determined as the fraction (%) of the total area of all measured peaks. The relative amounts of compounds varied greatly, indicating that the data are best described by non-parametric statistics, so it was decided to first classify the compounds according to their functional group which enables quantitative comparisons to be made between individuals. Also, the frequency of occurrence was expressed as the number of dogs in which the compound was found. It was considered that the most interesting compounds, and the most likely to act as semiochemicals, would be those that have the most frequency of occurrence in one group and the least in the other group (male and female). Therefore, there might be some compounds specified to one group and some scattered among males and females, for the latter case these compounds' carbon group formation differences were also checked. Finally, the literature was searched for reports of the presence and sex difference of the dog volatiles in other mammals to further ensure that the differences are significant. The search used SciFinder (https://scifinder.cas.org/). The compounds were also searched in the human metabolome database (https://hmdb.ca/), whether in excreta, blood or normal metabolism. General information about sources and uses of chemicals was obtained from the Pubchem database (https://pubchem.ncbi.nlm.nih.gov/#).

## Results

The chromatograms produced from the 10 different male and female subjects being expanded to highlight the profiles produced among the male subjects resulted in off-scale furan, butyrolactone, and oxirane peaks in chromatogram for males (Fig. 1), and the chromatograms grams expanded to highlight the profiles produced among the female subjects resulted in off-scale furan, decanoic acid, and acetic acid peaks in the chromatogram for females (Fig. 2). As can be seen from Figures 1 and 2, there are some common compounds present among the subjects and also some compounds which differ. Differences in abundance between the chromatograms are noted and may be due to concentration differences among the samples; however, similarities in the ratio patterns of the peaks are evident between the two figures. Sixty-four compounds were detected in the odor profiles of the 10 individuals (Table 1). The types of compounds determined to be in the odor profile include heterocyclics, esters, alcohols, aldehydes, ketones, aromatic compounds, lactones, terpenes, and amines.

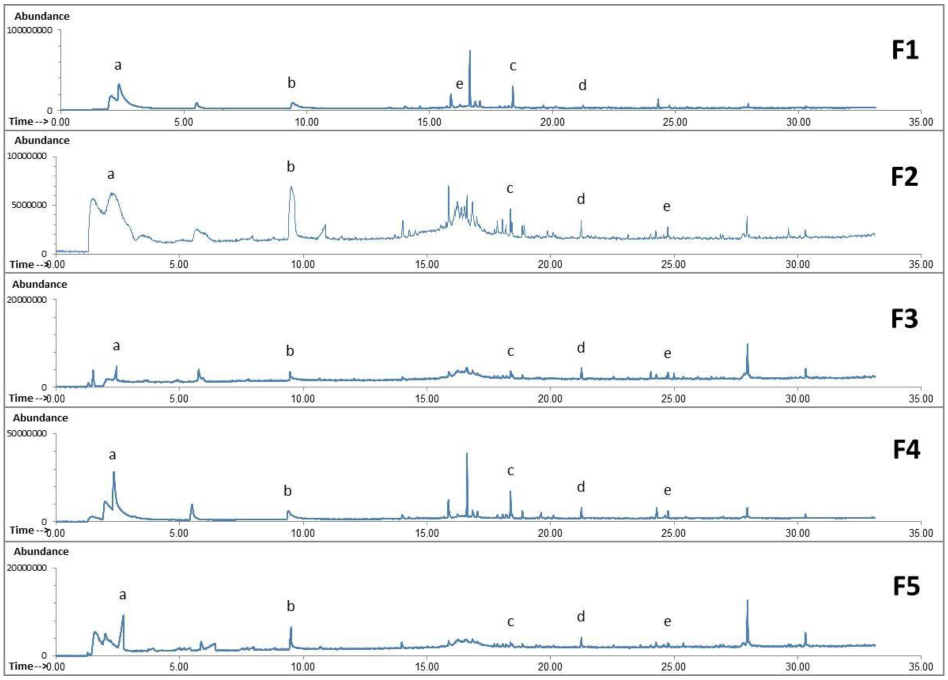


**Fig. 1.** Volatile compound odor profiles for five adult female dogs. a: Hydrofuran; b: Butyrolactone; c: Heptadienone; d: Decanol; e: Sulfuric acid. Chromatograms are from 1.50 to 33 min.

