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Chemical constituency and odor of semiochemicals: Studying the chemical composition and odor of volatile organic compounds of great cat marking fluid in an effort to aid tiger and lion conservation

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Chemical constituency and odor of semiochemicals: Studying the chemical composition and odor of volatile organic compounds of great cat marking fluid in an effort to aid tiger and lion conservation.

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DEDICATION

This dissertation is dedicated to my father, Lance Valentine Soso, who was my constant advocate and supporter. He was a staunch enthusiast of education, propelling the fields of science and math, and using knowledge as a tool to enhance oneself for the greater good of the global community.

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NOMENCLATURE

2-AP	2-Acetyl-1-pyrroline
CAR	Carboxen
Cryo	Cryotrapping
2,5-DMP	2,5-Dimethylpyrazine
DVB	Divinylbenzene
EAD	Electronic Antenna Detector
FF	Furfural
GC-FID	Gas Chromatography-Flame Ionization Detector
GC-MS	Gas Chromatography
3-HB	3- Hydroxy-butanal
HC	Heart-Cut
HC-Cryo	Heart-Cut-Cryotrapping
HPLC	High Performance Liquid Chromatography
HS-SPME	Headspace Solid Phase Microextraction
3-MB	3-Methylbutanamine
3-MCP	3-Methylcyclopentanone
mdGC-MS-O	Multidimensional Gas Chromatography-Mass Spectrometry-Olfactometry
MF	Marking Fluid
4-MP	4-Methyl phenol
NHC	No Heart-Cut

OAV	Odor Activity Value
PA	Propanedionic Acid
PDMS	Polydimethylsiloxane
SEP	Sample Enrichment Probe
SHC	Selective Heart-Cut
SPME	Solid Phase Microextraction
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
VOC	Volatile Organic Compound

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ABSTRACT

In conjoining the disciplines of ethology and chemistry the field of Ethochemistry has been instituted. Ethochemistry is an effective tool in conservation efforts of endangered species and the understanding of behavioral patterns across all species. Chemical constituents of scent-markings have an important, yet poorly understood function in territoriality, reproduction, dominance, and impact on evolutionary biology, especially in large mammals. Scent-markings are comprised of semiochemicals which are the key components in biota signaling. Sensory analyses of scent-markings could address knowledge gaps in ethochemistry and provide an insight into the animal‘s sensory perception. The overall objective of this research is to determine the chemical constituents of the African lion (*Panthera leo*) and Siberian tiger (*Panthera tigris altaica*) marking fluid scent-markings using simultaneous, state-of-the art chemical and sensory analyses. The specific objectives of this study were to: 1) develop a novel method for the simultaneous chemical and scent identification of lion and tiger marking fluid in its totality and 2) identify the characteristic odorants responsible for the overall scent of lion and tiger marking fluid. Solid-phase microextraction (SPME) for scent collection from mixed MF and urine and multidimensional gas-chromatography-mass spectrometry-olfactometry (mdGC-MS-O) for analyses were used. Simultaneous chemical and sensory analyses with chromatography-olfactometry hyphenation could potentially aid conservation efforts by linking perceived odor, compounds responsible for odor, and resulting behavior. To date, no study reported scent and composition of marking fluid (MF) from *P. leo or P. tigris altaica*.

2,5-Dimethylpyrazine, 4-methyl phenol, and 3-methylcyclopentanone were isolated and identified as the three compounds responsible for the characteristic odor of lion MF. Twenty-eight volatile organic compounds (VOCs) emitted from lion MF were identified, adding a new

list of compounds previously unidentified in lion urine. In addition, chemicals in nine new compound groups were identified: ketones, aldehydes, alcohols, amines, aromatics, sulfur containing compounds, phenyls, phenols, and acids. Eighty-nine VOCs emitted from tiger MF were identified. Additional odorants besides 2-acetyl-1-pyrroline, i.e. 3-methylbutanamine, R-3-methylcyclopentanone, propanedioic acid, urea, furfural, and 3-hydroxy-butanal were also identified as contributing to the characteristic MF odor. Simultaneous chemical and sensory analyses improved characterization of scent-markings and identified new MF compounds not previously reported in other tiger species.

This research will assist animal ecologists, behaviorists, zoo keepers, and conservationists in understanding how scents from specific MF compounds impact great cat communication and improve management practices related to animal behavior in captivity and in the wild. The analytical approach for simultaneous chemical and sensory analyses can be applicable to unlock scent-marking information for other species and potentially aid conservation and management. Likewise, the analytical approach for simultaneous chemical and sensory analyses can be useful to many aspects of animal production systems, such as breeding and behavior.

CHAPTER I

INTRODUCTION: ROLE OF CHEMICAL SIGNALLING IN WILDLIFE CONSERVATION

Status of Lion and Tiger Wildlife Populations

Over the course of the last century wild tiger ranges have been largely degraded and eliminated due to anthropogenic factors, climate change, and decline in prey abundance [1]. The resulting losses of great cat populations have impacted the ecosystems of their lands in Africa and Asia. A worldwide, multidisciplinary scientific effort is needed in order to prevent the complete eradication of the African lion (*Panthera leo*) and tiger species (*Panthera tigris tigris, Panthera tigris corbeti, Panthera tigris jacksoni, Panthera tigris amoyensis, Panthera altaica,* and *Panthera tigris sumatrae*). One of the areas of cross-disciplinary research focus is semiochemicals, i.e., chemicals used for communication with their kin, other species and environment by means of volatile and semivolatile compounds present at very low concentrations and associated with characteristic odors.

Improved fundamental knowledge of the composition and odor of semiochemicals and its relation to conservation is needed. Such knowledge will improve the effectiveness of conservation efforts for tigers, lions, and other endangered species. Understanding the fundamental mechanism controlling the environmental fate of semiochemicals will also broaden general knowledge of how wild and domestic animals communicate and the purpose of their chemical signaling.

At the beginning of the 20th century there were over 100,000 tigers in the wild. Currently there are fewer than 3,500 remaining in the wild [1] (Table 1) and about 7,200 in captivity. This represents a 97% percent decline since 1900. Lions have also seen a devastating decline of

nearly 30-50% in the past 100 years. The total wild lion population is estimated to be as low as 20,000 [2]. There are approximately fewer than 1,000 African lions residing in their original habitat in Western Africa. They have been driven out of their native land due to habitat destruction, hunting of their prey base, the bushmeat trade selling lion parts, use of lion body parts for medicine, and trophy hunting of African lion that reside primarily in Western Africa has reduced its population to nearly 1,000 animals [2].

Table 1. Estimated global wild tiger populations.

Tiger Species	Estimated Number	Conservation Status
Siberian Tiger *(Panthera tigris altaica)*	~500 [3]	Endangered [5]
	<1,500 [4]	
	~349 to 415 adult [5, 6]	
	Effective Population Size ~27 to 35 [7]	
Bengal Tiger *(Panthera tigris tigris)*	~254 to 432 [8]	Endangered [9]
Sumatran Tiger (*Panthera tigris sumatrae)*	~440 to 675 [10]	Critically Endangered [11]
	~400 [11]	
	Effective Population Size ~ 176 to 271 [11]	
Indochinese Tiger (*Panthera tigris corbetti*)	~ 202 to 352 [12]	Endangered [14]
	~ 7 to 71 adult and sub-adult tigers [12]	
South China Tiger *(Panthera tigris amoyensis)*	~57 [15, 16]	Critically Endangered [18]
	~0 Effective Population Size [17]	
Malayan Tiger *(Panthera tigris jacksoni)*	~ 493 to 1,480 [19]	Endangered [20]
	~250 adult tigers [19]	
Total	~1,370 to 2,607	

Studying semiochemicals can aid in great cat survival. Unlocking the relationship between semiochemicals and tiger and lion behavior may be the key to their conservation. Environmental factors play a critical role in the sustenance and composition of volatile organic compounds (VOCs) within semiochemicals [21,22].

Semiochemicals are exocrine excretions that communicate information between organisms [22]. Communication is a process in which animals use their sensory organs to send and receive information throughout their ecosystems [23]. Scent marks are social signals placed on a variety of objects in the environment, often in the absence of the receiver, and may only be detected much later, often in the absence of the signaler [24]. Gosling *et al*. (2001) describe scent-marking as the most ubiquitous form of chemical signaling in mammals.

Improved fundamental knowledge of the role of semiochemicals and its relation to conservation is needed. Such knowledge will improve the effectiveness of conservation efforts for many endangered species. Understanding the fundamental mechanism controlling the environmental fate of semiochemicals will also broaden general knowledge of wild and domestic animals in the areas of marking frequency, marking detection, and the purpose of their chemical signaling.

To date, chemosensory analysis has focused primarily on a limited number of species and specifically on understanding the role of chemical signaling in reproduction, kin recognition, and territoriality. By improving and expanding upon previous research, a greater understanding of the role of the animal-specific semiochemicals responsible for influencing tiger movement, reproduction, and social interactions is vital to tiger ethochemistry and survival.

This work will benefit the greater tiger and lion worldwide population within captivity and the wild. It will improve the chances of great cat survival. This research can lead to

collaborations amongst various facilities and conservation parks to use the knowledge gained in order to proliferate the species and reduce the human-wildlife conflict occurring in various countries. The approach used on this research can be used as a model for aiding conservation of other species.

Research Strategies for Wildlife Conservation

Conservationists have general approaches that are often regarded as limited in impact [25]. Some of these common practices are: habitat preservation, educating the public and behavioral research. Some novel chemical signaling tools are being utilized to understand the role of semiochemicals and the role of pheromones in reproduction. Studying tiger habitat to identify indicators of species richness of prey and ideal conditions for tiger fitness is also an area of focus [26]. Conservation research has speculated that ungulate and plant species diversity needs to be widely distributed in high density tiger and lion populations.

Recent studies have focused on estamating wild tiger populations either via modeling or visual confirmation (counting). Tiger population estimates have been performed using remote sensors and camera trapping in various locations. Modeling is currently utilized for predicting tiger survival rates [27].

Behaviors that are commonly studied in conservation efforts are: foraging, mating, reproduction, socialization patterns, methods of communication, and rearing of offspring. Animal dispersal and refuging systems are indicators of social status, habitat quality, and prey and predator abundance [28]. A common method used to measure dispersal and movement of tigers has been radio tracking.

Preserving the remaining tiger subspecies has led to the development of new reproductive and genetic techniques. Artificial insemination has been effectively used as a method of tiger

population growth in captivity [29, 30]. Tiger subspecies classification has been accomplished using nDNA and mtDNA [25]. This discovery has led to the proliferation of tiger subspecies through the genetic reinforcement from other subspecies. Genetic research has linked tiger emergence to the Pleistocene era (from 2,588,000 to 11,700 years B.C.) [31].

Social and economic values are critical components in determining the relationship that people have with tigers. Tiger poaching and distribution of tiger-related artifacts is well documented [32-39]. The need for curbing the demand for tiger parts and laws governing anti-poaching has risen [4,31,37-38,40-41]. Protecting tigers has become a global effort. There are several governmental and non-governmental organizations (NGOs) devoted to tiger conservation and protection. Major NGOs include: Exxon Mobile's Save the Tiger Fund [25,40], Convention on International Trade in Endangered Species [43], World Wildlife Fund [10], International Union for the Conservation of Nature [44], Conservation International [40, 45], Global Tiger Initiative [46], and Project Tiger [47].

Organizations and laws help to advocate for tigers and work with communities in areas where there are tiger habitats (Figure 1). The purpose of the community outreach is to discourage tiger poaching and possibly offer incentives for counteracting tiger poaching. Major laws that have been put in place to prevent trade and distribution of tiger parts in the U.S. are the: Lacey Act [47], Rhinoceros and Tiger Conservation Act [48], and Endangered Species Act [47]. India has also implemented a similar set of legislation known as the Indian Wildlife Protection Act [49].

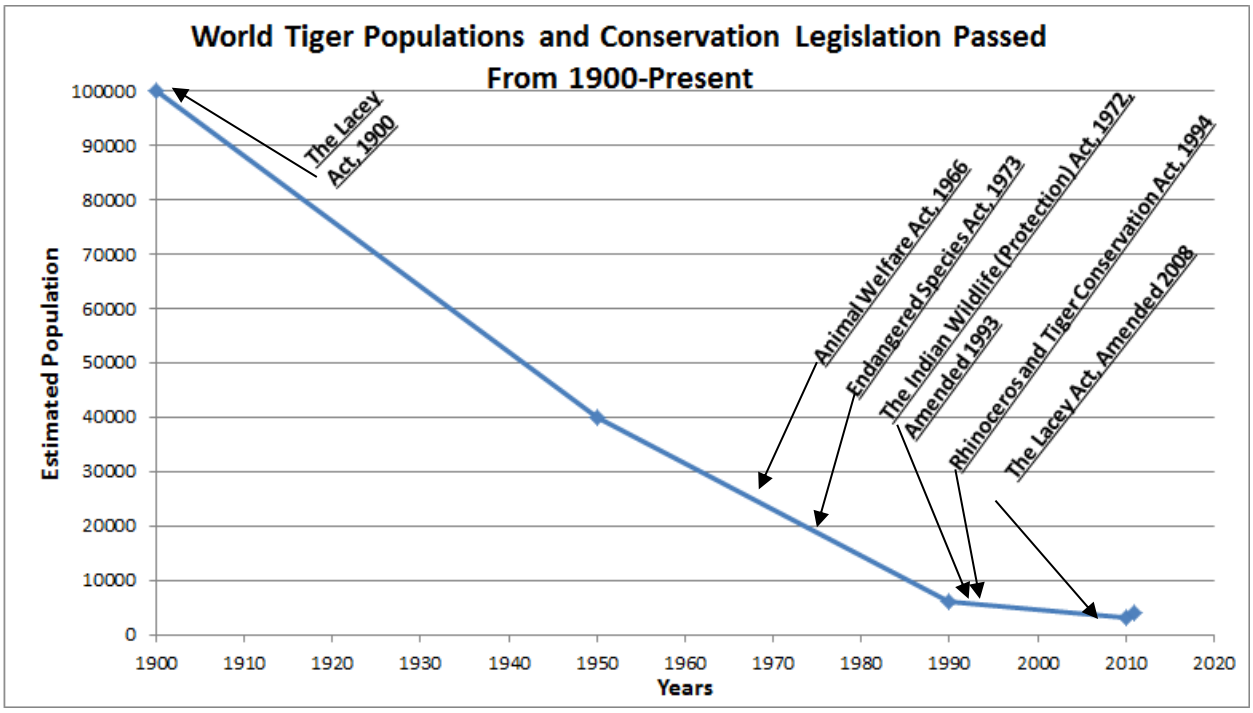

Figure 1. Rapid decline of the world's tiger population over the entire 20th and 21st century. The population is dwindled down to ~3,500 in 2011 from 100,000 in 1900 [9, 25].

Early Research on Chemical Signaling: Insects and Domestic Cats

Insects were the original models used to interpret the multiple purposes of semiochemicals [51,52]. Later, it has been demonstrated that rodents use scent marks for socialization, kin recognition, and territorial defense [53, 54]. Semiochemicals in domestic cats have been studied moderately are largely used for territorial, reproductive, and stress signaling [55-61]. The freshness of a mark explicates the frequency in which the animal enters this territory, whether or not another animal should risk being caught by the territorial ruler, the status (health and reproductive), strength, resources, and sex of the territory owner [24, 26, 62-64]. This is presumably why these animals remark their territories in an effort to convey messages to other animals. Research on chemical signaling and behavior in domestic cats is very relevant to the

chemical signaling of the tiger. The research indicates several behaviors such as urination, fecal deposition, pacing, are behaviors that both domestic and wild species have in common. Territorial behavior, pheromone release, urinary deposits, aggression, and chemosensory application are used by domestic cats in response to or in initiation of semiochemicals by other cats. Artificial marking sprays developed by Pagaet and Gaultier (2003) have also elicited calming behavioral responses from domestic cats and aid in the understanding of socialization behaviors [65].

Role of Chemical Signaling in Wildlife Conservation

Research in chemical signaling plays an important role in conservation of many endangered large animals. Elephants have been a major focal animal in the area of scent marking and its role in reproduction and socialization. They have been used to understand how influential scent marking is on mating and interaction of males and females of various ages and social levels within herds. Merte *et al.* (2009) found that male and female African elephants (*Loxodonta africana)* have developmental differences in chemosensory signal processing [66]. The exhibition of musth pheromone (frontalin) released by male elephants has been known to elicit female sexual responses to the male [67].

Wild cat scent markings have been studied to aid conservation, specifically focusing on territoriality. Great cats use scent marking as a method for distinguishing amongst other conspecifics, neighbors, territorial boundary markings, and as reproductive indicators. Behavioral studies of free-ranging tigers have determined that marking functions to establish and maintain territorial boundaries and advertises female reproductive status [26]. Scent marking behavior in snow leopards was used by researchers to determine taxonomical separation and classification [68]. One of the main function of cats' sense of smell is to decipher their own

scent marks and those of conspecifics, stimulate exploration, and territorial defense [69]. Genetic characterization and definition of Siberian tigers (*Panthera tigris altaica*) and the Amur leopard (*Panthera pardus*) is needed in order to restore the population, and felid research has led to their species and sex identification from fecal and hair samples [70]. Feces has also been used as an indicator of tiger population numbers and territorial distribution through dog scent-matching of individual Siberian tigers [71].

Chemical and sensory analyses of semiochemicals

Chemical composition of semiochemicals of tiger (*P. tigris tigris*), cheetah, and puma (*Puma concolor*) have been analyzed [26, 57, 71-74, 76]. Scent markings have also been used to determine population densities of tigers and pumas by abundance of scent marks in a given area.

Tiger marking fluid (MF), urine, and feces are the known sources of chemical communication in tigers. Ninety-eight volatile compounds have been identified in the MF of Bengal tigers [63]. 2-AP has been the only compound associated with the characteristic odor of tiger marking fluid [73]. It has been assumed that tigers use these volatile and non-volatile markings to convey olfactory signaling. However, what is inhaled and how it is processed has not been completely identified [26, 63, 73]. The use of gas and liquid chromatography has enabled characterization of MF, specifically its lipid component, volatile organic compounds, and a general characteristic odor of MF being similar to that of basmati rice.

Scent marking has been analyzed in Bengal tigers (*Panthera tigris tigris*), Marmoset monkeys (*Callithrix jacchus*), African elephants (*Loxodonta africana*), African cheetahs (*Acinonyx jubatus*), Indian leopards (*Panthera pardus fusca*), African lion (*Panthera leo*) Spotted hyenas (*Crocuta crocuta*) and humans (*Homo sapiens*). Common procedures used to chemically characterize scent markings include: headspace extraction, solid-phase

microextraction (SPME) for sample preparation and gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography, and thin layer chromatography (TLC) for sample analyses [75-78]. It has been previously found that chemical confirmation of semiochemical molecules influences elution order of semiochemicals using gas chromatography [79]. This work specifically focused on alkene elution. This has aided in understanding the configuration of Total ion chromatograms (TIC). Within the past decade, the leading technological method for scent marking characterization has been GC-MS.

In the case of the Bengal tigers two methods have identified the total lipid and urinary portions of the MF, i.e., TLC and GC-MS. There have been 118 compounds found in the MF of Bengal tigers [63]. TLC has been used for quantitatively determining lipid composition of Bengal tiger marking fluid [64]. GC-MS has been utilized to quantify both lipid and urinary components of Bengal tiger MF [63, 64]. Comparison of differences in the chemical composition and concentrations of marking fluid and urine of subspecies of tigers has never been conducted. Bramachary *et al*. (1990) identified the characteristic odor of Bengal tiger MF to be 2-acetyl-1-pyrroline (2AP), typically associated with the odor of basmati rice. The methods for identification of 2AP were based on the addition of hydrochloric acid for acidifying and preventing volatilization, followed by the addition of alkali for aroma identification, and addition of 2% KI to cleave the reactive methyl ketone group of the 2AP molecule [73]. Though 2AP is a characteristic odor compound of Bengal tigers it is not the only compound associated with the overall characteristic odor [78,79]. Determining all compounds responsible for the characteristic odor of tiger marking fluid beyond 2-acetyl-1-pyrroline, is necessary for accurate characterizing of Bengal tiger MF odor.

Andersen and Vulpius (1999) analyzed the chemical constituents of lion urine and found 55 chemicals responsible for comprising its total composition [62]. There have been a few other studies that researched the presence of specific compounds in lion urine. The only lion subspecies to have been analyzed for marking fluid volatile organic compound (VOC) composition was *Panthera leo persica*. However, the study focused on reporting the lack of 2-acetyl-1-pyrroline (2-AP) in anal gland excretion in the MF of Asiatic lions [72]. The focus on 2-AP stems from the earlier finding (Brahmachary, Poddar-Sarkar & Dutta, 1990) that it is a characteristic odor-imparting compound in tiger MF and thought to be in anal gland fluid [64].

Beaver (2009) suggested that the use of human simple olfactometry detection produces limitations making "it very difficult to appreciate the sensory ranges of animals" [82].
To date, there is no published research on domestic cats or wild cats reporting the chemical cause of specific odors associated with their scent marks. Thus, there is clearly a need to define characteristic odors by identifying key chemical constituents as responsible for odor in a more reliable approach using analytical tools. Several studies have established the importance of odor in scent mark detection and signalling in domestic cats [55-57]. Scent marks contain specific chemicals which signal to receiving animals an odor message about age, strength, dominance, relatedness, and reproductive status [83].

Studying the characterisitic odors of marking fluid and urine of tigers would behoove conservation efforts because understanding how animals perceive these odors will explain the importance of chemicals in signalling and duty cycle. The results of this study will aid in understanding the chemistry of semiochemicals associated with tigers and lions to aid in their conservation. In addition, the results will (a) improve our understanding of the role of semiochemicals in other species, (b) aid the development of semiochemical-based territorial

management techniques of wildlife; (c) aid the rate of success in mating in a plethora of animal species; and (d) aid in semiochemical-based regulation of aggressive behaviors in animals. Results will also have implications on strategies that could be used for conservation of endangered species worldwide.

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CHAPTER II

ANALYTICAL METHODS FOR CHEMICAL AND SENSORY CHARACTERIZATION OF SCENT-MARKINGS IN LARGE WILD MAMMALS: A REVIEW

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Abstract

In conjoining the disciplines of ethology and chemistry the field of Ethochemistry has been instituted. Ethochemistry is an effective tool in conservation efforts of endangered species and the understanding of behavioral patterns across all species. Chemical constituents of scent-markings have an important, yet poorly understood function in territoriality, reproduction, dominance, and impact on evolutionary biology, especially in large mammals. Particular attention has recently been focused on scent-marking analysis of great cats (Kalahari leopards (*Panthera pardus*), puma (*Puma concolor*) snow leopard (*Panthera uncia*), African lions (*Panthera leo*), cheetahs (*Acinonyx jubatus*), and tigers (*Panthera tigris*)) for the purpose of conservation. Sensory analyses of scent-markings could address knowledge gaps in ethochemistry. The objective of this review is to summarize the current state-of-the art of both the chemical and sensory analyses of scent-markings in wild mammals. Specific focus is placed on sampling and sample preparation, chemical analysis, sensory analysis, and simultaneous chemical and sensory analyses. Constituents of exocrine and endocrine secretions have been most commonly studied with chromatography-based analytical separations. Odor analysis of scent-markings provides an insight into the animal‘s sensory perception. A limited number of articles have been published in the area of sensory characterization of scent marks.

Simultaneous chemical and sensory analyses with chromatography-olfactometry hyphenation could potentially aid conservation efforts by linking perceived odor, compounds responsible for odor, and resulting behavior.

Introduction

Scope of this Review

To understand the ways in which animals interpret chemical messages, sampling, sample preparation, and chemical and sensory analysis must be performed to accurately define the odors and concentrations of chemicals within the signal. This developing field is limited in the scope of information available about chemosensory analysis of wild animal markings. The use of scent-markings as a method for aiding conservation has been reviewed [1], but lacked definition as to how these scent-marks and their chemical constituents were prepared and analytically characterized.

The objectives of this large mammal and great cat scent-marking review are to: (1) classify different sample preparation techniques for their analysis of scent-markings; (2) summarize existing information on the use of advanced analytical methods on these scent-markings; (3) identify different sensory techniques used to characterize odors of these scent-markings; and (4) classify different sample preparation techniques for the analysis of these scent-markings.

This review provides an overall perspective of literature on the subject of chemical and sensory analysis of large wild mammals, particularly great cats (i.e., leopards, snow leopard, lions, cheetahs, and tigers), scent-markings. Development in the area of sampling and analysis of semiochemicals aids in understanding animal behavior that can be used, for example, toward efforts such as conservation of great cats.

Animal Communication

Communication is a process through which animals use their sensory organs to receive information [2], aiding in the delivery of signals between various inter- and intra-species groups. These signals relay a plethora of information, such as alarm warning, reproductive status and mating, territoriality, and resource signaling [3]. Organisms can communicate through olfactory (chemical), auditory, electro, seismic, and visual communication [4]. The most commonly used method of communication; however, in large, wild mammals is chemical signaling, otherwise known as scent-marking.

Urination, scrapes, and species-specific exocrine secretions are frequently used as modes of chemical signaling for intra- and interspecies communication. Presumably, the chemical constituents of the scent marking convey information about the animal leaving the mark (sender) to the receptive animal (receiver) [5].

Scent-markings require accuracy of olfactory detection to send and receive the correct signal. Scent-markings contain a complex mixture of chemical compounds at varying concentrations based on its chemical message [6]. If an animal wishes to deter an interspecific interaction they can alter the chemical concentrations within their markings to deliver a counterfactual message. An example would be chemical mimicry of pheromones. This false cue/message may encourage attraction of prey species to the territory of predators.

Semiochemicals and Pheromones

Chemicals that act between organisms are called semiochemicals [7, 8]. In a system of producer-signal-recipient, the signal (semiochemical) is the central component. Semiochemicals are exocrine secretions, produced by one individual and acted upon by another.

Mammalian semiochemicals can be single compounds or mixtures of compounds that are quantitatively variable in coding individual identity based on concentration and specific chemical presence [9, 10].

In group living species, for example, it is essential that an individual can recognize members of its social group as individuals and distinguish them from non-group members. [11]. Limited research has been allocated to the chemical characterization of mammalian semiochemicals [9, 10], although analytical techniques used to identify semiochemicals in a variety of species have recently been reviewed [6, 9]. We build on these reviews by increasing coverage of more large mammals, specifically great cats, and by including sensory analyses techniques of scent-markings.

Semiochemicals can be classified as kairomones or pheromones [9, 12]. When the producer and recipient are of the same species, semiochemicals known as kairomones are used for communication. Allelochemicals, are specifically used when a producer and recipient belong to different species, mediate interactions that only benefits the receiver communication and are considered intraspecific and the signal is known as a pheromone [8]. Pheromones are released by one individual and are detected by conspecifics. Pheromones relay impactful messages about sex, species specificity, and reproduction to the receiver [13].

Pheromones are extensively used in territory marking by mammals. Although pheromones are often thought of as odorants (volatile organic compounds), they can be odorless (nonvolatile organic compounds) [13]. Often the volatile odorants are deposited as scents in the animal‘s dung, urine, scalp, hair, feet, skin, chest and/or breast, and/or may be produced by special glands [6, 14]. Examples of special activities for scent dispersal include the chin rubbing of rabbits, check rubbing in pronghorn (Antilocapra americana), cheek rubbing and interdigital

scrapping in domestic cats, interdigital scrapping in white-tailed deer (Odocoileus virginianus), and head rubbing in goats [15–18].

Pheromones are classified into two categories: (1) primers, which prolong a shift in the physiology of the recipient and (2) releasers, which trigger a rapid behavioral response [19]. Primer pheromones generate longer-term physiological/endocrine responses [14]. The course of a releaser is through the nervous system and its primary action generally involves the endocrine system, but is also regulated by the excretory system. Releaser pheromones are involved in four general types of communication: (1) alarm; (2) recruitment; (3) reproductive; and (4) recognition [7].

Alarm substances communicate that there is a possibility of danger. Recruitment pheromones are commonly found in social insects. They are generally employed by worker castes of social insects to guide their nest mates to a food source [7]. Reproductive pheromones come in the form of scents that influence reproductive behavior in many species. These chemical signals can act as an attractant, which links sexes together or increases aggression, or as an aphrodisiac to generate exact aspects of precopulatory or copulatory behavior [20, 21].

In many vertebrates mother-young recognition is contingent on chemical cues [22]. Territory and recognition scents are difficult to categorize because sometimes it is unknown if it is a territory scent, a scent that acknowledges social status, or a scent that identifies an individual [7]. For a thorough review of the functionality and origin of pheromones in animals refer to references [7, 14, 23].

Scent-Markings

Scent-marking is described as the most ubiquitous form of chemical signaling in mammals [5]. Chemical ecology, otherwise known as ethochemistry, is the study of these signals and the interactions they mediate [7]. Chemical signals and their resulting behavioral interactions are multifaceted and varied.

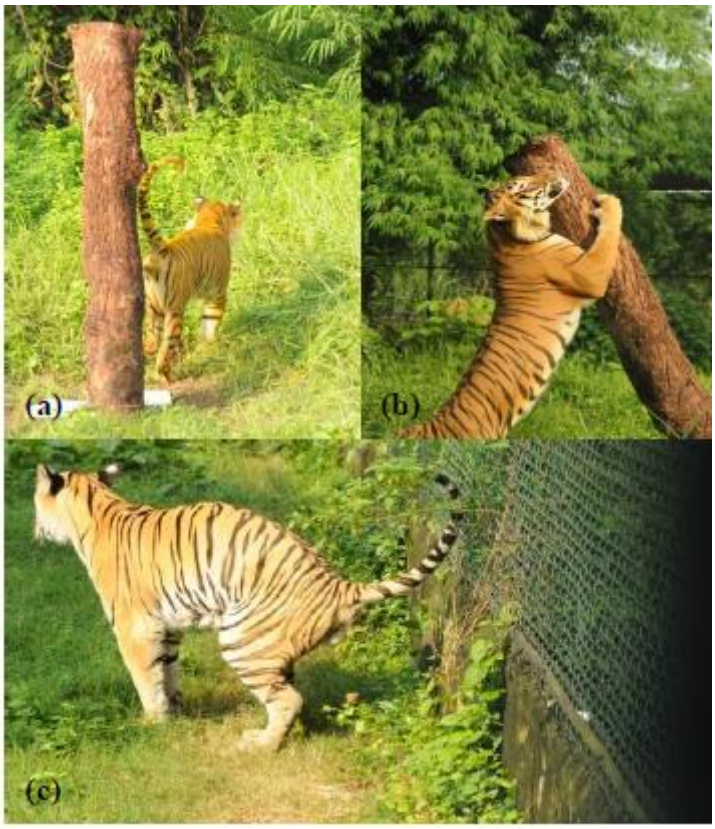


Figure 1. A Bengal tiger (*Panthera tigris tigris*) performing a variety of scent-marking behaviors in its outdoor enclosure at Khayebari Tiger Rehabilitation Project: (a) releasing marking fluid; (b) clawing/scratching (c) defecating.

Scent-marks are placed on objects in the environment, frequently in the absence of the receiver, and may only be detected later, in the absence of the signaler [5]. Senders are often not present to reinforce their scent signals and are unaware of whether the mark will be detected and

by whom. Scent-marks often degrade before they can be detected, as a result of environmental factors such as rain [11]. To counteract degradation, male mammals generally will remark active scent-markings. Compounds in scent-markings that have longevity under environmental conditions tend to have high molecular weights and low vapor pressures. Some examples of compounds that are found ubiquitously in scent-markings are: squalene, cholesterol, and long-chained carboxylic acids. These compounds are primarily in the secretions/excretions of mammals [24].

The most common form of marking is for resource defense territories. Scent-marking by resource holders presents an opportunity for competitor assessment [5]. Scent-marking has long been associated with male intrasexual competition [5, 25, 26]. Males appear to use scent-marking to obtain territories. Marking frequency is associated with social status and is placed in the areas of the territories where intrusion is the greatest (Figure 1). In some species, males usually leave scent-marks for females, but males often intercept these markings. Females use these scent-markings to assess mate quality through smelling direct body odors [27].

Detection of scent-marks is dependent upon the sensory neurons for olfaction within the vomeronasal organ (VNO) and the main olfactory epithelium (MOE) [13, 21]. Universally, mammals detect odorants and pheromones by the nasal olfactory epithelium via the main olfaction system and the vomeronasal organ [13, 21]. Sensory neurons that reside in the olfactory epithelium detect a plethora of chemicals. Within the olfactory epithelium there are two types of G protein-coupled receptors (GPCRs): (1) olfactory or odorant receptors (ORs) and (2) trace-amine associated receptors (TAARs) [28]. There are about 800–1500 OR genes that encode GCPRs, which are vital in odorant recognition in the olfactory epithelium [13].

According to the stereochemical theory of olfaction, mammals bind odorants to specific OR sites based on the size and shape of the molecule [29], which results in odor perception [13]. TAARs are a smaller family of receptors that define a specific population of canonical sensory neurons throughout one area of the olfactory epithelium, and are present in a wide variety of vertebrates [28]. It has been suggested that TAARs are located in the nose and have the ability to detect amine pheromones such as isoamylamine, 2-phenylethylamine, and trimethylamine [28]. Thus the olfactory epithelium appears to contain physically separate pheromone receptors than the vomeronasal organ.

The persistence time of the mark is the interval between deposition and the time when the mark can no longer be sensed [11, 30]. The persistence of the marks is heavily dependent on two factors: the relatively large size of its molecules and the lipid component [5, 11, 31, 32]. The large molecular mass is thought to result in lower volatility and increased persistence in the environment. The lipid portion of markings is known as a lipid fixative [31, 32]. In many great cat species it is comprised of free fatty acids, glycerides, esters, and phospholipid [31]. In the absence of this lipid component, aroma substances evaporate expeditiously [33].

Sample Preparation and Chemical Analysis of Scent-Markings

Sample preparation serves an important role in the efficient extraction of components of interest from the sample matrix. The results of this extraction process are later used with analytical instrumentation for target analyte: separation and isolation into constituents, identification, and quantitation [34]. Some biological samples are not suitable for direct analysis and therefore rely heavily on the efficiency of sample preparation and extraction procedures for future analytical analysis [35, 36].

Recent advancements in sample preparation and analysis of biological samples can aid in addressing needs and knowledge gaps when applied to scent-markings. Reduced sampling, sample preparation time, and faster, more sensitive and precise analytical procedures have the potential to help scientists working in the field of scent-marking analysis [37].

Sample Preparation Techniques

There are two main approaches to sample preparation techniques; solventless and solvent-based.

Solvent-Based Sample Preparation Techniques

Sample preparation methods are categorized by the compound‘s class, polarity, molecular weight (MW), volatility in which it can be extracted, the physical state (solid, liquid, aerosol and gas), and the analytical instrument used for chemical characterization [35, 37, 38]. Solvent-based preparation techniques are often used for the identification of peptides and proteins. Peptides and proteins tend to be polar and their MW is typically less than 5 kDa. This allows for techniques such as dried-droplet, double layer, and thin layer techniques to be used in conjunction with matrix-assisted laser desorption/ionization (MALDI) as an analytical method [36, 37]. Methanol- and ethanol-based solvents have also been widely used in the sample preparation of lipids in scent-markings [31, 39–41]. Solid phase extraction (SPE) has been used for the understanding of pheromone signaling and endocrine communication [42]. Dihydroxybenzoic acid is commonly used in characterizing carbohydrates and polar compounds with a mass greater than 3 kDa [43].

Solventless Sample Preparation Techniques

Modern day sample preparation has advanced dramatically in the area of solvent-free extraction processes [34, 44–49]. Solventless preparation methods generally require minimum steps, conserve time, minimize the use of toxic compounds, and minimize the interferences and

impurities introduced to samples with solvents. In the analysis of biological samples, the most commonly utilized solvent-free techniques are phase preparation methods, which include: solid phase microextraction (SPME), and solid-phase dynamic extraction [35, 37, 50]. SPME combines sampling and sampling preparation and is useful for non-destructive *in vivo* extractions from biota [51–53]. Reference [37] reviewed advanced methods of solventless preparation.

Analytical Instrumentation

Analytical methods are designed to separate, isolate, identify, and quantify analytes of interest within a sample. There are various techniques and reviews on the separation of these components, specifically in mammals [6, 54]. With regard to characterizing scent-marks of wildlife, the most frequently implemented analytical techniques are: gas chromatography (GC) [55], gas chromatography-mass spectrometry (GC-MS) [6,44,56–59], gas chromatography-flame ionization detector (GC-FID) [31,44], GC-time of flight mass spectrometry (GC-TOF-MS), nano-liquid chromatography-mass spectrometry (nano-LC-MS) [40], matrix-assisted laser desorption/ionization- time of flight mass spectrometry (MALDI-TOF MS) [42,60,61], electrospray ionization MS (ESI-MS) [60], gel electrophoresis [62], thin-layer chromatography (TLC) [31,33], gas liquid chromatography (GLC) [31], and tandem MS (ESI-MS/MS) [62].

In GC, the most widely used analytical tool, a mixture of volatile organic compounds (VOCs) is separated into individual VOCs and semi-VOCs, which are eluted out of the GC column at different times [63]. This allows for the quantification and qualification of the compounds within the mixture [63]. Another reason for the common implementation of GC is that it is capable of analyzing volatile compounds that can be detected via the olfactory system.

Identifying compounds using GC-MS is more efficient than other detectors because it has an extensive library available with over 200,000 entries (NIST EI-MS database) for comparison matching.

Sensory Analysis of Scent-Markings

Odor detection is a critical constituent in animal interpretation of scent-markings. Inferences into the actual chemicals and odors sensed by animals have been sought through the use of chemical and sensory analytical instrumentation and the use of animals. Rodents have been commonly used to measure the efficacy of the longevity of scent-marks [64–66]. Conservation studies have introduced the use of scent-matching dogs in order to estimate wildlife populations [67–70]. The use of simultaneous chemical and sensory analyses is an area of limited study with regard to mammal scents.

In recent years, the introduction of application-specific sensor array systems, otherwise known as ―electronic noses‖, were developed and combined with GC, MS, and infrared spectroscopy to mimic the sensitivity of the human (*Homo sapiens*) olfactory system‘s measurement of volatiles [71]. This can be applied to broaden the understanding of how animals use olfactory cues to understand chemical messages.

Animal Detectors

Over the last several decades, scent-marking odor classification of mammals has been limited in its ability to fully characterize the odorous volatile organic compounds (VOCs) within the marking and to detect their presence in the wild. Often this identification is performed via conspecific confirmation. Mice have been the primary models of olfactory detection and interpretation of markings, such as in deciphering the age and reproductive messages in urine

[27, 64, 72, 73]. Mice have also aided in the identification of 2-phenylethylamine as one of the kairomones responsible for avoidance behavior.

Dogs have also been used in the estimation of wild animal populations based on individual scent-mark recognition [68, 74]. The use of animal detectors, however, instead of sensory instrumentation can limit the amount of information acquired from the marking.
The human nose has been an olfactory detection system in various studies of animal pheromones. When m-cresol, 2-heptylpyridine, hexanal, (Z)-6-dodecen-4-olide, and α-terpineol were present in high concentrations, they were identified by human nasal detection as the compounds responsible for the pleasant herbal smell of bontebok (*Damilscus dorcas dorcas*) interdigital gland secretions [75]. The sensitivity of the human olfactory system permitted the detection of reproductive semiochemicals, 5α-androst-16-en-3-one (H5-down), 505β-androst-16-en-3-one (H5-up), and 3α-androstenol in pigs (*Sus scrofa*) [9,76]. Human sensitivity toward these compounds has been used to develop theory that such compounds could also be human pheromones [76]. Studying kin recognition olfactory cues in human neonates has determined that pheromones from their mother‘s breasts and underarm pad are used to distinguish their mothers from other women [77].

Simple human nasal detection was performed for the determination of the characteristic odor of tiger marking fluid [30, 33]. They described the odor as that of basmati rice caused by 2-acetyl-1-pyrroline (2-AP). This conclusion was based on personal and cultural experiences with this food item. This type of identification is useful, yet it could limit identification of all potential odorous compounds that may be contributing to the characteristic odor in highly complex scent mixtures.

Simultaneous Sensory and Chemical Analysis

The implementation of simultaneous chemical and sensory analyses is the modern approach to investigating the odors, tastes, and visual appearance of chemical compounds in biological samples. Based on their detection mechanisms, these systems can be classified into several categories, including chemical sensors, biosensors, GC-based systems, MS-based detectors, and hybrid GC/chemical sensors. Specifically, electronic noses‘(e-noses‘), multidimensional gas chromatography-mass spectrometry-olfactometry (md-GC-MS-O), electronic tongues‘, and visual analyzers are a few types of biosensory technologies available for the characterization of biological compounds. The reaction between odor molecules and the target sensing materials on the sensor surface triggers changes in mass, volume, or other physical properties. This reaction is then converted to an electronic signal by a transducer.

Widely used types of transducers include optical, electrochemical, heat-sensitive, and mass-sensitive. Some common chemical sensors are: surface acoustic wave sensor, quartz crystal microbalance sensor, metal oxide semiconductor sensor, and polymer composite-based sensor. An e-nose is an instrument that is designed to mimic the function of the natural nose. By definition, it uses a sensor array to not only detect but also discriminate among complex odors [71, 78, 79].

The ideal example for the detection of odors is the mammalian nose because of its ability to evaluate with both high sensitivity and specificity. Olfactory receptors make these properties possible, as they support combinatorial detection of odors at trace levels (e.g., 10−7 to 10−11 M in humans) [80, 81]. Exhaustive efforts have been devoted to exploiting these receptors in association with some electronic devices to develop biosensors that truly mimic biological noses [82–85].

The detection mechanism of these biosensors is based on the specific interaction between olfactory receptors and odorant molecules. Biosensors have been known to demonstrate better detection selectivity than chemical sensors. The bio-sniffer‘ is another example of a type of biosensor developed for VOC detection that is based on biochemical reactions between a biomolecule and a VOC, or a chemical reaction catalyzed by biomolecules [86,87].

MD-GC-MS-O is capable of removing the interference effect from non-target components. This system allows the users to separate components of interest, identify character defining compounds, and identify those components using modern mass spectral techniques [51, 88–94]. MD-GC-MS-O allows for the simultaneous analysis of compounds with the human nose as an odor detector and the MS as the chemical analyzer [93, 94]. Specifically, the mdGC-MS-O is used in the identification and characterization of VOCs and semi-VOCs in a variety of biological systems.

A few examples of research that have been performed using MD-GC-MS-O and simultaneous chemical and odor identification are: identification of compounds responsible for the characteristic odor of livestock and poultry manure and rumen of beef cattle; association of a specific odor with a volatile compound; the role of particulate matter as a carrier of odor; characterization of kairomones and characteristic odorants released by insects; and quantification of nutraceuticals in wine [51, 89–98].

This analytical tool is a state-of-the-art technology that is particularly suited for identification of chemical-odor association. This instrument can be used to explain the association between VOCs and their odors in wild mammal secretions and excretions. MD-GC-MS-O is capable of determining the concentrations of these compounds and evaluating the intensity and aroma of the odors of the entire scent-mark. Identification of compounds

responsible for specific odors and signaling could aid wild mammal conservation, and it would serve in giving some insight into how and why animals are detecting these scents.

Methodology of the Literature Review

Articles were obtained through searches on Science Direct, Academic Search Premier (EBSCO), and Google Scholar article databases. Keywords and phrases that were used in the searches included: conservation, GC-MS, GC-MS-O, gas chromatography, chromatography, endangered species, odor, chemosensory, simultaneous chemical and odor analysis, panthera, elephas, odocoileus, TAARs, olfactory receptors, scent-marks, urine, feces, mammals, scent-marking, conservation, animals, volatile organic compounds, sample preparation, analytical techniques, large mammals, pheromones, and marking fluid. Articles selected for this review focused on the use of modern analytical techniques to identify and/or quantify chemical compounds detected in scent-markings of large wild mammals and great cats for the purpose of sensory and chemical identification, conservation, behavioral understanding, and evaluation of sampling and sample preparation effectiveness.

Citations from the initial search were downloaded into EndNote, a reference management database. Duplicate citations were removed. Assessment of the identified studies for relevance was based on a standardized criterion developed by all co-authors: (1) the focal animal reported was a large wild mammal; (2) analytical techniques were utilized for chemical identification of scent marks; (3) sample preparation was defined; (4) the articles were peer-reviewed; (5) if sensory analysis was performed the method needed to be clearly defined; and (6) the co-authors had no objections, such as quality or topic focus of the articles.

If any of the five criteria were not met, the reference was omitted. For articles that remained in the review after applicability and quality selection, data were summarized and

reported. Data extraction from these articles was completed by one reviewer and when uncertain this reviewer consulted with the other authors. Data extracted from the research articles included: (1) sample preparation technique; (2) analytical methods; (3) animal species; (4) sensory analysis approach; (5) relationship to conservation; and (6) scent-markings being collected. Conclusions were based on a summary of the data.

Results and Discussion

Chemical and Sensory Characterization of Scent-Markings in Wild Mammals.

Sampling and Sample Preparation

This section summarizes sampling and sample preparation methods performed for the analysis of scent-markings of large mammals. It discusses solvent-free and solvent-based extraction methods and the advantages and disadvantages of these methods. The sampling and sample preparation section also explains the similarities and differences between the uses of various techniques for the identification of chemical constituents in scent-markings.

Solvent-free Extraction

Solvent-free extraction methods often reduce sample preparation time and eliminate multiple step procedures for the extraction of a component from a sample. Conventional solvent-free extraction methods implemented for wild mammal scent-marking characterization included: headspace extraction, direct injection, precolumn heaters, solid phase extraction (SPE), stir bar absorptive extraction (SBSE), and solid phase microextraction (SPME). Headspace extraction is the process of transferring a substance from a solid or liquid matrix to the vapor phase by heating, and removing analytes from the headspace in a carrier gas [99]. Direct injection is the direct insertion of an aqueous solution or aqueous extract from a sample matrix onto a GC column [100]. The precolumn heater (PH) technique is a solvent-free method to collect volatile

compounds. It consists of a glass cylinder heated to 100 °C with N2 being released simultaneously and driving the volatile material into a needle at the end of the cylinder [101,102]. SPE is performed by adding the test solution or solvents through a sorbent which is packed in a column and separation of both phases then occurs [103]. SPDE has been used to identify sulphur-containing hermiterpenoids responsible for the unique odor of maned wolves (*Chrysocyn brachurus*), when SPME was ineffective [104]. SPME is a combined sampling and sample preparation method that utilizes a fused-silica fiber coated with a thin polymeric film to passively diffuse compounds in a sample onto the SPME fiber via adsorption, absorption or capillary condensation [52]. In some cases, SPME extracts and collects samples from various environments without additional preparation before analytical separation [52, 92].

Headspace extraction results in the emissions of volatile compounds to the headspace, and thus provides some information about the fate of semiochemicals based on their physicochemical properties. This is particularly important when providing evidence of an animal‘s ability to identify compounds in the air from extreme distances. These volatile compounds are essential to our comprehension of animal communication. Headspace autosampling extraction of gases emitted from urine can provide information on compounds potentially detected by passing animals, specifically lions [59]. Headspace extraction can reduce sample preparation time and reduce impurities associated with solid or liquid matrix of a sample [49]. Reference [105] performed adequate headspace extraction on Asian elephant (*Elephas maximus)* blood volatiles in 35 min in comparison to other lengthier procedures.

VOCs in sternal secretions from koalas (*Phascolarctos cinereus*) were analyzed using a solvent-free technique [106]. The sternal secretions were collected and pipetted onto filter paper without solvents or additional extraction techniques. This extraction method was inexpensive,

rapid, and helped to find three additional nitriles (isobutyronitrile, 2-methyl-, and 3-methylbutyronitrile) suggested to be involved in odor cues, but never before detected [106].

The PH technique allowed for the identification of compounds in the interdigital glands of reindeer (*Rangifer tarandus)* [101,102] and was used to identify a recognition scent in the tarsal glands of male black-tailed deer (*Odocoileus hemionus columbianus*) and reindeer. This scent is recognized through tugging and licking the tarsal gland and is used to identify individuals by the scent associated with them [107]. The chemical responsible for the scent is cis-4-hydroxydodec-6-enoic acid lactone.

Solid phase dynamic extraction (SPDE) is an extraction process that can be utilized at ambient room temperature to extract semi-VOCs. When coupled with an automated sampling system that can regulate temperature, a higher number of volatile compounds can be extracted. Using a SPDE needle internally coated with a modified activated charcoal-polydimethylsiloxane (AC-PDMS) allowed for a small sample size of 0.5 mL of *Strepsirrhini* urine for characterization. This urine characterization led to the phylogenetic construction of the *Strepsirrhini* suborder [45]. Utilizing SPDE reduced the extraction time in comparison to a solvent-based procedure [45].

Stir bar absorptive extraction (SBSE) techniques have been advantageous in measuring small sample sizes and diluted media [108]. Volatile and semivolatile substances from aqueous and gaseous media have been extracted using a polymer-coated magnetic bar (Twister TM) [108–110]. The polydimethylsiloxane (PDMS) coating on the stir bar and constant stirring agitation allows for a more precise and reliable extraction, and decent analytical precision [108]. In SBSE, generally the phase volume is between 24 and 100 µl, exceeding the solid phase microextraction technique which is typically 0.5 µl. A few studies have utilized SBSE in the

detection of 26 volatile compounds of preputial glands of rodents [108,111]. Nonanol, benzaladehyde, several ketones, pyrazines, sulfur compounds, and heptanones have been reported as volatile characteristic compounds in mammal species using SBSE [108,111].

Solid phase microextraction (SPME) is particularly suited for characterization of volatiles from biota. SPME can be used for *in vivo* extractions of volatiles. SPME is a solventless extraction technology that incorporates fibers of assorted coatings and a fiber holder (Figures 2 and 3) that is either directly (e.g., by submersion in liquid) or indirectly (e.g., headspace) exposed to a sample. Different fiber coatings (Figure 3) can be used to optimize the type of compounds to be extracted from the sample. Volatiles and semi-VOCs passively diffuse onto the SPME fiber via adsorption, absorption or capillary condensation. SPME fiber coatings have very high affinity for VOCs and semi-VOCs [53].

Thus, the sampling results in high preconcentration and enrichment of compounds that did not require use of solvents and additional steps. Specific SPME coatings can be used for optimization of extraction processes favoring certain groups of compounds varying by MW, polarity, and functional groups. Often fibers with Carboxen polydimethylsiloxane (Car-PDMS) coating are used for the detection of VOCs with low MW. Divinylbenzene/Carboxen/PDMS coating is used on a broad range of analytes, specifically volatile and/or semi-volatile compounds. SPME combines sampling and sample preparation to minimize the sample preparation step with a process that is simple, reusable and efficient.

There are relatively few publications that report the use of SPME for characterization of scent- markings of large wild mammals [44,90], However, SPME has its strengths and challenges in regard to sampling, sampling preparation, and analysis of biological samples.

SPME has been found to be effective in the analysis of trace levels of analytes in the urine of Strepsirrhine families leading to a more exact characterization [112].

Automating headspace extraction with SPME was useful and a non-invasive method for monitoring reproductive status via the urine in elephants and other species [105]. African elephant (*Loxodonta africana)* urine analyzed with SPME used a chiral column to detect the pheromone, frontalin [44]. When SPDE and GC-MS analysis was performed with headspace extraction, however, it made the number of steps in the sample preparation and analysis of maned wolf urine diminutive in comparison to solvent-based techniques [104].

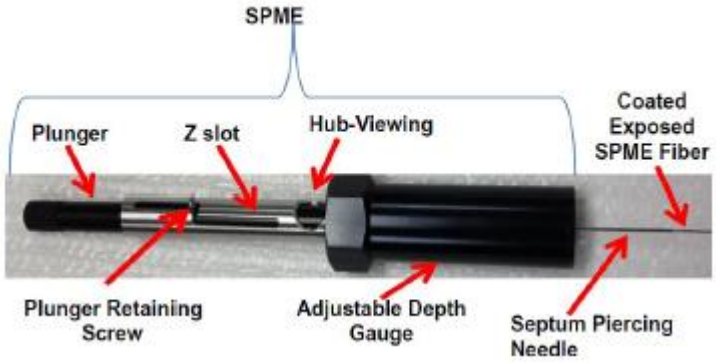


Figure 2. A manual SPME holder. SPME can be also used with any mainline autosampler for automated sample preparation.