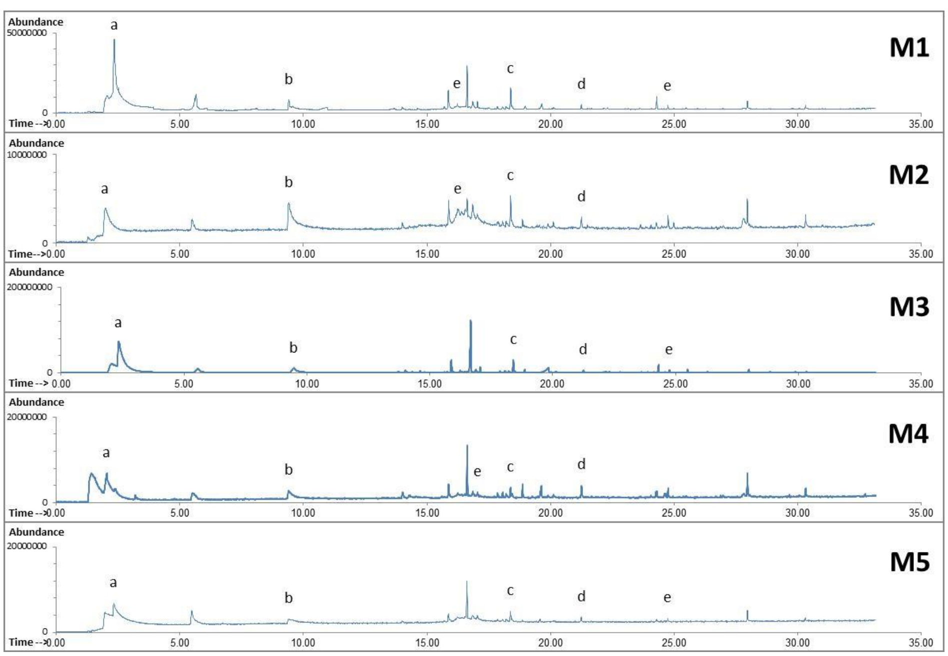


**Fig. 2.** Volatile compound odor profiles for five adult male dogs. a: Hydrofuran; b: Butyrolactone; c: Heptadienone; d: Decanol; e: Sulfuric acid. Chromatograms are from 1.50 to 33 min.

**Table 1.** Tentatively identified compounds [compound name and functional group listed for females (F) and males (M)]. The presence of the compound is indicated by "×" in the column corresponding to each sample.