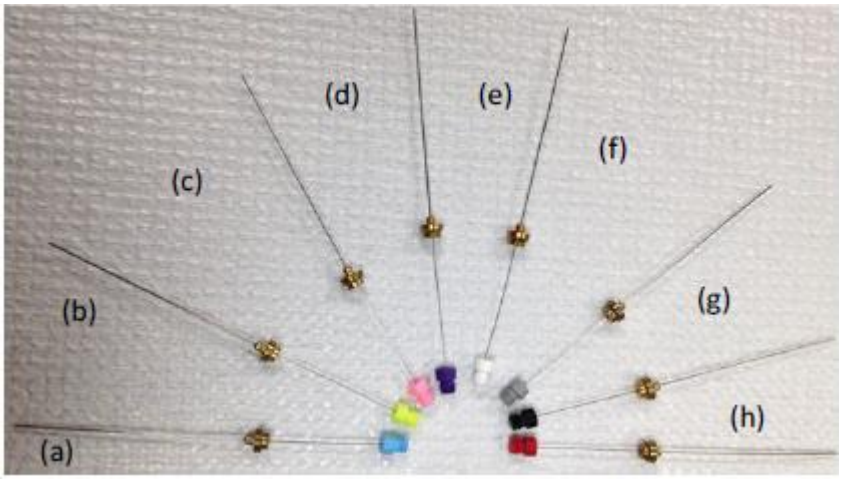


Figure 3. A variety of solid-phase microextraction fibers with different coatings used for the identification of non-polar and polar compounds, volatile odorous compounds, and/or compounds of different molecular weights: (**a**) 85 μm PDMS (**b**) 70 μm Carbowax/divinylbenzene (CW/DVB) (**c**) 65 μm PDMS/DVB (**d**) 50 μm CW/templated resin (**e**) 85 μm polyacrylic (**f**) 50/30 μm DVB/Carboxen/PDMS (**g**) 75 μm Carboxen/PDMS (**h**) 100 μm PDMS.

The use of ultrasound as a tool for compound separation has proven to be less effective than SPME. In the case of giant panda (*Ailuropoda melanoleuca*), ultrasound was used for 15 min to separate anogenital gland secretions from tampons [113]. The extract was then left to settle for 5 h resulting in 5 less VOCs in anogenital gland secretions than previous studies using SPME [113,114]. In the analysis of tiger urine and marking fluid, the use of headspace sampling with a _sample enrichment probe‘ containing a 28 mg PDMS rubber, reduced solvent preparation time and was possibly two orders of magnitude more efficient than SPME in general practice, dependent upon application [47,115]. The volume of the coating of an extraction fiber whether SPME or sample enrichment probe (SEP) determines the level of sensitivity and rate of extraction from a sample matrix [34]. In comparison to SPME the volume of the coating and extraction surface area of an SEP PDMS rubber is larger, potentially resulting in superior extraction efficiency.

Solvent-based Extraction

Territory and recognition scents are difficult to categorize because the scent may indicate territorial boundaries, social status, or individual animals, or incorporating all three factors [7]. Social status information is often associated with urination. To date, the majority of mammal urine extractions are accomplished via solvent-based extractions. Solvent-based extractions generally require a series of procedures and are time consuming.

Multiple bioassays and fractionation processes made the methods for detection of cycle stage, parturition, and estrous of elephants an extensive procedure [116].

Methanol extraction of koala sternal gland secretions required upwards of 8 hours [117]. The extraction process for black buck (*Antelope cervicapra)* urine used dichloromethane as the solvent and liquid N_2 to condense the extracted sample. This resulted in a total sample preparation time that was less than 1 h [118]. Solvent-based methods may have an impact on the chemical composition of a sample due to the interactions of chemicals within the scent mark and the solvent (or solvent impurities) used to extract the compounds of interest. The addition of methanol after sample collection and chloroform during tiger urine sample preparation, may have altered the results [31].

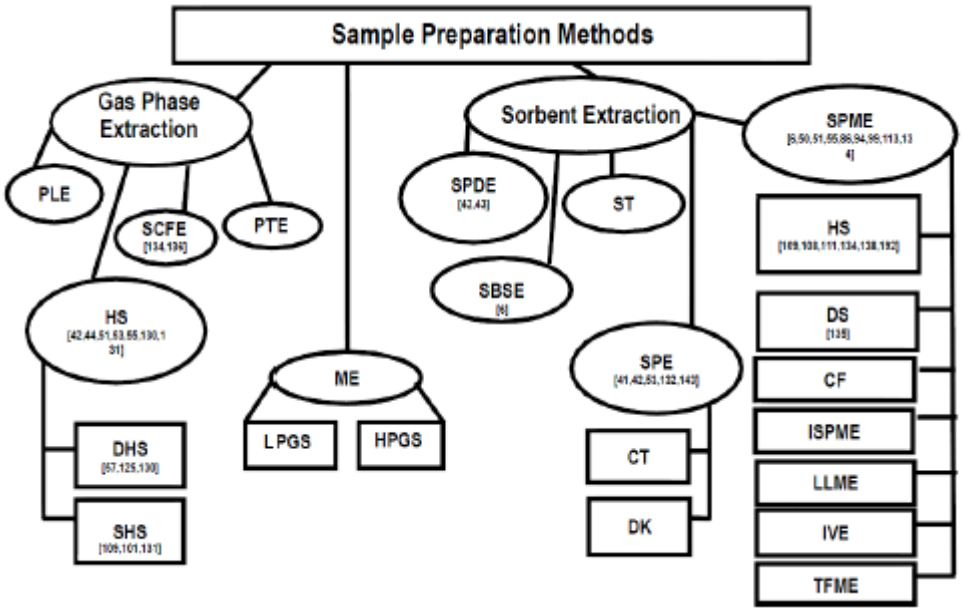
Summary of sampling and sample preparation techniques with references used for the chemical and sensory characterization of scent-markings in wild mammals is presented in (Figure 4). To date, the most frequently used sampling and sample preparation methods are: (1) solid-phase microextraction/headspace extraction; (2) solid-phase dynamic extraction; (3) static headspace extraction; and (4) solid-phase extraction.

It appears that in the last decade there has been a rise in the implementation of SPME for the sample preparation and sampling of scent-marks (Figure 4). This increase in SPME use may be due to the fact that it does not require the use of a solvent, can reduce sampling and sample preparation time by combining the two procedures, is very transportable for field analysis, and is highly efficient in extracting compounds of interest from biological samples [119].

Chemical Analysis

Research in chemical signaling plays an important role in the conservation of many endangered large animals. This section summarizes analytical methods performed for the analysis of scent- markings of large mammals.

The use of various GC- and high performance liquid chromatography (HPLC)-based techniques with an assortment of detectors is summarized with the advantages and disadvantages of each method.



* *Abbreviations*: SPDE-Solid Phase Dynamic Extraction; SPME-Solid Phase Microextraction; SPE-Solid Phase Extraction; ME-Membrane Extraction; PLE-Pressurized liquid Extraction; SCFE-Super-Critical-Fluid Extraction; PTE-Purge-and-Trap Extraction; SBSE-Stir Bar Sorptive Extraction; ISPME-In-tube Solid Phase Microextraction; ST-Sorbent Trap; LLME-Liquid-Liquid Microextraction; IVME-In Vivo Extraction; HPGS-High-Pressure; LPGS-Low-Pressure Gas Stripping; CF-Cold Fiber; DS-Direct Sampling; HS-Headspace; SHS-Static Headspace; DHS-Dynamic Headspace and TFME- Thin-film Microextraction; DK-Disk; CT-Cartridge

Figure 4. Summary of sampling preparation techniques with references used for chemical and sensory characterization of scent-markings in wild animals.

Gas Chromatography

Gas chromatography (GC) is a very useful analytical technique for the analysis of mammal scent- markings (Table 1). The use of GC resulted in finding high proportions of steroids and other chemicals that were not previously reported in gray wolf (*Canis lupus)* urine and feces volatiles [120]. Another example of the good utility of GC was reported in its use to characterize VOCs in human biological secretions and excretions. GC was fairly good at reproducibility in analyzing human urine, breath, and blood [46].

GC combined with a detector allows for the identification of compounds within the sample. The most commonly used detectors were: MS, FID, and FT-IR. MS was the most widely used because of its capability to perform a spectral search and match for over 200,000 compounds within its spectral library. Also, MS detection was preferred with GC analysis because of its compound

identification abilities and sensitivity [121,122]. The GC-MS spectral library comparison made chemical identification of Strepsirrhine families' urine uncomplicated [45,123].

Table 1. Summary of findings and knowledge gaps in the area of sample preparation and analysis techniques used to analyze large mammal scent-markings.

Sample Preparation Technique	**Chemical Analysis**	**Species**	**Type of Marking**	**Major Findings**	**Identified Needs/Gaps of Knowledge**
Solvent-based extraction [62]	MALDI-ToF MS; ESI-MS; ESI-MS/MS [62]	Lion *(P. leo persica);* Tiger *(P. tigris sumatrae);* Persian Leopard *(P. pardus saxicolor);* Snow leopard *(P. uncia);* Clouded leopard *(N. nebulosa)*	Urine	Cauxin was present in the urine of male cats; Intensity of cauxin in big cats was lower than domestic cats; Sequence in serum albumin signifies the relatedness of cat species; Felinine and its degradation products are putative pheromones	The exact role of cauxin as a catalyst in the conversion of dipeptide 3-methylbutanol-cysteinylglycine to glycine and felinine
Solvent-based extraction [41] SPME [124]	GC-FID, TLC [41] GC-MS [124]	Cheetah *(Acinonyx jubatus)*	Marking Fluid, Urine	3.87 ± 0.58 mg/ml total lipid extracted from cheetah MF; Composed of free fatty acids; Lipids have limited fixative property; Pantolactone found in urine	Development of analytical techniques should be performed for chemical i.d. of total marking fluid composition
Solvent-based extraction [118]	GC-MS [118]	Blackbuck *(Antelope cervicapra)*	Urine	28 major constituents were identified in the urine of all males; Three compounds were seen only in dominant males during the dominance hierarchy period	Functional role of compounds is needed to determine the role of compounds in social communication
SPME [124]	GC-MS, GC-PFPD, GC-FID [124]	African wild dog *(Lycaon pictus)*	Urine, Feces, Anal glands, Preputial glands	103 organic compounds detected; Squalene is a major component of urine, feces, anal gland; 11 compounds were species specific	Analytical methods not efficient in determination of chirality of identified compounds or positions of double bonds in unsaturated acids

Table 1 Continued

Solvent-based extraction [107,125,126]	GC [107], GLC-FID [125,126]	Black-tailed deer *(Odocoileus hemionus columbians)*	Interdigital scent, Tarsal scent	Tarsal gland plays a role in sexual isolation between deer subspecies; 5 unsaturated lactones elicit licking behavior, excitement	Identification of specific odor profiles of the scent marks responsible for eliciting behaviors using GC
Solvent-based extraction [113,127] SPME [114]	GC–MS [113,114], HPLC [127]	Giant panda *(Ailuropoda melanoleuca)*	Anogenital gland secretions, Urine, Feces, Blood serum	Anogenital secretions composed of steroids, fatty acids, aldehydes, alkanes, alkenes, amines, terpenes, and furans; Glucocorticoid hormonal levels rise during mating season	Behavioral bioassay is needed to unveil how these compounds mediate synchronization of breeding
Solvent-based extraction [128]; Headspace sampling [18]	GC-MS [128], GC [128]	White-tailed deer *(Odocoileus virginianus)*	Tarsal scent	Characterized 63 compounds in females and 55 in males; Alcohols, aldehydes, alkanes, alkenes, amines, ethers, furans, and ketones occurred in the urine of either sex	Additional chemical analyses and behavioral bioassays for screening of biologically important compounds
Solvent-based extraction [31,33,129]; SEP [47]	GC-MS [33,47], GC [47], TLC [31], GLC [31,33,129], GC-FID [31,129]	Bengal tiger *(Panthera tigris tigris)*	Marking Fluid, Urine	Average lipid content of MF is 1.88 ± 0.75 mg/moL; 98 volatile compounds confirmed including ketones, fatty acids, lactones	Quantitative derivatization of major unsaturated compounds; Confirmation of 2-Acetyl-1-pyrroline for odor characterization
Solvent-based extraction [130]; Headspace autosampling [59] SPME[124]	GC-MS [59,124,130]	Lion *(Panthera leo)*	Marking Fluid, Urine	55 compounds i.d. and 7 are potentially species specific; Males‘ markings more similar than females; Males have higher levels of 2-butanone and females have higher concentrations of acetone; Pantolactone found in urine	Only samples with lipid confirmation were analyzed for composition, limiting the results

Table 1 Continued

Solvent-based extraction [123,131], SPME [112], SPDE [45]	GC-MS [45,123], GC [112], GC-FID [131]	Strepsirrhine families	Urine	Acetone, 2-hexanone, 4-heptanone and 2-heptanone have a primal role in communication	Relationship between social and solitary species scent-markings; Quantitative differences between scent-markings of lemurs between seasons
Solvent-based extraction [132], Headspace sampling [132–134]	GC-MS [132,133], GC-FID [133], GC-FTLR [133], Reversed-phase HPLC[133]	Gray wolf *(Canis lupus)*	Feces, Urine	77 compounds in feces of adult wolves; Aromatic organic compounds, steroids, carboxylic acids, aldehydes, alcohols, squalene and α-tocopherol	Understanding of variations in chemicals related to sex, reproductive season, or social status
Solvent-based extraction [106,117], No-treatment [106,117]	GC-MS [106,117]	Koala *(Phascolarctos cinereus)*	Sternal gland secretion	Volatile fatty acids, aldehydes, ketones, mono- and sesquiterpenes were identified; Some volatile nitriles and oximes i.d. never determined in any mammalian skin gland	Incorporation of scent and chemical analysis to understand influence of age on marking detection and composition
Solvent-based extraction, micro-preparative GC [135]	GC-MS, GC-FID [135]	Brown-mantled tamarin *(Saguinus fusciollis)*	Scent mark	17 compounds responsible for the composition of marmoset scent-markings; 3 dienes, 1 squalene, 8 monoenes, 5 saturated compounds	Compounds at 0.01% concentration were omitted from analysis, possibly affecting the true total composition
Solvent-based extraction [116], Headspace extraction [105], SPME [55], SPE [42,55,136]	Radioimmuno Assay [105], GC-FID [105], GC-MS [105,116], GC [55], MALDI/TOF-MS [42], PAGE/electroblotting [42], MRS [116]	Asian elephant *(Elephas maximus)*	Urine	Combined headspace SPME and GC-MS determined 5alpha-androst-2-en-17beta-ol and -17-one to determine start of estrous and predict the period of parturition; 5 -androst-3 -ol-17-one and probably 5 -androst-3 -ol-17 -ol are generated from sulfate conjugates by a thermal process;	Influences of environmental, hormonal, and genetic factors of musth are unknown

Table 1 Continued

				Follicular LH2 identified as a preovulatory hormone in female elephants	
Solvent-based extraction [46,137], SPME [46,137,138], SFE [139], SDE [139], SWE [139]	GC[138], GC-MS [46,137–139], GC x GC, GC-MS-O [138]	Human (*Homo sapiens*)	Urine, Feces, Sweat, hand scent	The use of NaCl and KCl improved the extraction efficiencies of VOCs from urine, with NaCl being optimal	Additional qualitative and quantitative comparison of VOC profiles of multiple specimen samples collected simultaneously from individuals
Solvent-based [140]	GC, GC-MS, NMR [140]	American beaver (*Castor Canadensis*)	Castor sacs	5 phenolic compounds identified; 15 phenolic compounds previously identified in prior studies	Detection methods may have prohibited the confirmation of 10 phenolic compounds previously detected with TLC
SPME [124,141]	GC-MS [124,141]	Spotted hyena (*Crocuta crocuta*)	Feces	252 volatile compounds detected; Composition of scent marks indicate social status; Pantolactone found in feces	Use of GC-MS to measure the energy cost associated w/ specific compounds in scent marks
SPDE, SPME [44] CHS, IFE [142]	GC-FID, GC-MS [44,142]	African elephant (*Loxodonta africana*)	Urine	Frontalin pheromone was found in elephant urine; endo- and exo-brevicomin, similar to frontalin, are also beetle pheromones; IFE and CHS headspace methods were equally significantly effective in detecting ketones and acids	Continued investigation of optimal extraction method for chiral columns
Precolumn heater technique [101,102]	GC-MS [101,102]	Reindeer (*Rangifer tarandus*)	Tarsal scent gland, Interdigital gland	Two of the major constituents have been identified as 1-hydroxy-7-methyl-3-octanone and 7-methyl-1-octen-3-one	Relationship between season and scent- marking concentrations

Table 1 Continued

Precolumn heater technique [143]	GC-MS [143]	Bobcat (*Lynx rufus*)	Urine	Identified sulfide, disulfide, and trisulfide compounds	Further field studies on the role of dichloromethane in urine as an animal deterrent
Acid/steam distillation [144]	GC-MS [144]	Horse (*Equine caballus*)	Urine, feces, urine-marked feces	Fatty acids, alcohols, aldehydes, phenols, amines alkanes, tetradecanoic and hexadecanoic acids in feces differed based on maturity, sex, and reproductive stage	Lack of Chemosensory analyses could suggest role of marking cresol by stallions in masking mare feces odor.

* *Abbreviations*: GC/FTIR- gas chromatography/Fourier-transform infrared spectroscopy; RT-retention time, MALDI-TOF-MS matrix-assisted laser desorption ionization time of flight mass spectrometry; ESI-MS- electrospray ionization mass spectrometry; ESI-MS/MS-tandem mass spectrometry; GC-gas chromatography; VOC-volatile organic compounds; SPDE-solid phase dynamic extraction; AC-PDMS- activated charcoal (Carboxen)-polydimethylsiloxane; GLC-gas liquid chromatography, MRS-magnetic resonance spectroscopy; SEP-sample enrichment probe; SDE-simultaneous distillation-extraction; SWE-subcritical water extraction; SFE-supercritical fluid extraction; NMR-nuclear magnetic resonance; GC-PFPD-gas chromatography-pulsed flame photometric detector; CHS-contained headspace; IFE-Inverted funnel extraction; LH2-leutenizing hormone in luteal urine.

While GC-MS is a well-established and often preferred technology for detecting volatile compounds with MW below 300, it is not ideal for the detection of higher MW compounds [113,118]. The use of GC-MS resulted in the detection of low MW and nonvolatile compounds of giant panda (*Ailuropoda melanoleuca*) anogenital gland secretions, urine, feces, and blood serum [113]; all of which were not readily detected by HPLC [127].

In the case of urine from gray wolves, notable peaks from the GC were identified through matching GC retention times and MS spectral patterns [133]. The use of GC-MS for the extraction of aromatic compounds in urine and feces of *gray wolves* was deemed efficient [132]. SPME-GC-MS combined with GC-Pulsed Flame Photometric Detector dichloromethane extracts coupled with GC-FID resulted in the identification of 103 compounds in urine, feces, and anal gland secretions of African wild dogs *(Lycaon pictus)*. Out of all of the 11 species-specific compounds, 8 were confirmed. The confirmed compounds were: 1,3-propandiol, N,N-dimethylacetamide, 1-methyl-2,4-imidazolidinedione, 1-methylimidazole-5-carbox-aldehyde, and quinazoline. The aforementioned

compounds were at three times the level in urine than feces [124]. This analytical method, although beneficial, was lacking in its ability to conclude chirality issues with identified compounds and the position of double bonds in unsaturated acids.

Although GC is the modern system for separations and chemical composition determination, the use of variable detectors, in conjunction with the GC, may impact the ability to quantify or qualitatively define scent-markings. While GC-MS analysis allowed for quantification of the compounds in the scent-markings of brown-mantled tamarin *(Saguinus fusciollis)*, compounds with concentration levels of 0.01% were omitted from analysis, possibly excluding the incorporation of specific pheromone or semiochemicals that are essential in animal communication but present in very low abundance [135]. The use of GC-MS [118] resulted in detecting volatile compounds in black buck urine that had a MW of less than 300. White-tailed deer urinary lactone, (Z)-6-dodecen-4-olide, previously found in the tarsal gland of deer were not detected via GC-MS [128].

In addition, nondistillable compounds in the tarsal gland were also not identified through GC-MS detection [18]. In the case of bobcats (*Lynx rufus)*, MS and retention time identification allowed for first time confirmation of compounds in urine [143]. Nevertheless, the combination of the two methods of detection provided a true confirmation and multiple assessments of urinous compounds.

GC-based analyses had some additional drawbacks such as sample dehydration/alteration. Dehydration was observed when characterizing koala sternal gland secretions [106], *i.e.*, dehydration of the oximes occurred during the desorption of the swab in the GC injection port. In the identification of castoreum composition in the American beaver (*Castor canadensis*), GC analysis may have impacted the analysis of highly volatile phenol constituents [140]. Previous studies used alcohol and additional basic materials with fractionation for extraction and alumina chromatography for analysis. Using this method, cis-Cyclohexane-1,2-Diol was identified in beaver castor sacs [145]. GC-FID is highly efficient in the quantification of chemical compounds. GC-FID in combination with GC-MS has been efficient in the identification of 103 compounds in African wild dogs. It has

been suggested, however, that nonvolatile compounds in urine of Strepsirrhine families may not be detected via GC-FID [131]. The interdigital and tarsal scent compounds of black-tailed deer were identified through retention time and not with a mass spectral library database because gas liquid chromatography-flame ionization detector (GLC-FID) and GC were employed [107,125,126].

Elephants have been a major focal animal in the area of scent-marking and its role in reproduction and socialization. They have been used to understand how scent-marking impacts mating and interaction of males and females of various ages and social levels within herds [136,142,146,147]. Male and female African elephants have developmental differences in chemosensory signal processing [148]. The exhibition of musth pheromone (frontalin) released by male elephants has been known to elicit female sexual responses to the male [136]. The use of SPDE and SPME in conjunction with chiral column GC-FID and GC-MS were useful in the detection of frontalin [44]. Ketones such as 2-butanone, acetone, 2-pentanone, and 2-nonanone have been quantified using GC-MS and showed elevated levels during all periods of musth [142]. A series of alkan-2-ones and alkan-2-ols were identified in the urine of African elephants using GC-MS [146]. It was suggested that after performing analysis that GC-MS could serve as _time-release chemical signals to conspecifics [36,149].

For several chemical component identifications, a combination of capillary GC with Fourier-transformed infrared spectroscopy FTIR was essential for accurate identification of *gray wolf*' urine and feces volatiles [133]. MALDI has been used for the confirmation of the precursor pheromone felinine in the urine of domestic cats [61].

Sensory Analysis

A three step process is needed to fully comprehend the role of cues in scent-markings in animal behavior. First, an understanding of which chemical constituents constitute the marking must be determined. Next, an odor characterization of these specific compounds must be performed. Lastly, a behavioral analysis of how the animal reacts to these specific odorous compounds to

determine the relationship between behavior and scent must be completed. Without the input of sensory analysis, the interpretation of cues in scent-markings can be limited. The use of the human nose for sensory analyses, as opposed to the use of animal olfactory sensing further complicates this process. This section summarizes the limited information available on the use of chemical and sensory analysis for the characterization of large mammal scent-markings (Table 2).

Table 2. Summary of simultaneous sensory and chemical analysis of scent-markings from endangered large mammals.

Species	**Aim**	**Type of Marking/ Sample**	**Chemical/ Sensory Analysis**	**Findings**	**Identified Needs/Gaps of Knowledge**	**Advantages/ Disadvantages**
Lemur catta [131]	Demonstrate individual recognition of female genital marking in *Lemur catta*	Genital marking	GC-FID, Lemur olfaction	Only females have recognizable scent-markings	Further experiments on the occurrence of individual recognition	**Dis**- Animals showed a high variability in their motivation to investigate markings
Elephas maximus [44,146]	Review the response behavior by elephants to interpret chemical detection and ratio of enantiomers of frontalin based on sex, age, and stage of musth	Musth, Urine	GC-MS, Elephant olfaction	Compounds in urine and musth responsible for transport and behavior; Musth varies w/age and stage of Musth and/or frolatin component; Chirality in pheromones	Lack of information on pheromone variation over time of year and region; The interactions of pheromones with receptor proteins	**Adv**- SPE unlike headspace analysis, does not require the solute to be volatile to be extracted; **Dis-** Sample size of 6 males
Homo Sapiens [138,150]	Summarize the current knowledge on chemical and clinical aspects of body-derived VOCs.	Sweat, Urine, Feces, Breath	GC, GC-MS, GC x GC, GC-MS-O, E-noses	VOCs emitted from the body vary with age, diet, sex, physiological status and genetics	Minimal research on VOC diagnostic criteria for disease	**Adv**-GC-MS-O identified characteristic odorous VOCs that are in low abundance in various biological samples
Various Vertebrate and Invertebrate Species [151]	Review the history and developments in the area of olfactory biosensors that detect	Sub-tissue, Whole organisms	EOG, E-noses, SPR, FRET, SAW, FET, QCM	The ability to detect volatile compounds w/ the same specificity as nature‘s olfactory machinery is	SWCNT-based platforms will aid in developing a portable apparatus for olfaction in 10yrs	**Adv**- ORs in biosensors are more sensitive detectors of ligands than GC-MS and chemical noses; E-noses are

Table 2 Continued

	volatile compounds			applicable in environmental studies		**real-time methods; Dis-EOG provides no information about or molecular basis of olfaction w/o molecular analysis; Luminescence optical assays have low detection limits; E-noses lack biorecognition stability and portability**

Electronic/Chemical

GC-MS were able to generalize all compounds in spotted hyena *(Crocuta crocuta)* as being responsible for eliciting behavioral responses without detecting specific odorous compounds [141]. This study measured concentrations of VOCs from animals believed to be of different social status and age without the use of olfactometry. These results limit the amount of information associated with the odors that are being detected by the animal. An electronic-nose (E-nose) indicated that VOCs emitted from the body vary with age, diet, sex, physiological status and genetics (Table 2). The main findings in reference [151] are that electro-olfactograms and E-noses can act with the same specificity as the human nose in the detection of volatile compounds and may be applicable in environmental studies.

Animal Detection

Animals are frequently the objects of sensory evaluation (Table 2). Gray wolves return to their territory boundaries every three weeks to re-mark with various scent-markings, which are below detection level after 23 days, to counter the effects of the environment [152]. The detection of these markings is dependent upon how long the compounds in the marking remain odorous. The use of conspecifics, however, to detect olfactory changes in the scent marks of other brown-mantled tamarin made it impossible to qualitatively measure changes [135].

Odor detection thresholds for humans are different for each chemical (i.e., high concentration of virtually odorless compounds does not elicit any response). The same principle is thought to apply in wild mammals. In complex mixtures of scent-markings reside distinct odorous compounds responsible for the longevity of its scent availability. An example of a compound that constitutes a large mammal scent-marking is cyclohexanone. Cyclohexanone elicits flehmen responses from sub-dominant females, but in males there is no response [105]. Elephant detection of cyclohexanone in musth has led scientists to suspect that some musth signal messages in elephants may be single compounds [105]. In the case of cyclohexanone, with a boiling point of 161 °C and a slow volatilization period of hours is responsible for a relatively longer lasting signal than compounds of lower MWs.

Persistence of scent-markings in the environment has been recorded at a wide variety of lengths. In the case of dominant male mice, urine has been avoided by other males for up to 72 h. Klipspringer antelope (*Oreotragus oreotragus*) have scent marks that remain active for as long as 7 days [153]. Scent marks disappear in dwarf mongooses (*Helogale parvula)* after 10 days and in hamsters (*Mesocricetus auratus),* for 100 days. Even humans, however, can detect scent from anal gland marks of hyenids after 1 to 6 months [5]. Humans have utilized nasal detection to survey snow leopard (*Panthera uncia)* territories and marking behaviors by differentiating the age of different urine and scat markings over a period of months. Frequency of marking coincided with the

winter/early spring mating season. This marking rate potentially serves to maintain awareness of conspecific presence and also distance between snow leopards [154].

Simultaneous Chemical and Sensory Analysis

Multi-dimensional-Gas Chromatography

Multi-dimensional-gas chromatography (MDGC) has previously been defined as, ―the process of selecting a (limited) region or zone of eluted compounds from the end of one GC column, subjecting the zone to a further GC displacement‖ [121]. Two-dimensional chromatography utilizes two independent GC ovens equipped with proper switching system and column setup. Separation in multi column chromatography occurs by using (a) two columns with different polarity which are connected in series where the whole sample is eluting from the first to the second column; (b) two columns with different polarity connected in series that satisfy the conditions of orthogonality (*GC*×*GC*) (in this instance the whole sample is eluted from the first column to the second column in some specific time frame); and (c) by using practices, where only a small part of the sample elutes to the second column either via backflash, foreflash, and heart-cut [155]. Backflash is a method, where the specific portions of the sample eluted from the second column were previously washed from the first column by switching the direction of carrier gas flow to the opposite direction [155]. Foreflash is used for the removal of remaining solvent, derivatization agent, or other additives [155]. Heart-cut allows the assignment of one or more fractions from the first dimension to the second dimension with a different polarity. Transferring of the sample to the second dimension is carried out by an on-line cutting, which allows transfer for only specific analytes [156].

A series of detectors can be used for two-dimensional GC: flame ionization detector (FID), electron capture detector (ECD), atomic emission detector (AED), nitrogen-phosphorus detector (NPD), and olfactory detector and mass spectrometer (MS) [157,158]. MDGC can be combined with olfactory analysis in the form of an MD-GC-MS-O for the purpose of simultaneous sensory and chemical analysis.

The characteristic or overall aroma of a sample is an intricate combination of various odorants. Simultaneous analyses can potentially identify links between certain scents and the exact chemical compounds causing them. Simultaneous chemical and sensory analyses have the potential of linking both chemical and sensory analyses that are often analyzed independently. MD-GC-MS-O can be described as a two-way split detection system. In this arrangement, compounds are quantitatively trapped in a capillary column loop, which isolates them online from preceding and following peaks, and splits the target region into the second column for effective resolution from interfering matrix compounds [159]; this allows for MS and/or olfactory analysis. A small split flow (~10%) to the MS detector achieves correct timing to ensure target trapping in the loop which must be sufficiently cool to retain the trapped compounds of the target region [160]. Multidimensional GC-MS was applied to sensory and chemical characterization of odorous gases of swine manure and isolation of *trans*-resveratrol in red wine [89–91, 96].

Simultaneous chemical and sensory analysis is very rarely performed in the area of wild large mammal scent-markings. The only instances of sensory analysis were the use of conspecifics after chemical identification [5, 42, 55, 131, 161]. GC-MS-O was used to identify characteristic odorous compounds that were in low abundance in a complex mixture of VOCs from various biological samples (urine, breath, feces, and sweat) in humans [135]. Early development of human breath sampling and analysis protocol for clinical settings began through the practice of GC-MS-O instrumentation [138]. GC-MS-O (Figure 5) has also been used to determine odorous compounds released by humans suffering from various illness, such as cancer [138].

It has been reported that olfactory receptors in biosensors are more sensitive detectors of ligands than GC-MS and chemical ―noses [151]. An E-nose is considered a real-time detection technology. This also means that it can be used side-by-side with another system such as a GC-MS. E-noses, however, lack biorecognition stability and portability.

Figure 5. Multi-dimensional gas chromatography-olfactometry system at Iowa State University.

Electro-olfactograms (EOG) are ―electrical potentials of the olfactory epithelium that occur in response to olfactory stimulation‖ [162]. EOGs are the sum of generator potentials of olfactory receptor neurons [162]. An electro-olfactogram does not provide information about, or molecular basis of, olfaction without molecular analysis. Another type of biosensor, luminescence optical assay, lacks the ability to detect compounds that do not have low detection limits. This limits the range of compounds it is capable of detecting.

Chemical and Sensory Characterization of Scent Markings in Great Cats

Great cat markings have been studied to aid in conservation, specifically focusing on territoriality, dominance, and reproduction (Table 3) [31, 33, 41, 59, 130, 163–165]. Great cats use scent-markings as a method for distinguishing amongst other conspecifics and neighbors, as territorial boundary markings, and as reproductive condition indicators.

Although there is limited information about the analysis of great cat scent marks, conclusions can be deduced and used to aid in conservation.

Table 3. Number/percentage of articles that focus on categorizing scent-marking behaviors in wild cats and their relationships to conservation.

Species	Behaviors Associated with Scent-Marking				Relationship to Conservation
	Reproduction	Territoriality	Dominance	Other	
Tiger (*Panthera tigris*)	(5) 23.8% [31,33,130,163,166,167]	(4) 19.04% [130,163,166,168]	(4) 19.04% [130, 169–171]	(8) 38.09% [62,68,129, 172–176]	·Implement better wildlife management practices ·Provide adequate land and resources ·Increase lifespan of captive and wild tigers ·Determine populations ·Understand chemosignalling ·Indicator of reproductive status, territory, and physical condition
Lion (*Panthera leo*)	(1) 9.09% [59]	(3) 27.27% [163,177,178]	(3) (27.27%) [170,171,179]	(4) 36.36% [62,174,175,180]	·Taxonomical separation and classification ·Sex and identification ·Understand chemosignalling
Puma (*Puma concolor*)	(2) 18.18% [181,182]	(6) 54.54% [70,183–187]	(1) 9.09% [70]	(2) 18.18% [174,185]	·Population assessments ·Territoriality ·Phylogenetic reconstruction
Snow leopards (*Panthera uncia)*	(2) 25.00% [154,184]	(3) 37.50% [154,164,186]	(0) 0.00%	(3) 37.50% [174,188,189]	·Population estimates ·Phylogenetic reconstruction ·Distribution
Cheetah (*Acinonyx jubatus*)	(1) 16.67% [41,190]	(2) 33.34% [41,191]	(1) 16.67% [41]	(2) 33.34% [41,174]	·Marking fluid is an indicator of physical condition ·Population estimates
Kalahari leopards *(Panthera pardus)*	(2) 25.00% [165,192]	(3) 37.50% [192,193]	(1) 12.50% [192]	(2) 25.00% [174,194]	·Population assessments ·Territoriality ·Phylogenetic classification ·Diet

3.5.1. Characterization of Great Cat Scent-Markings

Behavioral studies of free-ranging tigers have determined that marking functions to establish and maintain territorial boundaries and advertise female reproductive status [166] (Table 3). There has never been a study, however, that analyzed changes in scent-mark composition over the reproductive cycle of tigers. This would help to identify why these markings are presented with such frequency during proestrus. The main function of cats' sense of smell is to decipher their own scent marks from those of conspecifics, stimulate exploration, and to defend territories [195].

The focus of previous studies has been on identifying total compound composition, neglecting the study of olfaction's relationship to scent-mark identification by animals. Application

of MD-GC-MS-O has the potential to measure the influence of odor in scent-marking detection in species that use chemical cues as their communication method.

Scent-mark constituents and/or behaviors have been analyzed in snow leopards, puma, African cheetahs, Indian leopards (*Panthera pardus fusca*), and African lions (Table 3). Pumas, leopards, and cheetahs do not contain a lipid component in their marking fluid, unlike in tigers and lions [127]. 2-acetylfuran, acetaldehyde diethyl acetal, ethyl acetate, dimethyl sulfone, formanilide, urea, and elemental sulfur were identified in cheetah urine [6, 196]. It has been suggested that elemental sulfur may be a cheetah pheromone, however further research is required [6]. Scent-marking behavior and markings (feces) in snow leopards, pumas, cheetahs, lions, caracals, tigers, mountain lion, and lynx was used to determine taxonomic separation and phylogenetic classification between cat species [174, 197]. Common procedures used to chemically characterize scent-markings include headspace extraction and solid-phase microextraction for sample preparation and GC, GC-MS, LC, and TLC for sample analyses [41,198,199]. Previous research suggests that the polarity of a solvent, specifically nonpolar solvents, as well as the geometric isomerism of a semiochemical molecule influences elution order of semiochemicals using gas liquid chromatography [200]. This work specifically focused on alkene elution. The elution orders of simple alkenes, especially those removed from the chain termini, eluted later than the cis-alkenes when the solvent was nonpolar. This has aided in understanding the configuration of total ion chromatograms (TIC). Within the past decade, GC-MS has been the leading technology for scent-marking characterization in great cats.

Chemical composition of semiochemicals of Bengal tigers, African cheetahs, and pumas have been analyzed [33, 41, 47, 68, 69, 161, 166, 183, 201]. Tiger marking fluid (MF), urine, and feces are the known sources of chemical communication in tigers. Analytical methods implemented in the detection of tiger semiochemicals include: GC, TLC, and GC-MS. Ninety-eight volatile compounds have been identified in the MF of Bengal tigers [47]. It has been assumed that tigers use these volatile and non-volatile markings to convey olfactory signaling. What is inhaled, however, and

how it is processed has not been completely identified [33, 47, 167]. 2-acetyl-1-pyrroline has been the only compound associated with the characteristic odor of tiger marking fluid [33]. The identification of this compound in Bengal tigers has been achieved by aroma identification; however the lack of a sniff GLC or GC-MS-O has prevented its analytical confirmation [33, 47, 167]. Burger et al. were never able to confirm 2-AP in Bengal tiger MF or urine [47]. The methods for the identification of 2-AP aroma was based on the addition of hydrochloric acid for acidifying and preventing volatilization, followed by the addition of alkali for aroma identification, and addition of 2% KI to cleave the reactive methyl ketone group of the 2-AP molecule [33,202]. These steps were followed by odor identification based on human olfaction, but its presence has never been confirmed with analytical tools. References [203, 204] suggested that the use of human simple olfactometry detection produces limitations making it very difficult to appreciate the sensory ranges of animals. Though 2-AP is a characteristic odor compound of Bengal tigers it may not be the only compound associated with the overall characteristic odor [205].

The use of GC and LC has enabled characterization of MF from Bengal tigers, specifically its lipid component, VOCs, and a general characterization of MF odor, similar to that of basmati rice. The useof MD-GC-MS-O could potentially define all odorous compounds and provide an all-encompassing and accurate overview of odorous compounds responsible for eliciting behaviors and tiger identity.

In the case of the Bengal tigers, two methods have identified the total lipid and urinary portions of the MF, i.e., TLC and GC-MS. TLC has been used for quantitatively determining lipid composition of Bengal tiger marking fluid [31,129], and GC-MS has been utilized to quantify both lipid and urinary components of Bengal tiger MF [47]. Comparison of differences in the chemical composition and concentrations of marking fluid and urine of subspecies of tigers have never been conducted.

The sebaceous glands contribute to the production of lipocalin protein molecules and fixative lipids in tigers which aids in the long term persistence of marking fluid (MF) in the wild [31]. Bengal tiger marking fluid compounds have been primarily identified using GC column retention time [31]. Retention times are not ideal as chemical co-elution can occur particularly in complex scent-related matrix. The age of the sample and presumed loss of compounds over time can make it impossible to detect volatile compounds, specifically 2-AP using GC-MS [33].

Genetic characterization and definition of Siberian tigers (*Panthera tigris altaica*) and the Amur leopard (*Panthera pardus*) are needed to restore their populations. Previous felid research has led to their species and sex identification from fecal and hair samples [169]. Reference [169] used scent-matching dogs to determine that each tiger has uniquely identifying scent-marks that can be detected by dogs 76% of the time [169]. This indicates that there is a strong association between characteristic odor and chemical composition of scent marks. Feces have also been used as an indicator of tiger population numbers and territorial distribution [68]. Scent-markings have also been used to determine population densities of tigers and pumas.

The volatile constituents of lion urine have been reported [59]. The use of GC-FID instead of GC-MS to analyze cheetah MF may have resulted in the absence of aldehydes and ketones found previously in tigers and leopards [41]. The use of gel electrophoresis made it difficult to identify cauxin in the following big cats: Asiatic lions (*Panthera leo persica*); Sumatran tigers (*Panthera tigirs sumatrae*); Persian leopards (*Panthera pardus saxicolor*); jaguar (*Panthera onca*); and clouded leopard (*Neofelis nebulosa*) because of its similar mass to urinary serum albumin [62].

To date, there is no published research on domestic or wild cats linking a chemical with specific odors associated with their scent marks. Thus, there is clearly a need to define characteristic odors by identifying key chemical constituents responsible for odor in a more reliable approach using analytical tools. Several studies have established the importance of odor in scent mark detection and signalling in domestic cats [161, 165, 206–208]. Scent marks contain specific chemicals which signal

to receiving animals an odor message about age, strength, dominance, relatedness, and reproductive status [5, 207]. The actual amount of time it takes to quantifiably determine differences in semiochemical composition of tigers is unknown, but it has been estimated that by human nose, a general decrease in detection has been noted after a period of two weeks [166].

Conclusion

Chemical and sensory analyses of semiochemicals can potentially aid wildlife conservation. These volatile compounds are essential to the comprehension of animal communication. Large mammal scent-markings are of particular interest because they have not been studied in as much depth as insects and small mammals (e.g., rodents). Great cats, specifically, are facing complete eradication and could benefit from alternative and improved conservation approaches. Scent-marking sample and analytical techniques have their pitfalls and advantages, but have evolved in efficiency over the last decade. The most frequently implemented analytical techniques for characterizing scent marks of wildlife are: GC [55], GC-MS [44, 56–59], GC-FID [31, 44], GC-TOF-MS, nano-LC-MS [40], MALDI-TOF-MS [42, 61, 62], ESI-MS/MS [62], gel electrophoresis [62], TLC [31, 33], GLC [31], and ESI-MS/MS [62].

Understanding of scent-marking constituency aids in the identification of key chemical markers responsible for behavior associated with mating, territoriality, and resource management. Without the input of sensory analysis, the last two steps in the understanding of ethochemistry cannot be executed. The use of animals, human olfaction, and simple GC analysis in the determination of odor composition is limiting at best. The implementation of MD-GC-MS-O, E-noses, and EOGs can help to bridge the knowledge gap about total odor composition of scent marks. This new found information can lead to wildlife management improvement and protection of large mammals and other groups of endangered species.

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CHAPTER III

CHARACTERIZING THE SCENT AND CHEMICAL COMPOSITION OF *PANTHERA LEO* MARKING FLUID USING SOLID-PHASE MICROEXTRACTION AND MULTIDIMENSIONAL GAS CHROMATOGRAPHY–MASS SPECTROMETRY-OLFACTOMETRY

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Abstract

Chemical signals are the primary transmitters of inter- and intra-species communication across all biota. Scent-markings are comprised of semiochemicals which are the key components in biota signaling. Lions (*Panthera leo*) use chemical signaling to indicate health, reproductive status, and territorial ownership. To date, no study has reported scent and composition of marking fluid (MF) from *P. leo*. The objectives of this study were to: 1) develop a novel method for the simultaneous chemical and scent identification of lion MF in its totality, 2) identify the characteristic odorants responsible for the overall scent of lion MF as perceived by human panelists, and 3) compare the existing library of known odorous compounds characterized as eliciting behaviors in animals in order to understand their functionality in lion behavior. Solid-phase microextraction (SPME) for scent collection from mixed MF and urine and multidimensional gas-chromatography-mass spectrometry-olfactometry (MDGC-MS-O) were used for analyses. 2,5-Dimethylpyrazine, 4-methyl phenol, and 3-methylcyclopentanone were isolated and identified as the three compounds responsible for the characteristic odor of lion MF. Twenty-seven volatile organic compounds (VOCs) emitted from lion MF were identified, adding a new list of compounds previously unidentified in lion urine. In addition, chemicals in nine new compound groups were identified: ketones, aldehydes, alcohols, amines, aromatics, sulfur containing compounds, phenyls, phenols, and acids. Twenty-three VOCs are known

semiochemicals that are intricate in attraction, reproduction, physiology, and alarm signaling behaviors in other species. SPME and simultaneous chemical and sensory analyses with MDGC-MS-O improved separating, isolating, and identifying MF compounds volatilized to air. This approach can assist ecologists, animal behaviorists, zoo keepers, and conservationists in improved understanding of which specific compounds are responsible for scents eliciting behaviors, creating stimulating and enriching environments, aiding conservation efforts for lions and other species.

Introduction

Wildlife survival of great cats is contingent on their use of olfaction and scents to identify prey, distinguish amongst conspecifics, and indicate reproductive status [1, 2-5]. Unlocking the scent of bodily excresions that are used as 'chemical messages' could lead to reducing human-wildlife conflicts, increasing endangered populations, improving zoological enrichment approaches, and reducing anxiety in captive and wild cat populations. Researchers have studied scent-marking behaviors and their importance in small cats [6], pumas [7], jackals [8], lions [9], leopards [10-12], tigers [11-13], and cheetahs [12] to understand the purpose of these markings in animal communication to prevent their extinction. The African lion (*Panthera leo, P. leo)* has experienced devastating decreases in its population over the course of the past 50 years [14].

The chemical composition of lion marking fluid (MF) in totality has yet to be investigated. Previously researchers have chemically characterized volatile constituents of other scent-marking excretions released from lions in their manes [1], foreheads and cheeks [15], and urine [16, 17]. Although marking behavior in lions has been studied [9], the chemical constituents of total marking fluid has never been performed.

Specific compounds are responsible for eliciting behavioral responses, yet studies have generated limited information (i.e., chemical content and scent) on these compounds. This study aims at connecting chemical content of MF with specific scents.

Scent-markings are comprised of semiochemicals, which are key components in biota signaling. Lion scent-marks are indicators of their territorial areas, copulation and health status, individuality, genetic variation, and sexual differentiation [12, 15-17]. Lion markings are excreted through feces, facial rubbing, urine and MF. However, marking fluid and urine are the most ubiquitous [15, 18-20]. Marking fluid in lions, tigers, leopards, and cheetahs comprises of urine and a lipid component [20-26]. Lipids are present in the bladder of lions and are released during urination and spray-marking [25]. Andersen and Vulpius (1999) suggested that in *P. leo* these two involuntary methods of marking produce the same range of chemical compounds. The lipid bilayer plays a role in release rate/emissions of volatiles from urine into air [12, 23, 26]. Chemical composition can also be potentially confounded by the direction of release and contact with interfering surfaces.

To date, no study has reported the composition of MF from *P. leo; however,* Andersen and Vulpius (1999) reported 55 VOCs in urine with potential traces of MF and saw dust bedding in cages [27]. That study was somewhat limited in the capability of analytical and sample preparation instrumentation because none of the compounds reported were positively confirmed with chemical standards. The only lion subspecies to have been analyzed for MF VOC composition was *Panthera leo persica* [20]. However, the main focus of that study was to report on the lack of 2-acetyl-1-pyrroline (2-AP) in anal gland excretion in the MF of Asiatic lions.

The focus on 2-AP stems from the earlier finding (Brahmachary, Poddar-Sarkar & Dutta, 1990) that it is a characteristic odor-imparting compound in tiger MF and thought to be in anal glad fluid.

This study focused on simultaneous chemical and sensory analyses of total MF, i.e., total as it is released and present in real environment, without separating into urine and lipid components. The aim was to construct the library of compounds emitted from *P. leo* MF using solid-phase microextraction (SPME) for improved volatiles extraction with minimal matrix interference and multidimensional-GC-MS-olfactometry (MDGC-MS-O) for a comprehensive (both chemical and sensory) and where feasible, standard-based analysis (S1A Sup Info). Therefore, the objectives of this study are to: 1) develop a novel method for the simultaneous chemical and odor identification of lion MF in its totality, 2) identify the characteristic odorants responsible for the overall scent of lion MF as perceived by human panelists, and 3) compare the existing library of known odorous compounds characterized as eliciting behaviors in animals in order to understand their functionality in lion behavior.

Materials and Methods

Experimental Site and Animal Subjects

This study was carried out in the Atmospheric Air Quality Laboratory of Iowa State University (ISU) in accordance with the Guide for the Institutional Animal Care and Use Committee. The protocol was approved by Iowa State University's Institutional Animal Care and Use Committee (IACUC Log # 4-11-7133-A) and by the Blank Park Zoo in Des Moines, Iowa. One male (4 yr old) and 1 female (6 yr old) African lion (*P. leo*) from the Blank Park Zoo had marking fluid collected.

Marking Fluid Sample Collection

The indoor lion enclosures of the Blank Park Zoo were power-washed with luke warm water only and scrubbed with a floor squeegee for 20 min to reduce sample background contamination. Water used to wash the floor was collected and analyzed to account for additional background contamination and its separation from MF volatiles. Direct live lion behavior observation was performed by one trained person to time the release of the scent marking. At the Blank Park Zoo, keepers identified that these lions released MF pin a downward direction. Lions were removed from their enclosures and MF samples were collected immediately from the floor and pipetted into 40 mL glass vials (Supelco, Bellefonte, PA, USA). The MF released from lions appeared yellow with a white lipid film on the top (S1A Fig) and the amount collected ranged from 10 to 20 mL. The vials were washed with a powdered detergent (Alconox, Inc., NY, USA), rinsed with hot water and deionized water for 10 min, then dried at 140 °C 14 hours prior to use to assure minimum interference with MF. Any polysiloxanes identified were not included in the total composition of the lion MF mass spectral results. These compounds are associated with SPME fibers and capillary GC column bleeds [28]. Any interfering compounds contributed strictly from the water were also not considered to be a component of total lion MF. These water composition compounds were previously unidentified in lion urine. MF samples were collected intermittently between January 1, 2015 through May 15, 2015. On collection days, samples were retrieved at peak lion activity (7 a.m. to 12 p.m). After collection, the samples were stored in a cooler with ice packs for transportation and upon returning to the laboratory, samples were further separated into 6 mL and stored in 40 mL vials (Supelco, Bellefonte, PA, USA) at -20 °C until analysis.

Headspace Solid Phase-Microextraction Sampling of Marking Fluid

Approximately 400 mL of lion marking fluid were utilized for this experiment from (n=20) equal number of female and male samples. Samples were run in triplicate for each experiment.Vial samples were brought to 39 °C (internal temperature of a lion) for 30 min using a digital hotplate (Fisher Scientific, model-1180049HPQ) and a Teflon coated 1.27 cm × 0.32 cm stir bar (Fisher Scientific) at 1200 rpm. Headspace SPME sampling was conducted with a manual fiber holder (Supelco, Bellefonte, PA, USA). After the SPME needle pierced the septum of the vial, the SPME fiber coating was exposed to the gases emitted from MF to the vial headspace and continuously adsorbed VOCs.

Effects of SPME Sampling Time

Four SPME coatings were tested (S1A Fig, S1A Table, S2A Table) using three gas sampling times for extraction and odor characterization efficiency (1 min, 1 h, and 24 h). The selected extraction time was 24 h (Fig 1) to maximize the number of odors and compounds identified. The four fibers that were compared were: 50/30 μm divinylbenzene/Carboxen/ polydimethylsiloxane (DVB/CAR/PDMS), 2 cm 50/30 μm (DVB/CAR/PDMS), 75 μm CAR/PDMS, and 65 μm PDMS/DVB. After the VOCs were extracted, they were then desorbed from the SPME fiber when inserted at 260 °C into the MDGC-MS-O injector [29]. The combination of one-step sample preparation and sampling in SPME offset overall process time.