| Groups | No. | Compound | F1 | F2 | F3 | F4 | F5 | M1 | M2 | M3 | M4 | M5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Esters** | 1 | Sulfuric acid esters | × | × | × | × | × | × | × | × | × | × |
| | 2 | Butyric acid esters | × | | | × | | × | × | | × | |
| | 3 | Carbonic acid esters | × | | | × | | | | | | |
| | 4 | Phthalate | × | | | | | | | | | |
| | 5 | Pentanoic acid esters | | × | × | | × | × | | | | |
| | 6 | Oxalic acid esters | | × | × | | | | × | | | × |
| | 7 | Acetic acid esters | × | × | × | × | × | | | | | |
| | 8 | Heptanoic acid esters | | × | | | | | | | | |
| | 9 | Chloroformate | | × | | | | | | | | |
| | 10 | Propionate | | × | × | | | | | | | |
| | 11 | Propanoic acid esters | | × | × | × | × | × | × | × | | |
| | 12 | Carboxylic acid esters | | × | × | × | × | | × | × | × | |
| | 13 | Citrate | × | × | × | × | × | | | | | |
| | 14 | Phthalic acid esters | | | × | × | | | | | | |
| | 15 | Butanoic acid esters | | | × | × | × | × | × | × | | |
| | 16 | Acetylingol | | | | × | | | | | | |
| | 17 | Decenoic acid esters | | | | | | | × | | × | |
| **Terpenes** | 18 | Octene | × | | × | | | | | | | |
| | 19 | Decane | | × | | | | × | × | | × | |
| | 20 | Diene | × | | | | | | | | × | |
| | 21 | Eicosane | | × | | | | | | × | | |
| **Aromatics** | 22 | Benzene | | | × | | × | × | | | | |
| | 23 | Phenol | | | × | | | | | | | |
| | 24 | Indene | | | | | | | × | | | |
| | 25 | Benzoate | | | | | | | | × | | |
| | 26 | Chloromethylcyclohexane | | | | | | | | | × | |
| **Amines** | 27 | Hydroxyurea | | × | | | | | | | | |
| | 28 | Diaminoethane | | | | | × | | | | | |
| | 29 | Oxime | | | | | × | | | | | |
| | 30 | Hydroxylamine | | | | | × | | | | | |
| **Alcohols** | 31 | Butanol | × | | | | | | | | | |
| | 32 | dimethylcyclohexanol | × | | | | | | | | | |
| | 33 | Heptanol | × | | × | × | | | × | | | × |
| | 34 | Oxiranyl | × | | | | | | | | | |
| | 35 | Methanol | × | | | × | | | × | | | |
| | 36 | Decanol | × | × | × | × | × | × | × | × | × | × |
| | 37 | dienol | × | | | | | | | | | |
| | 38 | nonenol | × | | | | | | | | | |
| | 39 | hexanediol | | × | | | × | | | | | |
| | 40 | cyclohexenol | | × | | | | | | | | |
| | 41 | Tetradecadienol | | × | | | | | | | | |
| | 42 | Ethyl alcohol | | | × | | | | | | | |
| | 43 | Octanol | | | × | × | × | × | × | | × | × |
| | 44 | Pentanol | | | | × | | | | | × | |
| | 45 | Dimethylpentenol | | | | × | | | | | | |
| | 46 | Butadienol | | | | | | × | | | | |
| | 47 | Galactitol | | | | | | × | | × | | |
| | 48 | hexanol | | | | | | × | × | | | |
| | 49 | Hexenol | | | | | | × | | | | × |
| | 50 | ooctadienol | | | | | | | | × | × | |
| | 51 | heptadienol | | | | | | | | × | | × |
| | 52 | Heptenol | | | | | | | | × | × | |
| **Ketones** | 53 | Carboxaldehyde | × | | | × | | | | | | |
| | 54 | Heptadienone | × | × | × | × | × | × | × | × | × | × |
| | 55 | Pentatriacontene | × | | × | × | | | | | × | |
| | 56 | Butylacrolein | | | | × | | | | | | |
| | 57 | Ethanone | | × | × | × | | | | | | |
| | 58 | Hexanone | × | × | | × | | × | | | | |
| | 59 | Butanone | | | | | × | × | | | | |
| **Lactones** | 60 | Butyrolactone | × | × | × | × | × | × | × | × | × | × |
| | 61 | Pantolactone | | | | × | | | | | | |
| **Heterocyclics** | 62 | Furan | × | × | × | × | × | × | × | × | × | × |
| | 63 | Oxirane | | × | | | | × | | × | | |
| | 64 | Pyran | | | | | | × | | × | | |

The compounds were identified by spectral library comparisons or by standard comparison. Five compounds were detected in the anal sac volatile profiles of all sampled dogs including hydrofuran, butyrolactone, heptadienone, decanol, and sulfuric acid. These five compounds make up an average of 55.00% of the compounds of each dog (the lowest amount in F5 with 12.67% and the highest amount in F1 with 84.77%). Hydrofuran, heptadienone and decanol peaks are attributed to the SPME fiber coating; although heptadienone and decanol are known as endogenous scent compounds of some carnivores' emanations, they are also commonly used laboratory solvents making their quantitation unreliable without special precautions against contamination.

Acetic acid and citrate were extracted only in the female group. Twelve compounds such as decenoic acid, indene, benzoate, and galactiol with different distributions were present only in males. However, 27 compounds such as phtalate, propionate, acetylingol, and carbonic acid with different distributions were extracted in some of the females, yet were not present in any of the males. Additionally, 18 compounds with high variance were scattered among the samples.