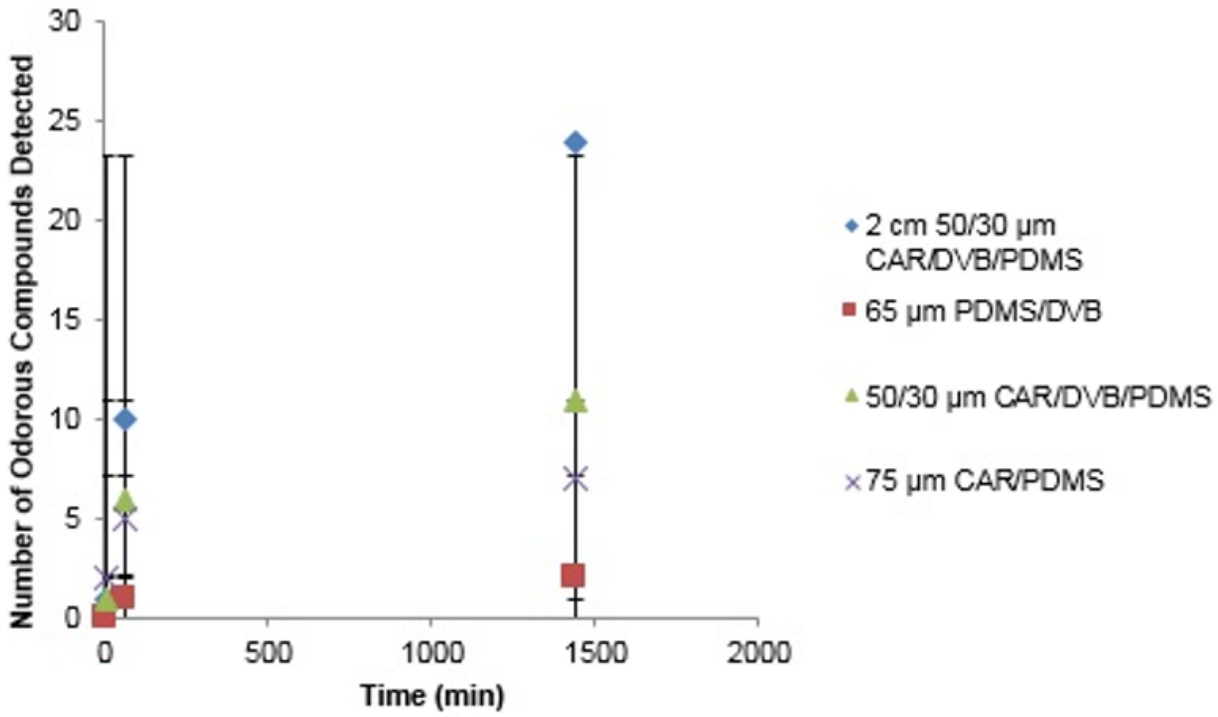

Figure 1. Effects of extraction sampling time. Effects of extraction sampling time (1 h, 2 h, 24 h) and SPME fiber type on the number of odorous compounds detected through sensory analysis (n=3 replicates).

The 1 min sampling time of MF headspace with SPME resulted in no detection of characteristic odors (Fig 1). Therefore, when determining an efficient extraction sampling time for selective SHC and SHC-Cryo, three additional MF headspace sampling times were compared (1 h, 2 h, and 24 h). The 2 h sampling time was used for both SHC and the SHC-Cryo modes because it was the shortest time that reliably resulted in the chemical and odor identification of the compounds of interest.

Olfactory Analysis

Olfactory evaluations were performed through the sniff port. Depending on the MDGC mode, separated compounds from one of the columns were split and delivered the sample to a

panelist via sniff port, while the remaining sample was sent at a 1:3 ratio to the mass spectrometer (MS) for identification. The temperature of the sniff port was set to 240 °C to minimize odorant loses due to condensation in the capillary leading to the sniff port. The tip of the sniff port had a custom nose cone designed at Iowa State University to better fit the panelists. Humidified air (99.997% purity, Praxair, Inc., Danbury, CT, USA) was delivered at 5.7 psi to offset the loss of humidity from panelists' mucous membranes during analyses. The results from the olfactory evaluations were recorded in the form of aromagrams using Aromatrax software (version 6.0, Microanalytics, Round Rock, TX, USA). The aromagram peak was recorded when an odor event was detected by panelists. During the odor event, panelists were responsible for recording (1) the time in which the odor originates and ends, (2) editable odor character descriptors, and (3) odor intensity as perceived by human panelists. The odor intensity was evaluated on a 0-100% scale with 0% indicating no odor and 100% indicating the strongest odor. Only odors that were consistently detected in every one of the three replicates were recorded. The panelists for this study trained extensively on a variety of samples with odorous VOC. Two trained panelists analyzed the VOCs of MF from lions.

Separation and Isolation of Odorous Compounds with Multidimensional GC-MS-O

The MDGC-MS-O has a two CG column system connected in series which operates utilizing two main modes: no heart-cut (separations on column 1 only, similarly to a common GC type) and full or selective heart-cut [30]. Heart-cut is defined as a transfer of a selected range of eluting compounds from column 1, the non-polar pre-column, to column 2, the analytical column. Compounds are 'heart-cut' from the switch valve (a.k.a. Deans' switch) and sent for further separations on column 2 connected in series with column 1.

The cryotrap (i.e., liquid CO_2 jet delivered to the outside jacket enveloping the front of column 2) can be used to trap selected heart-cut analytes from column 1 to enhance chromatographic separations on column 2.

The following sequence of approaches were used to maximize separation and isolation of odorous VOCs:

1) no heart-cut (NHC),

2) full heart-cut (HC),

3) selective heart-cut (SHC), and

4) selective heart-cut with cryotrapping between columns (SHC-Cryo)

In NHC mode, the sample was separated on column 1which was 24 m, 0.53 mm, film thickness; 0.50 µm with 5% phenyl methylpolysiloxane stationary phase (SGE BP5) and analyzed by the flame ionization detector (FID) and simultaneously by olfactometry at the sniff port. This allowed initial identification of eluting target odorants for further separation with HC-based modes. During HC mode, the midpoint heart-cut valve was opened for the pre-determined period that could range from seconds (SHC) to the whole GC run (40 min, 'full' HC) to allow transfer of compounds from column 1 to 2. The end of column 2 (30 m, 0.53 mm, film thickness, 0.50 µm fused silica capillary column coated with polyethylene glycol, WAX; SGE BP20) was always splitting effluent to the sniff port and MS for simultaneous chemical and sensory analyses. The panelist at the sniff port received separated analytes either from column 1 or column 2 depending on the mode of separation.

The selected HC time was based on the elution time ranges in which odors had been earlier identified by panelists in NHC mode. This allowed for a narrower range of separated compounds from the column 1 to be transferred to column 2 for better isolation, separation, and

compound-odor link identification. Standard C_6-C_{20} alkanes were separated in HC and NHC modes to aid selection of HC ranges, separation, and compound identification. Selecting particular odor-impacting compounds resulted in reduction of odorless, less important compounds associated with full HC mode. The use of MD-GC-MS-O has reduced the sample background and interferences caused by co-eluting compounds, resulting in improved spectral matches [31- 32, 33] and improved identification of key odorants is matrices such as animal waste.

Several 30 to 60 s wide ranges of HC were tested to narrow down the exact retention time in which the compound eluted on column 1 with subsequent separation in HC, SHC, and SHC-Cryo modes. Ultimately, separation and isolation improved for the key compounds resembling the overall lion MF odor were performed in the SHC-Cryo mode. Separated compounds that were identified as having a scent similar to the 'characteristic' (i.e., defined as 'nutty', 'sour', 'animal', and/or 'urinous') MF odor descriptors to that of the total MF odor.

Regardless of the heart-cut mode, the following GC and MS program was used. The GC-MS parameters used were: injector, 260 °C; FID, 280 °C; MSD inlet, 240 °C; sniff port, 230 °C; column, 40 °C initial, 3 min hold, 7 °C min^{-1}, 240 °C final, 8.43 min hold; carrier gas, GC-grade helium; total run time, 40 min. The GC operated in constant pressure mode where the mid-point pressure was held at 13 psi and the heart-cut sweep pressure was 7 psi. The FID connected to column 1 was maintained at 280 °C with a H_2 flow rate of 35 mL min^{-1}, an air flow rate of 350 mL $min^{-1,}$ and the makeup N_2 flow rate 10 mL min^{-1}. The FID acquisition rate was 20 Hz. Mass to charge ratio (m/z) range was set between 32 and 280 amu. Spectra were collected at a high scanning frequency of 7 scans s^{-1} and the electron multiplier voltage was set to 1400 V.

Multitrax (version 7.00, Microanalytics, Round Rock, TX, USA) software was used to control the timing of the HC valves in the MDGC-MS-O in all modes. A select set of criteria were used in the identification of the total list of compounds: 1) top five ion match confirmation, 2) odor descriptor matching (www.goodscentscompany.com and www.flavornet.org) 3) spectral confirmation with standards (Chemstation, Benchtop, and AMDIS_32 Software), 4) column retention time, and 5) NIST Library spectral matches. Chromatographic peaks without the standard confirmation of chemical compounds were not included in the analysis of this study. However, spectral signatures for the non-confirmed compounds were included in the Supporting Information section (S3A Table). The non-confirmed 54 peaks were recorded with their top 5 matching ions, retention times, odor descriptors observed by panelists, and measured odor intensities. Academic Search Premier and Web of Science scientific databases were used to search individual compounds identified in this study that were linked to behavioral studies of their functionality in animal species. The key words used were: “behavior”, “pheromone”, “animal”, “mammal”, and ‘the name of the compound of interest.’

Results

SPME Fiber Selection

Four SPME fiber coatings were compared for volatile organic compound (VOC) extraction efficacy of characteristic MF odorants (Fig 1 and S1A Table). The odor panelists detected 24 odorous compounds with a 24 h sampling time using a 2 cm 50/30 μm DVB/PDMS SPME fiber (Fig 1). The average number of compounds detected using a 1 h, 2 h, and 24 h sampling time was 1 ± 0.82, 5.5±3.69, and 11± 9.42 respectively. The 50/30 μm DVB/PDMS SPME fiber coating was the most efficient and on average extracted 24odorous compounds and it was selected for the rest of the experiments. There appears to be an increase of odorous

compounds detected similarly with the increase of mass extracted by the fiber with time (Fig 1) from 1 to 2 h extraction from the MF headspace. However, the odorant number increase behaves similarly to mass extracted, showing signs of limited fiber sorptive capacity with extended (24 h) SPME extractions from headspace. We, however, did not measure the quantitative differences in fiber efficiency to capture each of the characteristic compounds.

Identification of Volatile Organic Compounds and Odorants Using MDGC-MS-O

A total of 27 volatile organic compounds were identified in lion MF headspace through standard chemical confirmation (Table 1). An additional 54 VOCs without chemical confirmation have been determined to be volatilized from lion MF (S2A Table). This resulted in a total list of 81 compounds contributing to the total composition of lion MF. Twenty-four were identified through panelist olfactory confirmation and a 24 h SPME extraction (Table 1, S3A Table). Compounds were confirmed using forward and reverse mass spectral library matches with thresholds of 70% or higher retention times, and by matching the observed odors detected by panelists against the published odor descriptions for compounds. Out of these, odor character matching of compounds as perceived by human panelists accounted for matching of 7 of the 27 confirmed compounds. Odorous VOCs accounted for nearly a third of the total number of VOCs identified and half of the VOCs detected (Table 1, S3A Table). Also an assessor's breathing cycle can influence detection or sensitivity in olfactometry analysis [34]. Upon exhalation no odors are being perceived which can cause odor panelist to leave some compounds undetected [34]. The aqueous and oil mixture of the MF could be modifying the odor of compounds depending on the distribution of the odorants between the two components [35]. 3-Methylcyclopentanone (tentatively identified with 88% spectral library match) was the only odorous compound organoleptically identified by panelists at the sniff port as having an odor

without a published odor descriptor (S3A Table, retention time of 8.59 min). Identifying compounds without previously published odor descriptors allows for potential additions to odor databases. The fact that this compound is without a published odor descriptor does not diminish the impact that it has on the odor of lion MF. There were 17 compounds that had published odor descriptors and were not detected by the panelists (Table 1). The ability to detect scents of MF compounds by human panelists further underscores the notion that animals can detect and process a much wider range and even lower concentrations of the same compounds. Cataloging and analyzing scents can provide information for controlled experiments with surrogate scents comprised of odor-active compounds and test if and how they are being detected, recognized and responsible for lion signaling.

Previously published work on *P. leo* urine suggested that the same compounds are found in both urine and MF. That study [16] reported 55 compounds of which only 12 were found in this study. One possible reason for this apparent low number of common compounds in both studies, is that Andersen and Vulpius (1999) nor this present study could confirm the presence of all the compounds detected and they indicated that further confirmation of the compounds was necessary. It is important to compare the methods used by Andersen and Vulpius (1999) since sample preparation and analysis methods can affect results. Andersen and Vulpius (1999) collected lion urine samples directly from the floor of the night cages. However, due to sawdust contamination, they used a 'garlic press-like' device to extract the urine sample, then stored samples in plastic test tubes at -18 °C until analysis. All of these factors, including possible interfering compounds originating from sawdust, may have altered the outcome of earlier findings.

Table 1. Total VOC composition of *P. leo* marking fluid and its relationship to behavior.

No	RT (min)	Compound Name	CAS	R. Match (%)	Match (%)	Top 5 Ions and Their Relative Intensities	Odor Descriptors Observed by Panelists	Measured Odor Intensity	Published Odor Descriptors	Published Odor Detection Threshold (ppb)	Surrogate Odor Activity Value (PA/ODT)	Andersen and Vulpius (2009)	Cited relevance to behavior	
													Behavior	Species
1	1.48	Trimethylamine[a,†]	75-50-3	99	99	58(99), 59(68), 30(32), 42(25), 28(10)	Foul, fishy, rancid	100	Fishy, oily, rancid, sweaty[b,c]	3.70-16.00E-01[d]	1.10E+07	X		
2	1.70	Acetaldehyde[a,†]	75-07-0	85	82	44(99),43(57),42(17),41(7),40(2)	Pungent, chemical, ethereal, and musty	100	Pungent, etherial, fresh, lifting, penetrating, fruity and musty[b]	1.50-12.00E+01[d]	1.08E+04		Locomotion, Taste aversion, Anxiety	*Rattus rattus*[56-59; *Homo sapiens* [60]; *Mus musculus* [61-62]
3	2.01	Acetone[a,ᵠ]	67-64-1	89	81	43(99),58(64),15(23),42(9),27(6)			Solvent, ethereal, apple, pear[b]	5.00E+05[d]	5.74E-01	X	Locomotion, Sexuality, Irritation	*Rattus rattus* [63]; *Homo sapiens* [64]; *Panthera leo [14]*
4	2.56	2-Butanone[a,ᵠ]	78-93-3	94	82	43(99),72(25),29(17),27(8),57(8)			Etherial, diffusive and slightly fruity with a camphoreous nuance[b]	5.00E+04[d]	5.67E+01	X	Sexuality	*Panthera leo* [14]
5	3.50	2-Pentanone[a,ᵠ]	107-87-9	81	81	43(99),29(22),27(21),57(19),86(15)			Sweet, fruity, ethereal, wine, banana, woody[b]	7.00E+04[d]	9.12E-01	X	Concentration	*Odocoileus virginianus [65]*
6	4.78	3-Hexanone[a,ᵠ]	589-38-8	90	65	43(99),57(83),71(51),29(51),27(36)			Sweet, fruity, waxy, rum, grape[b]			X		

Table 1 continued

7	5.18	Dimethyl disulfide[a,†]	624-92-0	85	95	94(99),79(57),45(48),46(25), 47(19)	Foul, rotten, vegetable	60	Sulfurous, vegetable, cabbage, onion[b,c]	1.60-120.00E-01[d]	2.14E+06		Oviposition inhibition, Attraction, Sniffing	*Anopheles coluzzii* [66]; *Carollia perspicillata*[67], *Rattus rattus* [68]; *Delia radicum* [69]; *Glossophaga soricina* [67]
8	5.36	3-Methyl-butanal[a,ϕ]	590-86-3	87	66	44(99),43(93),41(90),58(81),29(46)			Ethereal, aldehydic, chocolate, peach, fatty[b]	2.50-3.00E+02[d]	8.40E+02	X	Attraction	*Harmonia axyridis [70]*
9	6.36	3-Penten-2-one[a,ϕ]	625-33-2	91	81	69(99),41(96),43(59),39(55),84(27)			Fruity, acetone, phenolic, fishy[b]	1.53E+00[d]	1.83E+05			
10	8.78	Heptanal[a,ϕ]	111-71-7	85	75	44(99),43(79),70(75),41(60),55(52)			Fresh, aldehydic, fatty, green, herbal, wine-lee ozone[b]	3.00E+00[d]	1.67E+04	X	Aggregation, Inhibited behavior, Excitation	*Locusta migratoria manilensis [71]; Culicoides nubeculosus [72]; Agrotis ipsilon* [73]
11	9.49	Cyclohexanone[a,ϕ]	108-94-1	82	86	55(99),42(75),98(45),41(37),39(36)			Minty, acetone[b]	1.20E+02[e]	2.80E+03	X	Attraction, Locomotion, Stimulation, inhibition	*Mus musculus* [74; *Hyphantria cunea* [75]; *Steinernema feltiae* [76]; *Steinernema carpocapsa* [76]; *Steinernema kraussei 76*]; *Heterorhabditis bacteriophora* [76]

Table 1 continued

12	9.56	Octanal[a,ϕ]	124-13-0	81	77	43(99),29(90),41(90),44(74),57(65)			Aldehydic, waxy, citrus, orange peel, green, fatty[b]	7.00E+01[d]	1.00E+04	X	Immobility	*Mus musculus* [74]
13	**10.39**	**2,5-Dimethylpyrazine[a,f]**	**123-32-0**	**97**	**91**	**108(99),42(99),39(42),40(29),81(16)**	**Nutty, potato, corn, earthy, taco shell, animal,**	**60**	**Musty, potato, cocoa and nutty with a fatty and oily nuance[b]**	**8.00-18.00E+02[f]**	**3.47E+02**		**Fear, Freezing, Aggression**	***Mus musculus* [77]; Locusta migratoria manilensis [71]**
14	11.49	2-Nonanone[a,ϕ]	821-55-6	94	91	43(99),58(91),41(29),71(22),57(22)			Fresh, sweet, green, weedy, earthy, herbal[b]	0.05-2.00E+02[d]	1.05E+04		Sex attraction	*Leptonycteris curasoae* [78]; *Rattus norvegicus* [79]; *Aegorhinus superciliosus* [80]; *Dendrosoter protuberans [81]; Cheiropachus quadrum* [81]; *Ahasverus advena [82]*
15	11.58	Nonanal[a,ϕ]	124-19-6	94	94	57(99),41(96),43(82),29(74),44(69)			Waxy, aldehydic, rose, fresh, orris, orange peel, fatty, peely[b]	2.00E-02[d]	4.66E+07	X	Sexual attraction	*Lycaeides argyrognomon* [83]; *Gravid culex* quinquefasciatus [84]; *Sitotroga cerealella [85]; Ephestia cautella [86]; Plodia interpunctella [86];Galleria mellonella [87]; Theraphosa spinipes [88]*

Table 1 continued

16	12.98	Acetic acid[a,♦]	5406 3-13-7	96	81	59(99),43(93),31(92),60(46),29(37)			Sharp, pungent, sour, vinegar[b]	6.00E+00[g]	3.62+04		Oestrus, Estrus, Attraction, Flight	*Bos Taurus* [89-90]; *Vespula maculifrons* [91]; *Drosophila melanogaster* [92]
17	13.94	Benzaldehyde[a,♦]	100-52-7	97	95	77(99),105(92),106(90),51(51),50(32)			Strong, sharp, sweet, bitter, almond, cherry[b]	3.50E+02-3.50E+03[d]	3.59E+03		Oviposition, Defensive, Aggression, Alarm recruitment	*Veromessor andrei* [93]; Scaptotrigona aff. Depilis [94]; *Nearctic messor* [95]; *Bombyx mori* [96]
18	14.46	Linalool[a,†]	78-70-6	86	70	41(99),43(99),71(90),55(64),93(64)	Citrus, grassy, green, herbaceous	80	Citrus, orange, floral, terpy, waxy, lavender rose[b,c]	6.3E+01[d]	1.60E+03		Alarm recruitment, Attraction	*Vespula maculifrons* [91]; *Bombyx mori* [96]; *Colletes cunicularius* [97]; *Corythucha cydoniae* [98]; *Mus musculus* [99]
19	14.52	1-Octanol[a,♦]	111-87-5	91	72	43(99),56(97),41(93),55(89),29(63)			Waxy, green, orange, aldehydic, rose, mushroom[1]	1.10E+02-1.30E+02[d]	3.03E+03	X	Foraging, Alarm recruitment, Sensory Perception	*Microplitis croceipes [100]; Apis dorsata [101]*
20	15.71	Butyrolactone[a,♦]	96-48-0	88	82	42(99),28(75),41(58),29(47),27(41)			Creamy, oily, fatty, caramel[b]				Appetite, Vomiting, and Temor Suppression, Estrus	*Papio anubis* [102]; *Sus scrofa* [103]; *Bos Taurus* [103]

Table 1 continued

21	16.01	Acetophenone[a,ϕ]	98-86-2	97	88	105(99),77(86),51(41),130(33),43(21)			Sweet, pungent, hawthorn, mimosa, almond, acacia, chemical[b]	6.5E+01[d]	6.75E+03		Anti-attraction, Attraction, Responsiveness	*Dendroctonus frontalis* [104]; *Microplitis croceipes* [105]; *Mus musculus* [106]; *Dendroctonus brevicomis leConte* [107]
22	16.83	Dodecanal[a,↑]	112-54-9	91	90	41(99),57(99),55(82),43(76),82(63)	Plastic, waxy	30	Soapy, waxy, aldehydic, citrus, green, floral[b]	2E+00[d]	7.68E+04		PhysiologicalResponses	*Culex quinquefasciatus* [108]
23	19.94	Phenylethyl alcohol[a,ϕ]	60-12-8	83	67	91(99),92(56),65(22),122(22),39(12)			Floral, rose, dried rose, flower, rose water[b]	1.70E+01[h]	1.21E+04			
24	21.29	Phenol[a,ϕ]	108-95-2	96	95	94(99),66(39),65(27),39(24),40(12)			Phenolic, plastic, rubber[b]	5.90E+03[d]	1.27E+03	X	Estrus, Oestrus, Sexuality	*Idea leuconoe* [109]; *Bos Taurus* [90]; *Mamestra brassicae* [110]; *Bubalus bubalis* [111]
25	**22.32**	**4-Methyl phenol[a,↑]**	**106-44-5**	**92**	**92**	**107(99),108(82),77(23),27(20),39(19)**	**Waxy, herbaceous, butter, sour, animal, barnyard, urinous**	**60**	**Phenolic, narcissus, animal, medicinal, mimosa[b,c]**	**5.50E+01[d]**	**1.28E+05**		**Sexuality, Estrus, Oestrus, Diestrus, Sexual attraction**	***Bubalus bubalis* [112]; *Alces alces* [113]; *Glossina spp.* [114-116]; *Stomoxys calcitrans* [117]; *Equus Callabus* [118--119]; *Bison bison bison [120]***

Table 1 continued

26	22.74	2-Piperidinone[a,‡]	675-20-7	85	75	30(99),99(80),42(78),41(72),43(69)					
27	26.64	Indole[a,*]	120-72-9	95	82	117(99),90(25),89(11),118(94),116(58)	Animal, floral, moth ball, fecal[b]	1.40E+04[d]	2.95E+01	Sexuality, Age differentiation	*Mus musculus* [121-122]

*Abbreviations: No-Number; R. Match-Reverse Match; RT-Retention Time; CAS-Chemical Abstract Service Number
**Compounds in bold are characteristic compounds and are displaying percentage matches from SHC-Cryo mode
* Odor descriptors observed by panelists do not match the published odor descriptors for this compound
¢ Compound does not have published odor descriptors, but odor associated with this compounds was detected by panelists
♦ No odors were detected by panelists, but odor descriptors have been published for this compound
‡ No odors were detected by panelists and no odor descriptors have been published for this compound
† Odor descriptors observed by panelists match the published odor descriptors for this compound
[a] Compounds verified with the retention time and ion confirmation match of standards
[b] Good Scents Company [36]
[c] Flavornet [37]
[d] Leffingwell [38]
[e] Indoor Air Quality Engineering: Environmental Health and Control of Indoor Pollutants [39]
[f] Detection thresholds for phenyl ethyl alcohol using serial dilutions in different solvents [40]
[g] Measurement of Odor Threshold by Triangle Odor Bag Method [41]

An improved characterization of compounds emitted from lion MF without interfering bedding material in this present work, using confirmation with standards and matching of odor descriptors to compounds, has been performed for the first time.

The use of SPME and MDGC-MS-O made it possible to identify 27 compounds. The following chemical compound groups (and percentages) were present in African lion MF: ketones (39.29%), aldehydes (25%), alcohols (7.14%), aromatics (7.14%), phenols (7.14%), amines (3.57%), sulfur containing compounds (3.57%), acids (3.57%), and phenyls (3.57%). Fig 2 shows that, in comparison with the published literature on *P. leo* urine (Andersen and Vulpius, 1999), three additional chemical compound groups: acids, phenyls, and phenols were identified in this study. Ketones constituted nearly 2x the percentage of the total composition of lion MF in this current study than Andersen and Vulpius (1999) originally identified. Aldehydes and amines contributed equally to the total composition of lion MF in this study and Andersen and Vulpius (1999). Andersen and Vulpius found (7) alkanes, (1) ester, and (2) ethers that were not detected in this study. Also, Andersen and Vulpius (1999) found twice as many alkenes and aromatic compounds compared with this study. One possible explantion is that there was potential contribution of compounds emitted from the saw dust used for cages bedding which was not separated from MF. Compound groups with the highest overlap between this and Andersen and Vulpius (1999) study were aldehydes. The compound groups with the highest number of overlapping compounds were amines and aldehydes. There were a total of 2 overlapping aldehydes between both papers. Overall, there were 12 compounds identified in MF within this study that were previously unidentified in Andersen and Vulpius (1999).

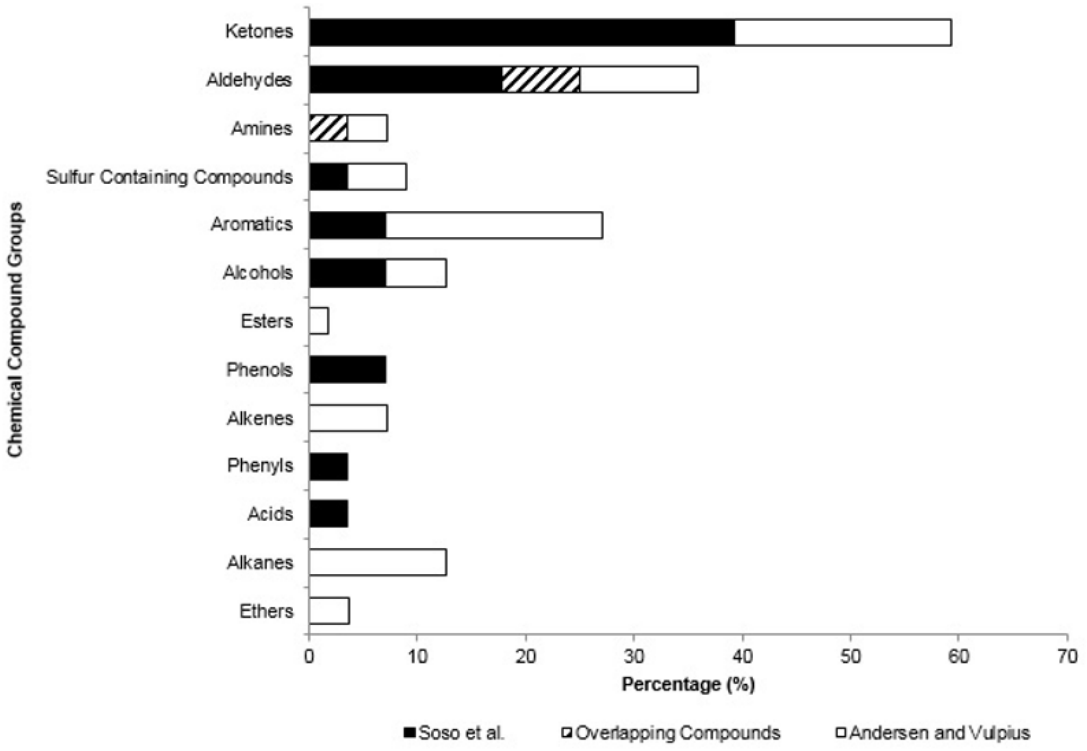

Figure 2. Comparison of marking fluid chemical compound groups**.** Comparison of the percentage of chemical compound group composition of identified with previously published lion urine compounds (Andersen and Vulpius, 1999).

Volatile Organic Compounds Responsible for Characteristic Smell of Lion Marking Fluid

Three VOCs define the characteristic odor of lion MF with the characteristic odor descriptors of ‘animal’, ‘urinous’, ‘nutty’, and ‘sour.’ These three characteristic compounds were identified as 2,5-dimethylpyrazine, 4-methyl phenol, and 3-methylcyclopentanone (Table 1). 2,5-dimethylpyrazine and 4-methyl phenol were confirmed with chemical standards and spectral matching, while 3-methylcyclopentanone was only tentatively identified using 88% forward and 84% reverse spectral matching (Fig 6). Two of these characteristic odorants (2,5-dimethylpyrazine and 4-methyl phenol) have high odor

intensities (Fig 4), yet 2,5-dimethylpyrazine is the only one that has a high surrogate odor activity value (Fig 3) defined as the ratio of peak area counts and odor detection threshold [30, 42].

Surrogate odor activity value (OAV) can be used to describe the impact of an individual compound on the total odor of a sample (as mixture of many compounds). Fig 3 ranks the top ten surrogate OAVs limited to those MF compounds for which odor detection thresholds are known.

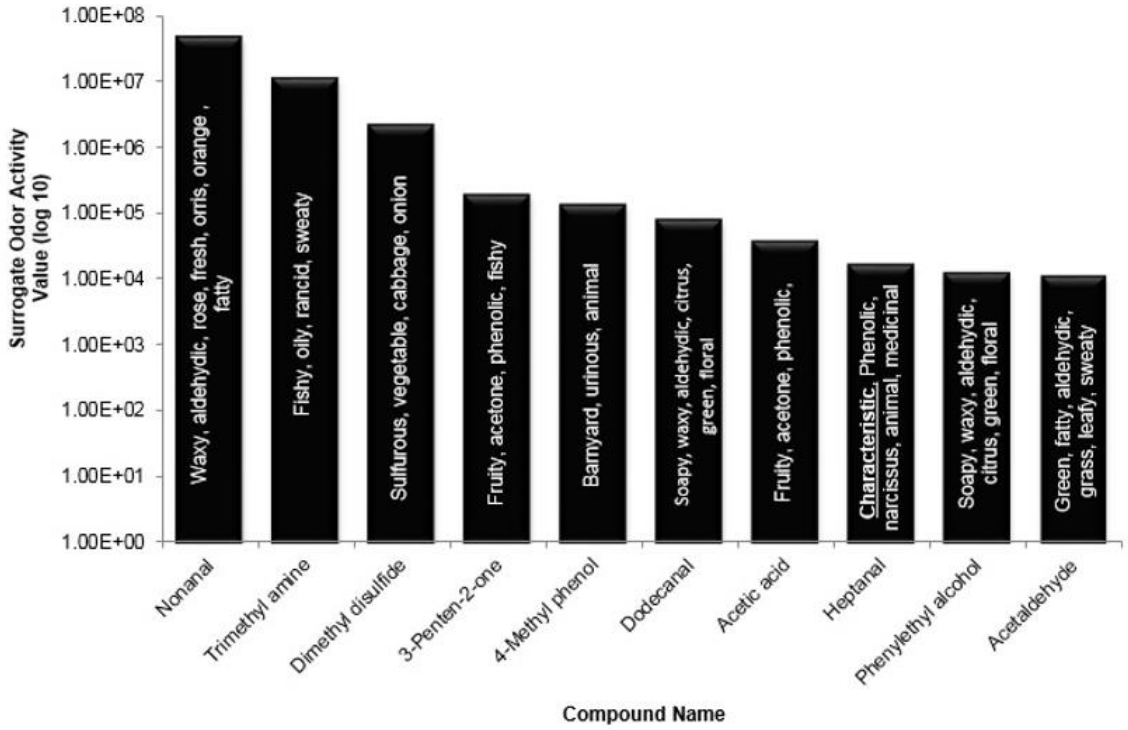

Figure 3. Top ten OAVs**.** Summary of top 10 identified compounds in lion MF with the highest surrogate odor activity values, OAV (OAV = peak area count/ odor detection threshold) and their odor character descriptors.

Based on the surrogate OAVs, nonanal, trimethylamine, and furfural are the top three contributing odors to lion MF. The compound with 5th ranked surrogate OAV (4-methyl phenol) is one of the characteristic MF odor compounds. Other characteristic

compounds were not the in the top 10 albeit that does not mean they are lower in odor intensity (Fig 4). For example, 3-methylcyclopentanone does not have a published odor detection threshold, thus making it impossible to rank its surrogate OAV. Comparison of the total ion chromatogram (TIC) using HC mode with the aromagram of lion MF with highlighted peaks with the top ten measured odor intensities within the sample is presented in Fig 4.

Fig 4 further highlights that high concentration does not necessarily result in a significant odor. Several of the intense scents originate from compounds associated with relatively small peaks (and low abundance).

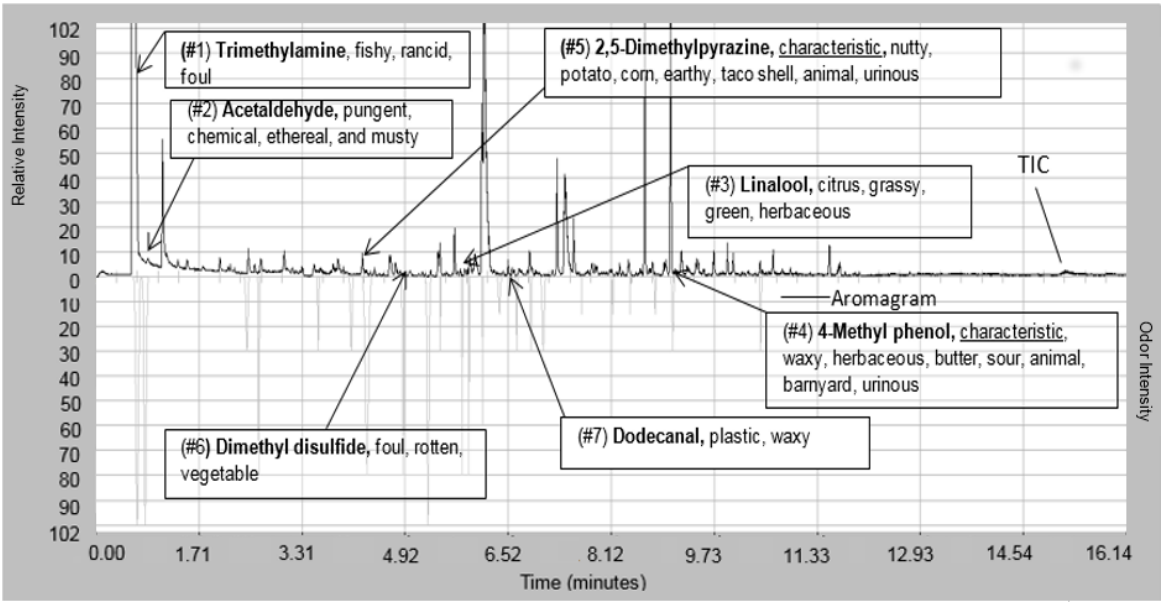

Figure 4. Top seven most odorous compounds in lion marking fluid.

Simultaneous chemical and sensory analyses of compounds and scents in lion MF headspace. Chromatogram (top) highlighting identified compounds in lion MF in order of odor intensity. The odor characters listed are based on observed panelists' evaluations. Aromagram (bottom) was created by panelists during sensory analyses, recording odor character, intensity and start-end detection times.

The seven odorous compounds in order of rank of odor intensity were: 1) trimethylamine, 2) acetaldehyde, 3) linalool, 4) 4-methyl phenol, 5) 2,5-dimethylpyrazine, 6) dimethyl disulfide, and 7) dodecanal. Two (i.e. #4 and #5) of the characteristic odorous compounds were present in the list of highly odor intense compounds demonstrating their importance in imparting the overall odor and likely also affecting mammals' ability to detect and interpret lion MF. The additional 5 compounds had more of the 'herbaceous', 'fruity', and/or 'pungent' odor descriptors. Although speculative at this point, they too may be responsible for general detection of lion MF, lion individuality, territoriality, aggression, and indication of desire to copulate.

The Role of VOCs in Animal Behavior

Although mixtures of compounds produce behavioral responses, some of these individual marking components play a role in altering behavior singly. "Differences in perception or processing of single compounds might reflect differences of their ecological relevance" [43]. Twenty-three of the 27 compounds identified in lion MF have been defined as semiochemicals in other animal species. These VOCs play a role in sexual reproduction, sexuality, gender and age differentiation, aggression, attraction, anti-attraction, defense, and locomotion [44-50, 51-60]. Most semiochemical studies focus on the impact VOCs have on insect behavior. Very few articles indicate the effect individual VOCs have on large mammal behavior. Studies, however, indicate that one of the characteristic compounds of lion MF, 2,5-dimethyl pyrazine, can elicit 'Freezing' behavior in *Mus musculus* [61]. This could be indicative of its role in inter- and intraspecies communication among mammals. The same compound results in aggression in *Locusta*

migratoria manilensis, which could be suggestive of a different role in insect communication [62]. 4-Methylphenol's effect on behavior has been thoroughly researched in many animal species (*Bubalus bubalis* [51]; *Alces alces* [52]; Glossina spp. [53-55]; *Stomoxys calcitrans* [56]; *Equus callabus* [57-58]). It plays a primary role in signifying status of the female in the estrus cycle in *Equus callabus* and *Bubalus bubalis* [51] and sexual receptivity in male *Equus callabus* [57]. 4-Methylphenol is a common component in the urine of many mammals. The role of this ubiquitous compound could potentially be used to improve lion reproduction. Acetic acid is also used as a detector of estrus cycle state and copulation signaling in a variety of species [63-66]. Alcohols such as linalool and 1-octanol have been linked to 'Alarm recruitment' behavior and 'Attraction' in animals [65, 67-72]. These compounds could be linked to lion territoriality scent-markings. They may be used as deterrents for other animals attempting to infiltrate their territory.

The top 3 compounds with the highest surrogate odor activity values in lion MF (4-methylphenol, nonanal, and dimethyl disulfide) were also the compounds with most researched olfactory functions and animal behavioral studies [73-76, 77-82, 51-59]. The interest in studying these highly odorous compounds could be due to their pungent smell and frequency in wing secretions, faecal and urine markings. Their high odor intensity in lion MF could be revealing their importance in lion communication.

Improved Separation and Isolation of Characteristic Marking Fluid Odorants

Identification of the three key characteristic compounds was performed utilizing 4 different MDGC-MS-O modes: 1) no heart-cut, 2) full heart-cut, 3) selective heart-cut and

4) cryotrap (Fig 5). Fig 5 shows the improvement in peak resolution as a result of using the four MDGC-MS-O modes. The NHC mode resulted in the aroma identification of 3-methylcyclopentanone and 4-methyl phenol. Although the NHC mode produced an odor for 3-methylcyclopentanone and 4-methyl phenol, no peak was present in the total ion chromatogram for 3-methylcyclopentanone. HC modes were then performed for improved separation and detection of any additional odorous compounds not found in the NHC mode. 2,5-Dimethylpyrazine was identified in addition to 4-methyl phenol and 3-methylcyclopentanone in HC mode.

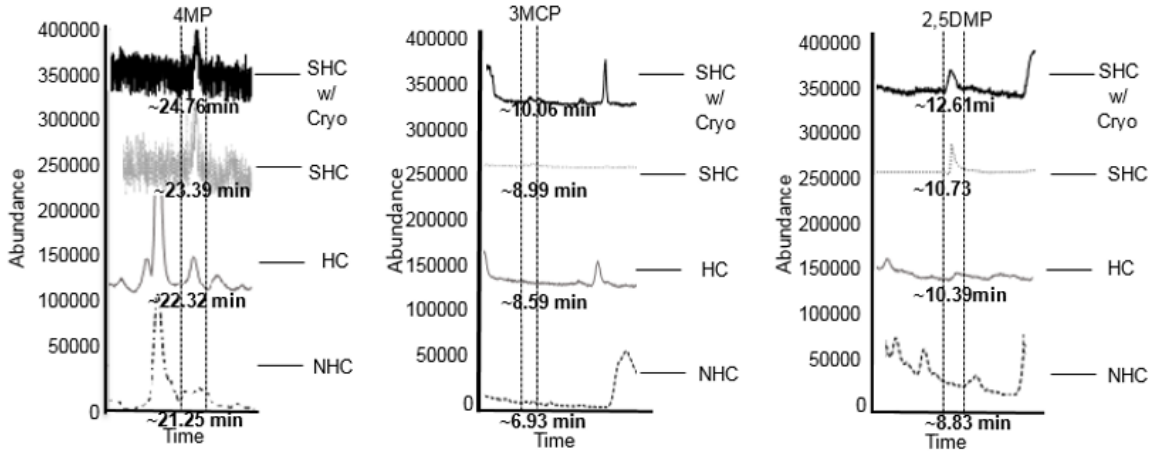

Figure 5. MDGC-MS-O mode for separation and identification of characteristic compounds of lion marking fluid. Separation and enhanced isolation of three characteristic odor-defining compounds extracted from lion urine using four subsequent mdGC-MS-O modes: no heart-cut (NHC), heart-cut (HC), selective heart-cut (SHC), and selective heart-cut with cryotrap (SHC-Cryo).

The presence of the aromas at specific retention times indicated where the responsible chromatographic peak should be eluting. The use of n-alkanes aided in determining the ranges in which to perform SHCs for the selected compounds. Selective

heart-cutting progressively improved compound identification match with spectral libraries by reducing the background from the sample matrix when the sample was transferred to the analytical (2^{nd}) column. The use of HC mode resulted in low percentage matches for 3-MCP (0%), 4-MP (54%), and 2,5-DMP (71%). SHC mode improved the spectral matches, increasing them to 67%, 60%, and 86% for 3-MCP, 4-MP, and 2,5-DMP, respectively. HC-Cryo mode produced the highest percentage matches of all of the three GC-MS modes for 3-MCP, 4-MP, and 2,5-DMP at 84%, 92%, and 97%, respectively. Selective HC was performed in 30s increments. The 3 time SHCs occurred at 6.70-7.20 min, 8.60-9.10 min, and 21.00-21.50 min on column 1. This experimental step is essential to properly isolate the odors and identify areas where the chromatographic peaks may not be evident but odors are (i.e., they are being detected simultaneously by panelist at the sniff port). This step was also necessary to determine if detected odors are not belonging to more than one coeluted compound. 3-Methylcyclopentanone required the use of cryotrapping to perform peak identification.

Cryotrap mode, when activated, was maintained at -40 $^{\circ}$C and cooled the short portion of the external front of the analytical column. This cooling process resulted in a peak separation for 3-MP that improved identification with Chemstation, AMDIS, and Benchtop Software (Fig 6). Without the SHC-Cryo mode, the identification of 3-MP would be less likely. Chemstation, AMDIS, and Benchtop Software programs with a NIST Library found high ion and forward and reverse matching for all of the characteristic compounds (Fig 6). 3-Methylcyclopentanone was the only characteristic compound that was not able to be confirmed through standard confirmation or published odor descriptors.

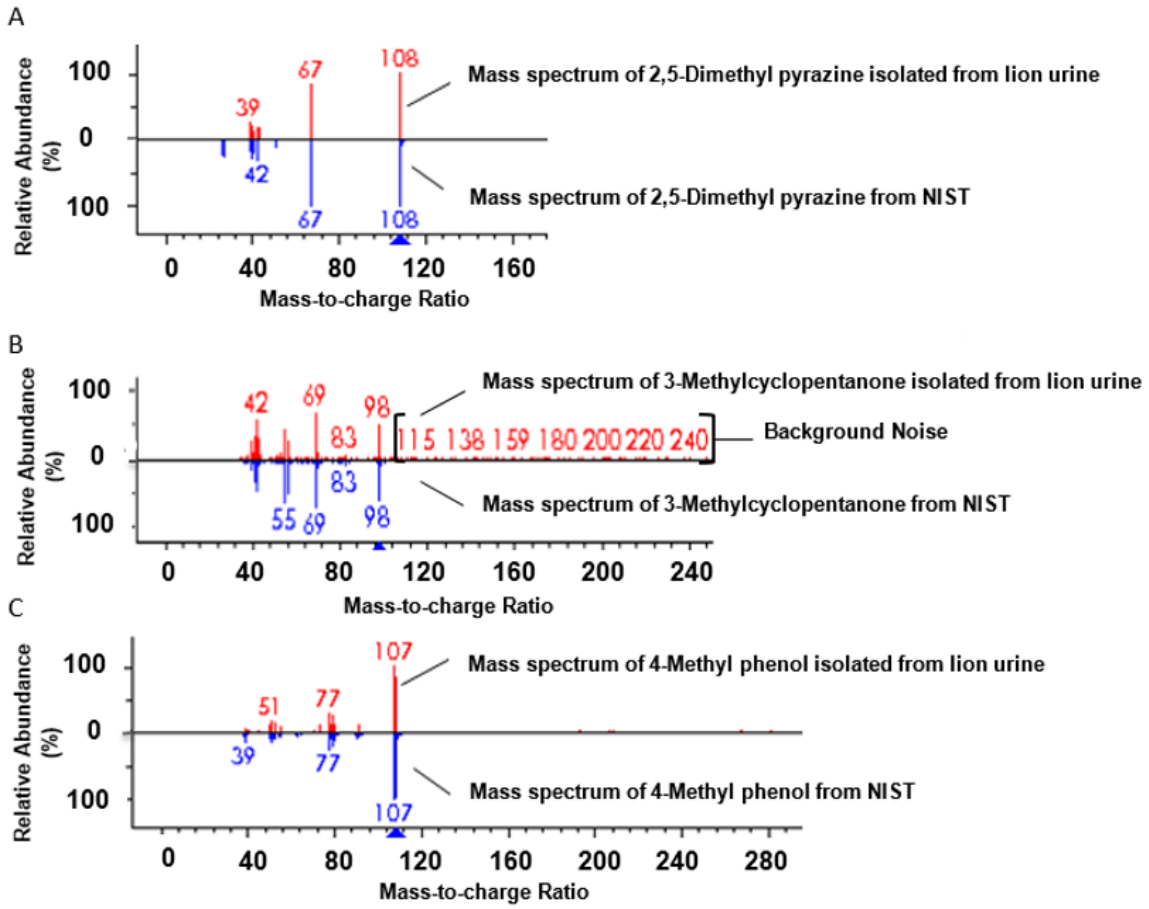

Figure 6. Confirmation of characteristic odorous compounds of lion marking fluid. Mass spectral confirmation of the three compounds responsible for the characteristic odor of lion MF in SHC-Cryo mode using the NIST mass spectral library. The horizontal axis is measuring the relative abundance. The relative abundance gives a proportional difference in ions detected of different masses.

Discussion

Exploiting lion chemical ecology is a potentially advantageous approach to reducing the continued devastation to *P. leo* populations. Scent has a bearing on the daily activities of lions in and outside of captivity. The introduction of scents to lions for enrichment in captivity has been known to alter behaviors such as flehmen, body rolling, and alertness [9, 83-84]. This study developed a novel method for the simultaneous chemical and odor

identification of lion MF to explore its characteristic odorants responsible for its overall odor. Combining chemical and sensory analysis allowed for the identification of lion MF volatiles that would otherwise be difficult to isolate using a typical GC-MS and GC-FID instrumentation. This novel method was able to determine that lion MF is potentially composed of 81 volatile organic compounds, 45 odorous compounds, and 3 characteristic compounds. The aroma detection of only a third of these confirmed compounds could have been due to potential interference from non-volatile components within the sample, the lipid portion of the marking fluid, or from the background contaminants of the enclosure's floor [35]. The VOCs identified in lion MF play a role in sexual reproduction, sexuality, gender and age differentiation, aggression, attraction, anti-attraction, defense, and locomotion in a variety of species. The top 3 compounds with the highest surrogate odor activity values in lion MF (4-methylphenol, nonanal, and dimethyl disulfide) were also the compounds with most researched olfactory functions and animal behavioral studies. The interest in studying these highly odorous compounds could be due to their pungent smell and ubiquitous nature. Their high odor intensity in lion MF could be revealing their importance in lion communication.

The use of solid phase microextraction and MDGC-MS-O with standard compounds allowed for the additional identification of phenols, phenyls, and acids. These chemical groups were previously unidentified. The identification of these compounds in the African lion MF could indicate that urine and marking fluid contain different volatile compounds.

The characteristic odor of lion MF was defined using organoleptics. The characteristic ‘sour’, ‘urinous’, ‘animal’ aroma of lion MF is primarily due to three key compounds. The three characteristic odorants are 4-methyl phenol, 2,5-dimethylpyrazine, and 3-methylcyclopentanone. Andersen and Vulpius (1999) were unable to detect and identify these three characteristic compounds in urine. This could be indicative of the difference in urine and marking fluid composition. The use of selective heart-cutting with cryotrap allowed for the probable identification of 3-methylcyclopentanone. Selective heart-cutting created more defined peaks for 3-MCP and 2,5-DMP improving their spectral matches, 84% and 97% respectively. Future studies could test the standard of 3-methylcyclopentanone to determine its presence within lion MF. This study did not focus on quantifiably measuring the concentrations of the chemical components of lion MF, therefore future studies could be performed to determine the exact concentrations of these VOCs. This would aid in understanding at what concentrations the signals are being detected by lions and potential differences among sexes, reproductive status, and animal individuality among others.

Future research should focus on performing an animal behavior study to test the effects of these volatile organic compounds on the eliciting of specific behaviors. This could be accomplished by measuring changes in hormones (i.e. cortisol) and behavioral responses to the introduction of individual compounds both identified in lion MF and are known behavior modifying semiochemicals in other animal species (i.e. 4-methyl-phenol and acetalaldehyde). This could indicate the particular role of each compound in lion behavior modification. Berns et al. 2015, utilized functional magnetic resonance imaging

(fMRI) to study how the canine brain responded to specific scents. This type of research should be further explored to understand how the brain processes and responsds to smell. The simultaneous chemical and sensory analyses using MDGC-MS-O method can be potentially useful for identification of odor-causing components in scent-markings of other animals. The use of SPME to collect samples in the field and captivity can also be explored. This unique and novel methodology combining SPME and MDCG-MS-O could be used to further understand the way animals perceive scent-markings and potentially prevent the eradication of many large endangered species.

Conclusions

The development of a novel method for SPME and simultaneous chemical and sensory analyses with MDGC-MS-O improved separating, isolating, and identifying MF compounds volatilized to air in lion total MF. This method led to the confirmed identification of 27 VOCs of which 7 were identified by odor panelists. Previously unidentified chemicals in the following nine chemical groups were identified: ketones, aldehydes, alcohols, amines, aromatics, sulfur containing compounds, phenyls, phenols, and acids. Using multidimensional-gas chromatography-mass spectrometry modes of cryotraping and selective heart-cutting, 2,5-Dimethylpyrazine, 4-methyl phenol, and 3-methylcyclopentanone were isolated and identified as the three compounds responsible for the characteristic odor of lion MF. Twenty-three of the 27 compounds identified in lion MF are characterized as eliciting behaviors in a plethora of animals. These compounds have been shown to influence reproduction, locomotion, freezing behavior, sexuality, gender and age differentiation, aggression, attraction, anti-attraction, and defense in a

mammals, including horses, cattle, and swine, as well as a host of insects. This could be a great indication of their role in lion behavior. Simultaneous chemical and sensory analysis methods of scent markings can help scientists to understand wildlife behavior and assist in conservation.

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CHAPTER IV

ANALYSIS OF ODORANTS IN MARKING FLUID OF SIBERIAN TIGER (PANTHERA TIGRIS ALTAICA) USING SIMULTANEOUS SENSORY AND CHEMICAL ANALYSIS WITH HEADSPACE SOLID-PHASE MICROEXTRACTION AND MULTIDIMENSIONAL GAS CHROMATOGRAPHY-MASS SPECTROMETRY-OLFACTOMETRY

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Abstract

Scent-marking is the most effective method of communication in the presence or absence of a signaler. These complex mixtures result in a multifaceted interaction triggered by the sense of smell. The objective was to identify volatile organic compound (VOC) composition and odors emitted by total marking fluid (MF) associated with Siberian tigers (*Panthera tigris altaica*). Siberian tiger, an endangered species, was chosen because its MF had never been analyzed. Solid phase microextraction (SPME) for headspace volatile collection combined with multidimensional gas chromatography-mass spectrometry-olfactometry for simultaneous chemical and sensory analyses were used. Thirty-two VOCs emitted from MF were identified. 2-acetyl-1-pyrroline, the sole previously identified compound responsible for the "characteristic" odor of *P. tigris* MF, was identified along with two additional compounds confirmed with standards (urea, furfural) and four tentatively identified compounds (3-methylbutanamine, (*R*)-3-methylcyclopentanone, propanedioic acid, and 3-hydroxybutanal) as being responsible for the characteristic aroma of Siberian tiger MF. Simultaneous chemical and sensory analyses improved characterization of scent-markings and identified compounds not previously reported in MF of other tiger species. This research will assist animal ecologists, behaviorists, and zookeepers in understanding how scents from specific MF compounds impact tiger and wildlife communication and improve management practices

related to animal behavior. Simultaneous chemical and sensory analyses is applicable to unlocking scent-marking information for other species.