Figure 3A shows the percentage of compounds in each functional group for male and female dogs. This varied from 0 - 30 (median 10); while, the number of compounds found in individual dogs varied from 29 - 70 (median 41). Figure 3B shows the relative amount of compounds in each functional group found in individual dogs. Esters, alcohols, and lactones were the only groups of volatile compounds found in all dogs, and in most (9/10), they were the most abundant of the compounds whose peaks were measured (Fig. 3B). The dog (F2) in which these functional groups were least abundant (37.86%; Fig. 3B) had the greatest relative amounts of amine compound (26.00%), i.e., hydroxyurea followed by dog F5 (14.00%), i.e., diaminoethane, oxime, and hydroxylamine. Other animals lacked such amine compounds in their anal sac secretions. From 18 compounds which were scattered among samples with a high variant, 14 compounds had been formed by similar carbon groups and four compounds had been formed by different carbon groups; this included butyric acid ester with five different carbon groups (ethylhexyl, tridecyl, pentadecyl, neopentyl, and propylpentyl) from which butyric acid tridecyl ester was only found in F1 and butyric acid neopentyl ester was only found in M4. Also, carboxylic acid had two different carbon groups (cyclopropane and benzene) from which cyclopropane carboxylic acid ester was only found in M3; pentenyl-formed of butanoic acid was only detected in some males (M1, M2, and M3), and from 3 types of octanol (butyl, methyl, and isooctanol), isooctanol was only found in M5. For all sex differences, online database was searched using the method as described above.

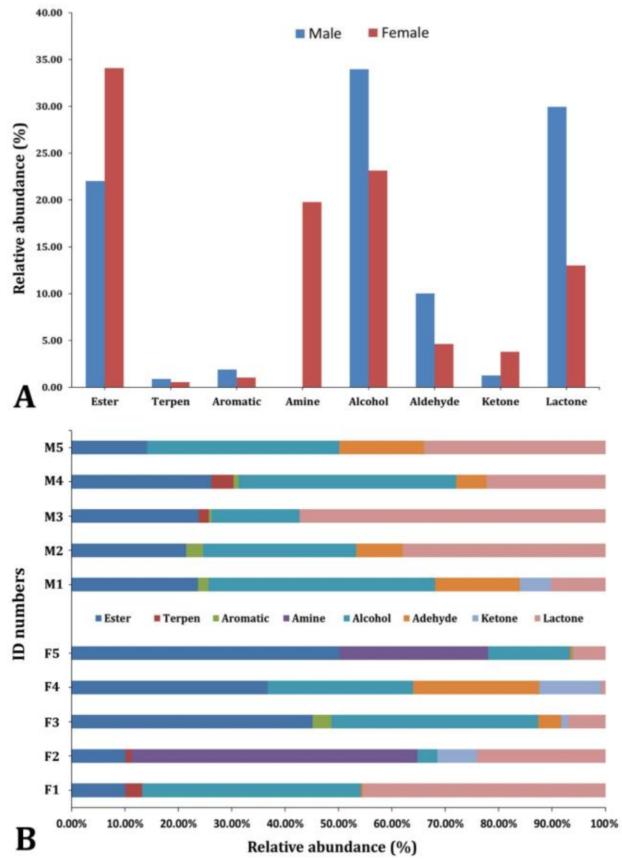


**Fig. 3.** Chemical profiles of volatile compounds emitted from anal sac secretions of domestic dogs. **A)** The average of relative abundance (%) of compounds in each functional group for male and female dogs. **B)** Individual relative abundances (%) of each functional group found in dogs except heterocyclics have been individually coded as shown. The colors indicate the chemical groups in Table 1 and Fig. 3A.

## Discussion

The SPME-GC/MS headspace analysis proved to be an effective method for determining odor profiles of anal sac secretions in both male and female domestic dogs. Both qualitative and quantitative differences were observed among the males and females. Various types of compounds were extracted from males and females, including a variety of aldehydes, organic fatty acids, ketones, and alkanes. Our study showed greater amounts and diversity of esters in females, and on the contrary, more alcohols in males supporting the theory that non-oxidative free fatty acid clearance through re-esterification is higher in women than men, suggesting that women tend to store; whereas, men tend to oxidize circulating free fatty acids.[28]