Introduction

At the beginning of the 20th century there were over 100,000 tigers in the wild, which constituted nine *Panthera tigris* subspecies. Currently there are fewer than 3,500 remaining in the wild [1] and about 7,200 in captivity. This represents an approximate 97% decline since 1900. This reduction in population is primarily due to a plethora of anthropogenic factors including poaching, which has resulted in small effective population sizes and degradation of reproductive output; loss of habitat; decline in number of prey species; and climate change [1]. Recent estimates put the number of Siberian tiger population to be critically endangered, with approximately 350 remaining in the wild [1]. A worldwide scientific effort is required to prevent the complete eradication of the six remaining tiger subspecies (*Panthera tigris tigris*, *Panthera tigris corbeti*, *Panthera tigris jacksoni*, *Panthera tigris amoyensis*, *Panthera altaica*, and *Panthera tigris sumatrae*) [1,2].

Scent-marking is described as the most pervasive form of chemical signaling in mammals [3]. This complex mixture of numerous chemicals can result in a multifaceted interaction. Great cat markings have been studied, limitedly, to benefit conservation, specifically focusing on territoriality, dominance, and reproduction [4–15]. Researching these markings has led to a greater understanding of how great cats use scent markings: as a method for distinguishing amongst other conspecifics, neighbors, territorial boundary markings, and as behavioral and reproductive indicators [16,17].

Scent marking plays an integral role in animal identity. Scent marks have been used as key indicators of tiger population numbers and territorial distribution [14]. Previous research on *Panthera* has led to their species and sex identification from fecal and hair samples [18]. Scent-matching dogs used in the identification of tigers in the wild have

proven to be 76% accurate [14]. This may be indicating that scent marks play a role in individuality and suggests that there is a strong association between characteristic odor and chemical composition of scent marks. Investigating scent marks could provide insight into the relationships between evolutionary changes and divergence across tiger subspecies which would assist with conservation and recovery efforts.

There has been limited research in the area of chemical and sensory analysis of great cat markings (Table 1). Scent marking has been analyzed in the African lion (*Panthera leo*), African cheetahs (*Acinonyx jubatus*), Indian leopards (*Panthera pardus fusca*), and puma (*Puma concolor*). Common procedures used to chemically characterize scent markings include: solvent-based extraction, headspace extraction, and solid-phase microextraction (SPME) for sample preparation and subsequent sample analyses using gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography (LC), and thin layer chromatography (TLC) [4,7–11,19–26]. Over the last decade, GC-MS has been the leading analytical technology for scent mark characterization.

Fifty-five volatile compounds were identified in lion urine through GC-MS analysis, but thirty-two were positively identified through chemical standard confirmation using multidimensional gas chromatography-mass spectrometry-olfactometry (mdGC-MS-O) [21,26]. The use of matrix assisted laser desorption ionization time of flight (MALDI-ToF) MS was useful to differentiate between the two compounds that migrated at nearly the same position in the gel electrophoresis used to identify cauxin in big cats [27].

The chemical composition of Siberian tiger (*Panthera tigris altaica*) MF has never been studied. To date, the MF composition of another species, *P. tigris tigris* (Bengal tiger) is by far best known. It is unique in that its chemical composition is very complex and it is the only subspecies of tiger MF ever to be studied for a comprehensive list of volatile organic compounds. Comparison of differences in the chemical composition and resulting odor of MF of subspecies of tigers has also never been conducted. Much of what is known

about chemical composition of MF stems from chemical analyses [5,9,10,11,20]. The use of GC, GC-MS, and LC has enabled characterization of MF from Bengal tigers, specifically its lipid component [4,9,11,14,20]. Banks et al. [19] used GC analyses to identify trimethylamine, ammonia, methylamine, dimethylamine, 2-phenylethylamine, propylamine, triethylamine, and butane-1,4-diamine in Sumatran and Bengal tiger MF. Historically, confirmation of MF compounds identity has been attempted using GC column retention time [9]. However, this method of identification has its limitations and may be less accurate due to chemical co-elution in multifaceted scent-related matrices.

Poddar-Sarkar and Bramachary [20] utilized Bligh and Dyer's [31] methanol-based solutions for the extraction of volatile compounds in Bengal tiger MF [8,20]. One hundred and fourteen volatile compounds (Table 1) have been identified in the MF of Bengal tigers [11]. With the exception of one study, Burger et al. [11], all previous tiger marking sample preparation techniques employed solvent-based extractions [4,9,20,28,29]. Burger et al. [11] used a "sample enrichment probe" (SEP) for the sample preparation of *P. tigris tigris* urine consisting of a short sleeve of 28 mg polydimethyl siloxane rubber affixed to a thin rod of an inert material [11].

Much thinner than the SEP, conventional SPME fibers consist of either a thin sorbent, polymer, or sorbent and polymer combined coating on a (e.g.,) fused silica glass fiber. This 1 or 2 cm fiber is attached to a ~200 µm o.d. inert wire supported inside a hollow needle. In comparison to commercial SPME fiber the volume of the coating and extraction surface area of a SEP PDMS rubber was likely larger, suggesting it has a superior extraction efficiency [10]. Besides the active compounds in MF, fixative lipids expelled with MF, assist in its long term persistence in the wild [20]. Thin layer chromatography determined that the lipid component constitutes 1.88 ± 0.75 mg/mL of MF and contains phospholipids, esters, free fatty acids, and glycerides [10,20].

Table 1. Comparison of sample preparation, chemical, and sensory methods used to characterize scent markings, and the relationship between chemical constituents of marking fluid and urine of great cats.

Species	Reference	Type of Marking	Sample Preparation	Chemical Analyses	Sensory Analyses	Identified Compounds	Commonality in Composition of MF and Urine
Panthera tigris tigris	Poddar-Sarkar, M. and Brahmachary, R.L. [9]	Marking fluid	Solvent-based extraction	GC-FID, GC, TLC, GLC, PC	Not conducted	Free fatty acids	Not conducted
	Poddar-Sarkar, M. [20]	Marking fluid	Solvent-based extraction	GC-MS, GC-FID, GC, TLC, GLC, PC	Not conducted	Total lipid of MF consists of sterol ester, wax ester, triglyceride, free fatty acids, free sterol, diglyceride, and monoglyceride	Not conducted
	Brahmachary, R.L. and Dutta, J. [28]	Urine	Solvent-based extraction	PC	Orqanoleptic testing with human nose detection	2-Phenylethylamine defined as the characteristic odor compound and biochemical marker of urine	Not conducted
	Brahmachary, R.L. et al. [4]	Marking fluid	Solvent-based extraction	PC, GC	Orqanoleptic testing with human nose detection	2-Acetyl-1-pyrroline identified as characteristic compound of marking fluid	Not conducted
	Burger, B.V. et al. [11]	Marking fluid and urine	SEP	GC-MS	Not conducted	98 volatile compounds confirmed including ketones, fatty acids, lactones in MF	Major constituents of urine fraction and of the whole MF were ketones and nitrogen compounds; 2-Acetylpyrroline was not detected in urine or marking fluid; 48 common compounds between urine and MF; Variability in polarity and volatility of compounds identified in urine; MF contains seven times as many VOCs as urine
	Brahmachary [29]	Urine	Solvent-based extraction	TLC	Not conducted	Putrescine and cadaverine components of urine were identified, but later studies (Burger et al. [11] did not report them	Not conducted
Panthera tigris sumatrae; Panthera tigris tigris	Banks, G.L. et al. [19]	MF and anal sac secretion	Solvent-based extraction	GC	Not conducted	Trimethylamine, ammonia, methylamine, dimethylamine,2-phenylethylamine, propylamine, triethylamine, and butane-1,4-diamine were found in Sumatran and Bengal tiger MF	Not conducted

Table 1. Continued

	Andersen, K.F. and Vulpius, T. [21]	Urine	Solvent-based extraction	GC-MS	Not conducted	55 compounds found; several amines, aldehydes, ketones, alkenes, and dienes; acetone, 2-butanone, 1-pentene, 2-pentylfuran, heptanal, 1,2-cyclooctadiene and diethylbenzene potentially responsible for species identity	Not conducted
Panthera leo	Albone, E.S. and Gronnerberg, T.O. [23]	Anal sac secretions	Solvent-based extraction	GLC-MS, TLC	Not conducted	1-alkylglycerols and 2-hydroxy fatty acids, phenylacetic, 3-phenyl-propionic, and related hydroxylated acids were identified	Not conducted
	Soso, S.B. and Koziel, J.A., Manuscript in Review [26]	Marking fluid	SPME	mdGC-MS-O	mdGC-MS-O	81 volatile organic compounds comprise marking fluid; 19 volatile organic compounds were detected using olfactometry; 2,5-dimethyl-pyrazine, 3-methylcyclopentanone and 4-methylphenol responsible for characteristic odor of marking fluid	MF was analyzed in totality with urinous component and compared with previous literature analyzing the same content; 26 additional compounds were identified along with characteristic odorants
Panthera leo persica	Brahmachary, R.L. and Singh, M. [30]	Marking fluid	Solvent-based extraction	PC, TLC	Not conducted	Amines and free fatty acids are putative pheromones of MF; Minor differences between lipid composition of lion and tiger MF; Anal gland fluid is not found in MF	Not conducted
Acinonyx jubatus	Poddar-Sarkar, M., and Brahmachary, R.L. [8]	Marking fluid	Solvent-based extraction	GC-FID, TLC	Not conducted	C_2-C_8 free fatty acids	Not conducted
Panthera pardus fusca	Poddar-Sarkar, M. and Brahmachary, R.L. [24]	Marking Fluid	Solvent-based extraction	GC-FID	Not conducted	C_2-C_9 free fatty acids in the acidic fraction of steam distillate of marking fluid; Several amines were detected in the basic fraction of marking fluid; The amount of lipid extracted from MF is 1.15 mg/mL	Not conducted

Note: SEP = sample enrichment probe; SPME = solid phase microextraction; TLC = thin layer chromatography; GLC = gas liquid chromatography; GC-FID = gas chromatography-flame ionization detector; PC = paper chromatography; GC-MS = gas chromatography-mass spectrometry; mdGC-MS-O = multidimensional gas chromatography-mass spectrometry-olfactometry; and GC = gas chromatography.

Analytical techniques have unlocked a major purpose of scent marking, conspecific and interspecies communication [32]. Chemosensory analysis of scent markings has explained how they are vesicles which contain information that aids in the distinctions between animals of different sexes, ages, and social status and define the time during which a scent marking can be detected in tigers and other great cat species [6,12,20,21,28]. However, what an animal inhales and how it is processed has not been completely identified or understood [4,11,15,33].

2-Acetyl-1-pyrroline (2-AP) and phenylethylamine are the only compounds that have ever been associated with the characteristic odor resembling basmati rice, of Bengal tiger MF [4,28]. The methods for the identification of 2-AP aroma were based on simple yet robust human olfaction, which is limited in its ability to only detect odors at trace levels, e.g., 10^{-7} to 10^{-11} M in humans [34,35]. This method is also limited in identifying other compounds that may be contributing to the overall odor, so the improved sensory characterization with simultaneous chemical and sensory analyses can still be explored. The age of the sample and presumed loss of compounds over time can make it impossible to detect volatile compounds, specifically 2-AP using GC-MS [4]. The inability to identify 2-AP in Bengal tiger MF and urine was believed to be due to its rapid decay, and therefore limited period of odor identification [5]. Also, 2-AP is thought to be formed by a Maillard reaction during previous solvent-based sample preparation and not necessarily by natural occurrence [4,5,36].

Presently, no published research reports characterization of specific odorous chemical markers within scent marks to determine precisely which compounds are responsible for eliciting behaviors in tigers. Thus, there is a need to define characteristic odors by identifying key chemical constituents responsible for odor in a more reliable approach using analytical tools. Simultaneous chemical and sensory analysis is a powerful

tool that could present a novel approach to odor characterization of MF of various mammals. The use of mdGC-MS-O could potentially define all odorous compounds and provide an improved library of odorous compounds contributing to eliciting behaviors and tiger identity. Multidimensional-GC-MS-O is a modern system that is utilized for the separation of volatile organic compounds (VOCs) and semi-VOCs. It utilizes multiple columns for the separation of polar and non-polar compounds and accounts for co-elution of compounds and chemical odors [37–42]. These are common problems associated with single column GC analyses [38,39]. Application of mdGC-MS interfaced with olfactometry (O) has the potential to accurately measure the influence of odor in scent marking detection in species that use chemical cues as their communication method.

Simultaneous chemical-sensory analyses have the potential to be more comprehensive, *i.e.*, yielding valuable information about compound-scent links. In addition, methods based on mdGC-MS-O have very low method detection limits, e.g., 0.020 $ng \cdot L^{-1}$ to 0.022 $ng \cdot L^{-1}$ [40]. MdGC-MS-O has the capability, through its heart-cut mode, to improve the isolation and separation of complex mixtures, enhance odor characterization, and identify compounds [37,38]. Simultaneous chemical-sensory analysis has enabled the following findings: compounds responsible for the characteristic odor of live *H. axyridis* [37]; compounds contributing to the characteristic odor of livestock and poultry manure, rumen of beef cattle; linking specific odor with a volatile compound; the role of particulate matter as a carrier of odor; characterization of kairomones and characteristic odorants released by insects; and quantification of nutraceuticals in wine [37–50]. Application of mdGC-MS-O has the potential to measure the influence of odor in scent mark detection in species that use chemical cues as their communication method.

Solid phase microextraction (SPME) is particularly suited for characterization of volatiles from biological sources. SPME is a solventless extraction method that combines

sampling and sample preparation. SPME fibers with assorted polymeric coatings can be either directly (e.g., by submersion in liquid) or indirectly (e.g., headspace) exposed to a sample. Different SPME coatings target specific categories of compounds based on their molecular weights, polarities, and functional groups. Volatiles and semi-VOCs passively diffuse onto the SPME fiber via adsorption, absorption or capillary condensation. SPME fiber coatings have a very high affinity for VOCs and semi-VOCs [45]. Thus, the sampling results in high pre-concentration and enrichment of compounds without the use of solvents and additional steps. There are relatively few publications that report the use of SPME for characterization of scent markings of large wild mammals [39,46–48]. SPME has been found to be better for the analysis of trace levels of analytes in the urine of Strepsirrhine families [51]. Automating headspace extraction with SPME was useful and a non-invasive method for monitoring reproductive status via the urine in elephants and other species [52–54].

The main objective of this study was to identify VOCs and odors of total MF associated with *P. tigris altaica* (Siberian tigers) with simultaneous chemical and sensory analyses using SPME and multidimensional GC-MS-olfactometry. Specifically, this study focused on: (1) Developing a sampling and analysis method for the identification of VOCs and semi-VOCs of Siberian tiger MF; (2) Determining which VOCs and semi-VOCs in Siberian MF are odorous and compare findings with literature; and (3) Developing an improved list of VOCs and semi-VOCs responsible for the characteristic aroma of tiger MF.

The use of SPME and mdGC-MS-O is a novel approach for improved characterization of odors of total tiger MF. The results of this study will: (a) aid in the development and improvement of semiochemical-based sample preparation and analytical techniques; (b) advance the understanding of the role of semiochemicals in other subspecies

of tigers; (c) benefit the greater tiger worldwide population, in captivity and the wild; (d) determine the efficacy of mdGC-MS-O in the detection of 2-AP and other odor characteristic compounds; (e) determine the efficiency of SPME in extracting volatiles from MF of tigers; (f) potentially aid the rate of success in managing reproductive and social behaviors in a variety of species; and (g) improve semiochemical-based regulation of aggressive behaviors in animals; and (h) compare differences in the concentration, chemical composition, and odor of Siberian tiger MF in comparison to Bengal tigers. In the long-term, it may improve the chances of tiger survival. Investigating the MF of Siberian tigers could: provide insight into evolutionary modifications and/or adaptations, explain the importance of specific chemical compounds and their environmental persistence, and explain the role of these chemicals in species and gender differentiation and gender specific behavior.

Materials and Methods

Standards and Solutions

The present study was carried out in the Atmospheric and Air Quality Laboratory of Iowa State University. Confirmation of the MF compounds was performed through identification with standards (if commercially available and feasible), GC column retention time, matching with Version 2.0 NIST Mass Spectral Search Program library, and matching of odor with odor data bases (e.g., Flavornet and Human Odor Space, The Good Scents Company, and Leffingwell & Associates).

2,4,6-Trimethylpyridine can be used as an internal standard for the confirmation of 2-AP. Previous studies of Grimm et al. [69] and Ying et al. [70] used 2,4,6-trimethylpyridine as an internal standard for the quantitative and qualitative analyses of 2-AP in rice (*Oryza sativa L.*) [70] and additional aromatic rice and Panda (*P. amaryllifolius*)

[69]. The conditions for analysis of 2-AP from *Oryza sativa L*. and *P. amaryllifolius* were optimized using HS-SPME/GC-FID and GC-MS. 20 mg of 2,4,6-trimethylpyridine and 20 μL of deionized water were inserted into a 22 mL vial at 80 °C for 30 min. One cm of the 50/30 DVB/CAR/PDMS fiber was exposed to this shaken vial to adsorb volatile compounds for 20 min [48–50].

Animal Subjects

We collected scent-marking samples from one male and one female adult Siberian tiger (*Panthera tigris altaica*) from the Blank Park Zoo. At the time of sampling, the female tiger was approximately 16 years old and the male was 19 years old. The animal subjects were fed and monitored daily by keepers and veterinary staff within the zoological grounds. Animals were cared for by the standards indicated by the Institutional Animal Care and Use Committee for Iowa State University and the Blank Park Zoo. No animals were harmed during the course of this study.

Marking Fluid Collection Processes

The development of a sampling and analysis method for the identification of VOCs and semi-VOCs of Siberian tiger MF required the proper collection of samples. The indoor enclosures were used as the areas for collection. The floors and walls of the enclosures were power washed and scrubbed to reduce background in the sample. A 20 mL sample of the water used to wash the surfaces of the enclosure was collected to account for potential contamination. MF was collected using two different collection devices (e.g., collection trays and aluminum foil) (S1B Fig). Four MF collection devices were hung varyingly on the portions of the caged wall of the indoor enclosure that are ≥0.90 m (≥3 ft) high (Figures 1, S1B, and S2B) at the Blank Park Zoo.

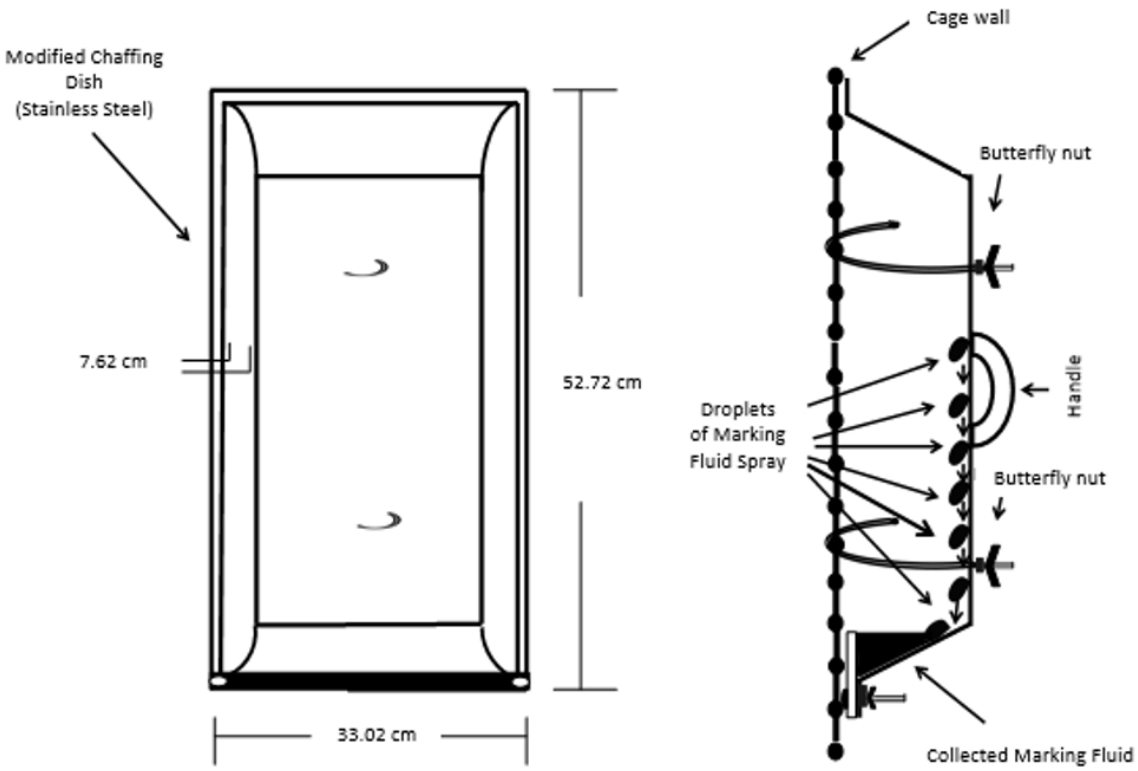

Figure 1. Marking fluid collection device and mode of collection.

The wall behind the caged area was covered in aluminum foil to prevent the loss of MF sample. Separately, the animals were in the enclosure with the collection devices and allowed to roam freely between two enclosures simultaneously. Upon a marking event (S3B Fig) the Pasteur pipettes were used to remove the MF from the collection devices (S1B Fig and S2B Fig) and the MF was pipetted into a 22 mL clear glass screw cap vials with a polytetrafluoroethylene (PTFE)-lined silicone septa vial that was properly labeled and stored in a portable cooler with ice packs. Approximately 80 mL of MF samples were collected. The collection process occurred over a 1-month period to reduce animal stress. After returning from the field, the samples were placed in a −20 °C freezer before analysis based on Burger et al. [11].

3.4. Sampling and Sample Preparation of Panthera tigris altaica Marking Fluid and Urine

Solid-phase microextraction method development was implemented to determine the most efficient parameters to extract the highest number of odorous volatile compounds. Five treatments (time-1 h and 24 h, sample size-0.25 mL and 0.50 mL, agitation method-static or magnetic stirring, and temperatures 25 °C and 37 °C) were applied to five SPME fiber coatings (85 and 75 µm CAR/PDMS, 50/30 µm DVB/CAR/PDMS, 100 µm PDMS, 65 PDMS/DVB). Fiber conditioning was based on manufacturer's requirements. Fiber coating selection was based on the coating's ability to attract and adhere to volatile and aromatic compounds previously identified in the chemical constituents of Bengal tiger MF and urine [42–49]. The experimental design is defined in Table 3.

Table 2. Experimental treatments and the different fiber types used in the experimental design.

Treatments				
Fiber Type	**Sample Size**	**Temperature**	**Time**	**Sample Agitation**
85 µm CPDMS	0.25 mL	25 °C	1 h	None
	0.50 mL		24 h	0.20 cm × 0.50 cm Stir bar @ 1000 rpm
	0.25 mL	37 °C	1 h	None
	0.50 mL		24 h	0.20 cm × 0.50 cm Stir bar @ 1000 rpm
75 µm CPDMS	0.25 mL	25 °C	1 h	None
	0.50 mL		24 h	0.20 cm × 0.50 cm Stir bar @ 1000 rpm
	0.25 mL	37 °C	1 h	None
	0.50 mL		24 h	0.20 cm × 0.50 cm Stir bar @ 1000 rpm
50/30 µm DVB/CPDMS	0.25 mL	25 °C	1 h	None
	0.50 mL		24 h	0.20 cm × 0.50 cm Stir bar @ 1000 rpm
	0.25 mL	37 °C	1 hour	None
	0.50 mL		24 h	0.20 cm × 0.50 cm Stir bar @ 1000 rpm
100 µm PDMS	0.25 mL	25 °C	1 h	None
	0.50 mL		24 h	0.20 cm × 0.50 cm Stir bar @ 1000 rpm
	0.25 mL	37 °C	1 h	None
	0.50 mL		24 h	0.20 cm × 0.50 cm Stir bar @ 1000 rpm
65 µm PDMS/DVB	0.25 mL	25 °C	1 h	None
	0.50 mL		24 h	0.20 cm × 0.50 cm Stir bar @ 1000 rpm
	0.25 mL	37 °C	1 h	None
	0.50 mL		24 h	0.20 cm × 0.50 cm Stir bar @ 1000 rpm

Abbreviations: CAR/PDMS = Carboxen polydimethylsiloxane; DVB/CAR/PDMS = divinylbenzene/ Carboxen polydimethylsiloxane; PDMS = polydimethylsiloxane; PDMS/DVB = polydimethyl-siloxane/divinylbenzene.

Prior to their use, all of the vials (2 mL Supelco®, Bellefonte, PA, USA), septa (polytetrafluoroethylene (PTFE)-lined silicone, Supelco®), and stir bars (0.20 cm × 0.50 cm, Fisher Scientific®, Rockville, MD, USA) were cleaned with sodium hydroxide (NaOH) and placed in the oven at 225 °C overnight to off-gas the impurities and prevent cross-contamination. For each experiment a defined quantity of sample was inserted into a 2 mL vial with a stir bar (agitation studies) or without one. These samples were kept in a −20 °C freezer until analyzed. Upon analysis, the sample was retrieved and brought to the desired temperature with a Fisher Scientific Isotemp Heated Magnetic Stirrer/Hotplate for a period of 30 min. For agitation studies, the magnetic stirrer was set to 1000 rpm for optimal vortical flow. This allows for the mass transfer of VOCs and semi-VOCs into the headspace. The selected fiber was inserted and pierced the septum remaining in a vertical position for the determined extraction period, removed immediately and manually injected into the GC injection port for analysis. Each experiment was replicated three times (n = 3) for each animal in the study. Each replicate used a separate 0.25 mL sample.