Compounds including 2,3-dihydrofuran, butyrolactone, oxirane, and 3- hydroxypropyl along with various esters are reported here for the first time as components of dog odor. A majority of the compounds in the odor profiles of both males and females was esters and alcohols, and this is in contrast with research previously conducted on the volatile compounds of anal sac secretions in bengal cat.[29] The common compounds found among both females and males included alcohols, esters, heterocyclics, and aldehydes. The common compounds were present in differing ratio patterns between the males and females, indicating qualitative similarities among individuals with quantitative differences. In the present study, two types of sulfurous compounds including sulfurous acid, octyl 2-pentyl ester and sulfurous acid, cyclohexylmethyl hexyl ester were detected being considered to act as pheromone.[30,31] Although no sex differences were found, sulfurous compounds can still work as inter-species signals. Previous studies on Siberian weasels (*Mustela sibirica*) suggested that 2-ethylthietane was a volatile compound specific to females and can be used to communicate information about sex in weasels, and acetic acid and citrate were detected specific to females in our study.[14]

The sex difference in the composition of secretions is generally regulated by hormones.[32] For example, sex hormones significantly affect some volatile constituents in the anal gland secretion of the wolf.[33] In this context, some studies suggest that male sex hormones play an important role in the sex differences in gut microbiota.[34] Citrate was one of the two compounds identified in our study which was only present in the female dogs. It has been shown in *Drosophila melanogaster* that gut-derived citrate controls food intake and sperm maturation in male fly through a gonad-gut axis.[35] Although citrate is necessary for a normal sperm production and maturation in mammals, it remains to be seen whether such axis really exists in mammals and additionally if utilization of citrate by testes in carnivores is a reason that this compound cannot be traced in anal sac secretions.

On the other hand, it has been reported that microbes, and particularly bacteria, are adept at influencing the behavior of animals. These studies have discovered a number of molecules produced by microbes mediating changes in animal behavior.[36-38] It has been shown that acetic acid, which was detected in our study as a female-specific secretion, is produced by *Tessaracoccus* which is a genus in the *Propionibacteriaceae* family.[29] This is consistent with the possibility that the anal sac secretion is maintained at least in part by bacteria producing volatile compounds for the host. Interestingly, recent studies suggest *Propionibacterium* to be specific of gut microbiota in women.[39] One molecule, in particular, indole carboxaldehyde detected in odor profiles of two female dogs in our study closely resembles serotonin and plays a role in whole-body aerobic metabolism by interacting with iron in heme-containing oxygenases. Moreover, indole by modulating the release of luteinizing hormone regulates the female reproduction.[40]

In our study, the compound acetophenone was found in the odor profiles of three of the females and two of the males, and this compound has been previously reported in the odor profile of female foxes.[30] Compound dimethylcyclopentyl ethanone, which is known for its pungent musty odor and is proved as a novel adenosine deaminase inhibitor,[41] was found in odor profiles of three of the studied female dogs. A recent study on mice has indicated that the levels of adenosine in red blood cells (RBCs) are age- and sex-dependent; so that, adult female mice RBCs were characterized by the lower basal adenosine exposure levels.[42]

The results of our research not only indicate sexual differences but also individual and inter-species differences when compared to similar studies. The presence of butyrolactonein, heptadienone, and decanol in dogs' odor might be used for species-related recognition, since they are present in all dogs and have not been reported for other species in previous studies.

Overall, the SPME-GC/MS headspace method developed for the analysis of carnivore odor profiles allowed us to identify the volatile organic compounds present in dogs' anal sac secretion samples. Through this method, a combination of the relative ratios of common compounds and the presence of differing compounds made a chromatographic distinction among individuals. The results of our study indicate that the volatile organic compounds are the key compounds in anal sac secretion playing an important role in dog's odor related social life; however, other substances that have a relatively low volatility or are present in low concentrations may also make significant biological contributions.

Although we have determined differences in terms of compounds and their relative concentration in volatile organic compounds of dog's anal sacs, future studies should focus on the specific role of every and each compound in dogs' social life taking into account the possible effect of age, breed, and diet. Additionally, the limitation of low sample size in our study can be overcome by a robust statistical analysis in a future study being conducted within a larger population.

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## Conflict of interest

The authors declare no competing financial interest.

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