Sample Analysis

Simultaneous chemical and sensory analyses of MF was performed using two modes (full Heart-cut and Selected Ion Monitoring) on a mdGC-MS-O instrument (Microanalytics, Round Rock, TX, USA). The MF was used to develop the SPME methodology for the analysis of *P. tigris altaica* MF. During SPME method development, the samples were run on the mdGC-MS-O in full Heart-cut mode (full HC). During this mode the heart-cut valve was open between 0.05 and 35 min run-time. The run parameters used were: injector, 240 °C; FID, 280 °C, column, 40 °C initial, 3 min hold, 7 °C·min^{-1}, 240 °C final, 8.43 min hold; carrier gas, GC-grade He. The GC operated in a constant pressure mode, maintaining the mid-point pressure at 8.5 psi. During full HC mode, the midpoint heart-cut valve was opened for the pre-determined period that ranged the whole GC run (40

min) to allow transfer of compounds from column 1 to 2. This was controlled by the automation system MultiTrax ™ V. 6.50 (Microanalytics). Spectra were collected in three scan groups. Scan group 1 ran from 0 to 8 min collecting compounds with molecular weights ranging from 0–150 at 10.26 scans/sec. Scan group 2 ran from 8 to 20 min collecting compounds with molecular weights ranging from 150–280 at 5.53 scans/s. Scan group 3 ran from 20 to 40 min collecting compounds with molecular weights ranging from 280–350 at 4.43 scans/s. Selected ion monitoring (SIM) mode was utilized for the detection of 2-acetyl-1-pyrroline. SIM was run at 1.6 cycles/s. The mass channels were m/z = 111, 69, 43, 41, 42 for 2-AP. The end of column 2 (30.00 m, 0.53 mm, film thickness, 0.50 µm fused silica capillary column coated with polyethylene glycol, WAX; SGE BP20) was always splitting effluent to the sniff port and MS for simultaneous chemical and sensory analyses. The sniff port was turned to the "On" position to insure all odors eluting from column 1 ventured to column 2. The split ratio between the MS and the sniff port was 1:3. The sniff port temperature was set at 240 °C to eliminate condensation. Humidified air (99.997% purity, Praxair, Inc., Danbury, CT, USA) was delivered at 5.7 psi to maintain constant humidity for panelists' mucous membranes. The tip of the sniff port had a custom panelist designed nose cone developed at Iowa State University. AromaTrax™ V. 8 (Microanalytics) and ChemStation™ (Agilent, Santa Clara, CA, USA) software programs were used for data acquisition (S4B Fig). The aromagram was formed when an odor event occurred and was defined in an area of chromatographic separation. During the odor event panelists were responsible for recording the period in which the odor originates and ends, editable odor character descriptors, and perceived odor intensity. The aroma intensity was evaluated on a 0%–100% scale with 0% indicating no odor, 15% indicating a questionable odor, 30% indicating a faint odor, 60% indicating a medium odor, 80% indicating a strong odor, and 100% indicating an intense odor.

Determination of Chemical Composition and Odor of Siberian Tiger Marking Fluid

SPME fiber selection was based on its efficiency in the number of compounds detected, retention time (RT), total peak area counts using the ChemStation integration tool [54–56]; number of odors detected using AromaTrax™ V. 8, Microanalytics©, Round Rock, TX, USA) tools and highest average odor intensities [56,71]; and detection of characteristic odorants resembling tiger MF aroma. To account for potential subjective bias, an odor panel (2 mdGC-MS-O experts) judged and compared odor character and intensity, but only one panelist was responsible for odor determination in the study. The data sets collected were analyzed using AromaTrax™, Benchtop/PBM (Palisade Corp., Ithaca, NY, USA), Automated Mass-Spectral Deconvolution and Identification System (AMDIS), the NIST library (NIST, 2005), and MSD ChemStation (Agilent). Confirmation of the presence of these chemicals was based on the use of standard chemicals (when available), Flavornet and Human Odor database [57], MSDS data, THE LRI and Odour Database [71], and http://www.leffingwell.com confirmation, as well as panelist odor identification confirmation. Changes in the number of odorous compounds, retention time, integration (number of compounds), peak area counts (via ChemStation), changes in odor intensity and descriptors (via Aromatrax) were measured.

Isolation of Characteristic Odorants with GC-MS-O System

The use of multi-dimensional GC-MS-O allows for all compounds to be on the pre-column (column 1, non-polar) to be transferred to the analytical column (column 2, polar) for better separation. This resulted in the development of an improved list of chemicals responsible for the characteristic aroma of tiger MF. Compounds that were identified as having similar characteristic (nutty or urinous aromas) descriptors to that of the total aroma of MF were selected as compounds of interest in defining the characteristic aroma. These

seven compounds were identified via olfaction and spectral confirmation. Multitrax (Microanalytics) software was used to control the timing of the valves in the GC-MS-O mode so that full HC mode could be run.

Results and Discussion

Selection of Marking Fluid Extraction Parameters

Extraction efficiencies using five fiber types (50/30 µm divinylbenzene/Carboxen/polydimethyl siloxane (DVB/CAR/PDMS), 85 µm Carboxen/PDMS (CAR/PDMS), 75 µm CAR/PDMS, 100 µm PDMS, and 65 µm PDMS/DVB, two temperatures (25 °C and 37 °C), two sample quantities (0.25 mL and 50 mL), and two extraction times (1 h and 24 h) were compared (Figures 1, S5B–S7B). Extraction parameters for the MF were based on the number of total and characteristic compounds detected, and peak area count comparisons of key compounds (S5B–S8B Figs). Based on these results, the 75 µm CAR/PDMS fiber with a 0.25 mL sample quantity, 24 h extraction at 37 °C was selected as the most efficient to characterize the VOCs within tiger MF. The 75 µm CAR/PDMS fiber was the only fiber coating that extracted enough mass for detecting the matching signature molecular ions and characteristic odors of all the "nutty" and "urinous" compounds emitted from tiger MF. Although the 65 µm PDMS/DVB SPME fiber was efficient at extracting enough mass for the detection and chromatographic identification of 2-AP, it was inefficient at the extraction of mass necessary for the detection of all 14 confirmed odorous compounds with a total of 32 odorous events detected with the 75 µm CAR/PDMS SPME fiber (S1B Table, S8B Fig, Table 2). Compared with the 75 µm CAR/PDMS SPME fiber, the 65 µm PDMS/DVB SPME fiber was only able to extract about half the number of compounds resulting in odorous events (18). In addition to 2-AP, the use of the 75 µm CAR/PDMS SPME fiber resulted in the

identification of two (confirmed with chemical standards) compounds (urea, furfural), and four compounds tentatively identified as ((*R*)-3-methylbutanamine, 3-hydroxybutanal, propanedionic acid, and (*R*)-3-methylcyclopentanone)) responsible for characteristic odor in tiger MF (S8B Fig, Table 2).

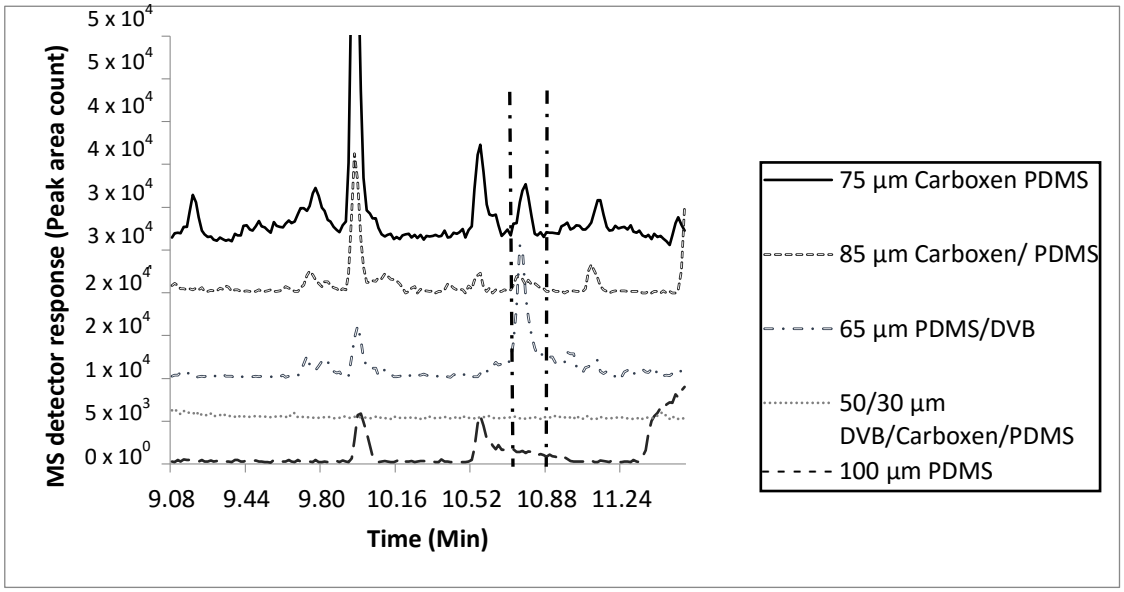

Figure 2. Effects of fiber coating type on SPME adsorption of 2-acetyl-1-pyrroline, the characteristic odorant compound released from marking fluid of *P. tigris altaica* with 85 µm CAR/PDMS, 50/30 µm DVB/CAR/PDMS, 100 µm PDMS, 65 µm PDMS/DVB, and 75 µm CAR/PDMS SPME fibers. Marking fluid (0.25 mL) and a stir bar were inserted into a 2 mL glass vial with a PTFE coated septa for a period of 30 min for equilibration. Samples (n = 3) were extracted at a temperature of 37 °C for 1 h. MS scan mode was total ion scan. Two min of the 40 min total scan is shown. Identification of 2-AP was accomplished with two fibers, the 75 µm CAR/PDMS and 65 µm PDMS/DVB SPME fibers. The 75 µm CAR/PDMS fiber had a peak area of 3.5×10^5 counts and the 65 µm PDMS/DVB SPME fiber had a peak area of 8.9×10^5 counts.

2-AP was used as a reference compound to measure changes in peak area counts between different sample volumes and SPME extraction times. There were no statistical differences in concentrations of 2-AP between the 0.25 mL and the 0.50 mL sample size and extraction times using the 75 µm CAR/PDMS (S7B Fig). Due to the limited number of samples available, the 0.25 mL quantity was selected as the sample size for this study. The 24 h extraction time was selected because the number of detectable odorous compounds increased two-fold with the 23 h increase in extraction time (S8B Fig).

Identification of Volatile Organic Compounds in *P. tigris altaica* Marking Fluid

Thirty-two compounds were identified by chemical standards (except for 2-AP), peak area, odor detection, retention time, spectral matches with top five ion relative intensities (Table 2). An additional 48 unconfirmed unidentified peaks were determined to be present within *P. tigris altaica* MF (S1B Table). Identification of four of these peaks was attempted because they were characterized as having 'nutty', 'urinous', and/or 'corn-like' aromas by the odor panelists. These compounds (2-acetyl-1-pyrroline, (*R*)-3-methyIbutanamine, 3-hydroxybutanal, propanedionic acid, and (*R*)-3-methylcyclopentanone) were considered to be 4 of the 7 characteristic compounds tentatively identified through spectral match with top five ion relative intensities, odor panelists' detection, and published odor descriptors. *P. tigris altaica* MF was comprised of nine chemical groups. These include ketones (9), aldehydes (5), amines (1), amides (1), alcohols (7), acids (2), phenols (1), sulfur-containing compounds (2), and nitrogen-containing compounds (4). All of these compounds were matched with an MS NIST spectral library match of 80% or higher and with olfactory detection by a trained panelist.

Table 3. A list of all the VOCs in the marking fluid of *P. tigris altaica* identified using GC-MS-O. **Bolded entries** are compounds that are characteristic of the total aroma of tiger MF. Compounds were identified using spectral matches with the top five ions, odor descriptor matching, chemical standard confirmation (except for 2-AP), retention time, and the NIST library spectral matching.

No.	Compound Classification	RT (min)	CAS	Top 5 Ions and Relative Intensities (%)	R. Match Factor (%)	Aroma Descriptor by Panelist	Published Odor Descriptors	MOI (%)	PA	ODT (ppb)	SOAV
Nitrogen containing compounds											
1	2,5-Dimethyl-pyrazine [a]	10.47	108-50-9	42(99),108(92),39(31),40(25),81(18)	80		Cocoa, Roasted Nuts, Roast Beef, Coffee [b]		1.85×10^4	8.00×10^2–1.80×10^4 [c]	1.02×10^0–2.31×10^1
2	**2-Acetyl-1-pyrroline** [ϕ]	10.76	99583-29-6	43(99),41(54),42(24),83(13),39(11)	84	Basmati rice, Taco Shell, Nutty, Corn	Nutty, Popcorn, Toasted, Grain, Roasted, Basmati Rice, Malty [b,d]	80	1.05×10^4	0.10×10^0 [e]	1.05×10^5
3	Indole [a]	26.9	120-72-9	117(99),90(43),89(20),63(9),118(9)	96		Animal, Floral, Moth Ball, Fecal, Naphthelene [b,f]		4.79×10^3	1.40×10^2 [c]	3.42×10^1
4	**Urea** [a]	28.98	57-13-6	17(99),60(92),44(75),16(17),43(16)	96	Urinous, Ferret, Foul	Ammonia [g]	30	8.35×10^3		
Ketones											
5	Acetone [a]	2.04	67-64-1	43(99),58(30),42(10),15(17),27(8)	96		Solvent, Ethereal, Apple, Pear [b]		1.51×10^6	5.00×10^5 [c]	3.02×10^0
6	2-Butanone [a]	2.56	78-93-3	43(99),73(32),29(18),57(10),27(8)	99		Acetone-like, Ethereal, Fruity, Camphor [b]		6.98×10^5	5.00×10^4 [c]	1.40×10^1
7	3-Pentanone [a]	3.62	96-22-0	43(99),57(54),44(35),86 (32),41(27)	90	Body Odor, Plastic, Citrus, Bleach, Medicinal	Ethereal, Acetone [b]	30	1.00×10^6	7.00×10^4 [c]	1.43×10^1
8	2,3-Butanedione [a]	3.77	431-03-8	43(99),86(20),42(8),44(8),41(4)	93	Butter, Sweet, Cake Batter	Sweet, Buttery, Caramellic nuance [b]	30	8.99×10^5		
9	2-Methyl-3-pentanone [a]	3.91	565-69-5	57(99),43(77),29(38),100(27),71(45)	90	Chemical	Mint[b]	30	1.59×10^5	5.00×10^3 [h]	3.18×10^1
10	4-Heptanone [a]	6.36	123-19-3	43(99),71(85),41(18),27(17),11(17)	92		Fruity, Cheese, Sweet, Cognac, Pineapple [b]		5.54×10^5	0.82–4.10×10^1 [i]	1.35×10^4
11	2-Heptanone [a]	7.62	110-43-0	43(99),58(40),27(35),71(12),29(12)	95		Soapy, Fruity, Spicy, Sweet, Herbal, Coconut, Woody [b]		1.26×10^6	0.14–3.00×10^3 [c]	4.20×10^2–9.02×10^3

Table 3 Continued

12	2-Nonanone [a]	11.63	821-55-6	58(99),57(28),43(27),41(26),55(16)	81	Earthy, Grassy, Skunky, Foul, Onion, Rancid, Green pepper	Earthy, Herbaceous, Weedy, Green, Dirty [b]	80	3.78×10^4	$0.05–2.00 \times 10^2$ [c]	1.89×10^2
13	2-Undecanone [a]	15.22	112-12-9	58(99),43(58),59(32),71(29),41(18)	93		Waxy, Fruity, Creamy, Fatty, Orris Floral [b]		2.90×10^4	7×10^0 [c]	4.15×10^3
Amines											
14	Trimethylamine [a]	1.37	75-50-3	58(99),59(70),30(35),42(25),28(12)	95	Fish, Onion, Foul, Rancid, Skunky	Fishy, Oily, Rancid, Sweaty, Fruity [b]	100	7.12×10^7	$3.70–10.60 \times 10^{-1}$ [c]	$6.71 \times 10^7–1.92 \times 10^8$
Aldehydes											
15	Hexanal[a]	5.56	66-25-1	44(99),56(82),41(71),43(77),57(39)	83		Green[b]		3.42×10^5	$4.50–5.00 \times 10^{-2}$ [c]	$7.61 \times 10^3–6.84 \times 10^4$
16	3-Methylbutanal [a]	5.77	590-86-3	44(99),43(86),41(49),57(41),39(26)	95		Ethereal, Aldehydic, Chocolate, Peach, Fatty, Nutty [b,d]		8.49×10^5	$0.20–2.0 \times 10^0$ [c]	$4.25 \times 10^5–4.25 \times 10^6$
17	Nonanal [a]	11.82	124-19-6	57(99),41(92),43(91),56(80),44(76)	88		Fatty, Floral-Rose, Waxy [b,c]		1.52×10^4	1.00×10^0 [c]	1.52×10^4
18	**Furfural** [a]	13.23	98-01-1	97(99),96(98),39(65),38(22),29(20)	97	Potato, Body odor, Earthy, Nutty	Sweet, Woody, Almond, Fragrant, Baked Bread [b,f]	80	3.25×10^4	$3.00 \times 10^3–2.30 \times 10^4$ [c]	$0.14–1.10 \times 10^1$
19	Benzaldehyde [a]	14.04	100-52-7	106(99),77(97),105(97), 107(80),39(63)		Fruit loops, Fruity, Sweet	Almond-like, Fruity, Cherry, Sweet, Bitter, Sharp [b]	100	1.75×10^5	$3.50 \times 10^2–3.50 \times 10^3$ [c]	$5.00 \times 10^1–5.00 \times 10^2$
Alcohols											
20	Ethanol [a]	3.02	64-17-5	31(99),45(55),29(32),27(24),46(21)	97		Strong, Alcoholic, Ethereal, Medical [b]		1.58×10^6	1.00×10^5 [c]	1.58×10^1
21	1-Butanol [a]	7.16	71-36-3	56(99),31(98),41(90),43(70),27(58)	95		Medicine, Fruit, Wine [f]		4.43×10^5	5.00×10^2 [c]	8.86×10^2
22	3-Methyl-1-butanol [a]	8.77	123-51-3	55(99),42(90),41(82),43(84),70(73)	96		Fusel, Alcoholic, Pungent, Etherial, Cognac, Fruity, Banana and Molasses [b]		9.37×10^4	2.50×10^2-3.00×10^2 [c]	$3.12 \times 10^2–3.74 \times 10^2$
23	1-Hexanol [a]	11.13	111-27-3	56(99),43(83),41(59),55(58),42(57)	86		Pungent, Etherial, Fusel Oil, Fruity and Alcoholic, Sweet with a Green Top Note [b]		7.36×10^3	2.50×10^3 [c]	2.94×10^0
24	1-Octanol [a]	14.76	111-87-5	56(99),55(88),41(81),73(75),70(61)	80	Roasted, Earthy, Grassy, Green Pepper	Waxy, Green, Orange, Aldehydic, Rose, Mushroom [b]	30	2.78×10^4	1.10×10^2 [c]	2.53×10^2

Table 3 Cont'd

25	Benzyl Alcohol [a]	19.68	100-51-6	79(99),77(57),108(90),107(70),51(22)	92		Floral, Rose, Phenolic, Balsamic [b]		1.94×10^4	1.00×10^4 [c]	1.94×10^0
26	Phenylethyl alcohol [a]	20.16	60-12-8	91(99),51(64),39(75),92(60),77(48)	91	Citrus, Sweet	Rose, Floral [b]	30	9.04×10^4	7.50×10^2–1.10×10^3 [c]	8.22×10^1
Sulfur containing compounds											
27	Dimethyl disulfide [a]	5.39	75-18-3	94(99),79(58),45(50),46(25),47(20)	97		Sulfury, Onion, Sweet, Corn, Vegetable, Cabbage, Tomato, Green, Radish [j]		4.71×10^5	2.00–1.20×10^0 [c]	3.93×10^4–2.94×10^6
28	Dimethyl trisulfide [a]	11.47	3658-80-8	126(99),79(56),45(33),47(23),111(18)	92	Onion, Skunky	Foul, Sulfur, Fish, Cabbage [f]	60	1.08×10^4	0.50–1.00×10^{-2} [c]	1.08×10^6
Acids											
29	Valeric acid [a]	17.6	109-52-4	60(99),73(37),41(15),29(14),27(12)	98	Rancid, Foul, Unknown	Rancid, Sickening, Putrid, Acidic, Sweaty, Sour, Cheese-like [b]	30	8.90×10^3	3.00×10^3 [c]	2.97×10^0
30	Octanoic acid [a]	22.53	124-07-2	60(99),73(62),43(42),41(39),55(37)	93		Fatty, Waxy, Rancid Oily, Vegetable, Cheesy [b]		1.55×104	3.00×10^2 [c]	5.18×100
Amides											
31	Acetamide [a]	17.94	60-35-5	59(99),44(89),43(60,42(29),18(27)	98		Mousy [b]		1.87×10^5		
Phenols											
32	4-Methylphenol [a]	22.6	106-44-5	107(99),108(85),77(32),79(21),51(16)	97	Barnyard, Chemical, Animal, Earthy	Phenolic, Narcissus, Animal, Mimosa [b]	80	2.97×10^3	5.50×10^1 [c]	5.40×10^1
33	Phenol [a]	21.54	108-95-2	93(99),66(39),65(28),39(25),40(15)	97		Phenolic, Plastic, Rubber [b]		2.12×10^4	5.9×10^3 [c]	3.60×10^0

Abbreviations: CAR/PDMS-Carboxen polydimethylsiloxane; DVB/CAR/PDMS-divinylbenzene/Carboxen polydimethylsiloxane; PDMS-polydimethylsiloxane; polydimethylsiloxane/divinylbenzene; GC-gas chromatography; RT-retention time; CAS- Chemical Abstracts Service Numbers; RA-relative abundance; SOAV- surrogate odor activity value; MOC-Measured Odor characters; MOI- measured odor intensity; ODT- Odor detection threshold; bolded entries are compounds that are characteristic of the total aroma of tiger MF; 2-AP- 2-acetyl-1-pyrroline – is placed in Table 3 because it was implicated as a characteristic odorant in Bengal tiger MF [4,5, 32]; [*] Compounds verified with spectral matches with the top five ions, odor panelists' detection, and published odor descriptors; [a] Compounds verified with retention time and ion confirmation of standards (except for 2-AP); [b] Good Scents Company [55]; [c] Leffingwell & Associates [56]; [d] Flavornet [57]; [e] Flavor Chemistry and Odor Thresholds [58]; [f] Encyclopedia *Britannica [59];* [g] *Urea (Ultra-Pure Grade) Safety Data Sheet [60];* [h] Measurement of Odor Threshold by Triangle Odor Bag Method [61]; [i] Fenaroli's Handbook of Flavor Ingredients 5th Edition [62]; [j] *Flinn Scientific, Inc. Safety Data Sheet (SDS) [63];* [k] Haz-Map [64].

Fourteen of the total compounds had human-detectible aromas that matched their published odor descriptors (Table 2). Those compounds with no detectable odors were identified through retention time, spectral match with top five ion matching, and chemical confirmation (Table 2). An additional set of 21 odor events were detected by panelists, but the identity of the compounds was not confirmed with chemical standards, due to feasibility. Four of the 21 odor events were comprised of odorous compounds with 'characteristic' aroma notes.

There have been few reports published on chemical constituents of tiger MF. The majority of them focus on the Bengal tiger (*P. tigris tigris*) and the Sumatran tiger (*P. tigris sumatrae*) [5,11,14,20,65]. Previous studies on tiger MF have identified the constituents based on the analysis of separated MF into two separate fractions, the "lipid fixative" and "urine fraction" [11,20]. Burger et al. [11] is the only study published on Bengal tiger MF that analyzes both fractions, but separately. Compared to Burger et al. [11], the present study was able to detect equal number of sulfur-containing compounds in Siberian tiger MF (Figure 2). We also found five nitrogen-containing compounds in Siberian tiger MF, which is identical to the number previously determined in Bengal tigers [11]. Although the number of sulfur-containing compounds and nitrogen-containing compounds is the same, they were different in each subspecies. There were twice as many phenols in Siberian tiger MF than Bengal, but half of them were common to both. Aldehydes and ketones constitute similar numbers of compounds in tiger MF. Also, we determined the presence of 2-AP, previously undetected in Bengal tiger MF. The two groups with the highest number of common compounds were the alcohols and the aldehydes. Both studies identified 2-phenylethylamine as a constituent of tiger MF, albeit the identification in present study is preliminary (*i.e.*, without chemical standard confirmation). 2-Phenylethylamine is found in the urine of carnivores and is one of the amine molecules that activates the trace amine-

associated receptor in the epithelial tissue of the nasal cavity in bobcats and several other animals [66,67]. 2-Phenylethylamine is found in highest concentrations in the urine of tigers and lions [28,67]. Trimethylamine was identified and is a common compound identified in the MF of Bengal and Sumatran tigers, and African lions [19,20].

Odorous Volatile Organic Compound Detection

Addition of olfactometry to gas chromatography-mass spectrometry has enabled the detection of compounds in tiger MF that would otherwise not be identified. There were a total of 35 odors detected in Siberian tiger MF (Tables 3 and S5B). They ranged from "faint" to "intense" on the odor intensity scale (0%–100%). The overall characteristic scent of tiger MF can be characterized as "nutty" and "urinous." Surrogate odor activity value (SOAV) measures the odor impact of a compound to the total odor of a sample. It is defined as the concentration (measured in chromatographic peak area count) of a single compound divided by the published odor detection threshold for that compound [68].

Based on the compounds identified in the sample, the top ten SOAVs were trimethylamine, 3-methylbutanal, dimethyl disulfide, dimethyl trisulfide, 2-AP, hexanal, nonanal, 4-heptanone, 2-heptanone, and 2-undecanone (Figure 4). 2-AP, trimethylamine, and dimethyl trisulfide were the only compounds included in the top ten SOAVs that were organoleptically identified by panelists. The solitary use of SOAV for the determination of highly odorous compounds may not be inclusive of all highly odorous compounds being detected by animals. The determination of SOAVs is not applicable for compounds without published odor detection thresholds, leaving those compounds with potential odor influence unaccounted for. Organoleptic detection of scent- markings produces a list of odors that are detectable within the MF matrix. When determining the top ten most odorous compounds based on odor intensities selected by trained odor panelist, the list changes drastically (Figure 5).

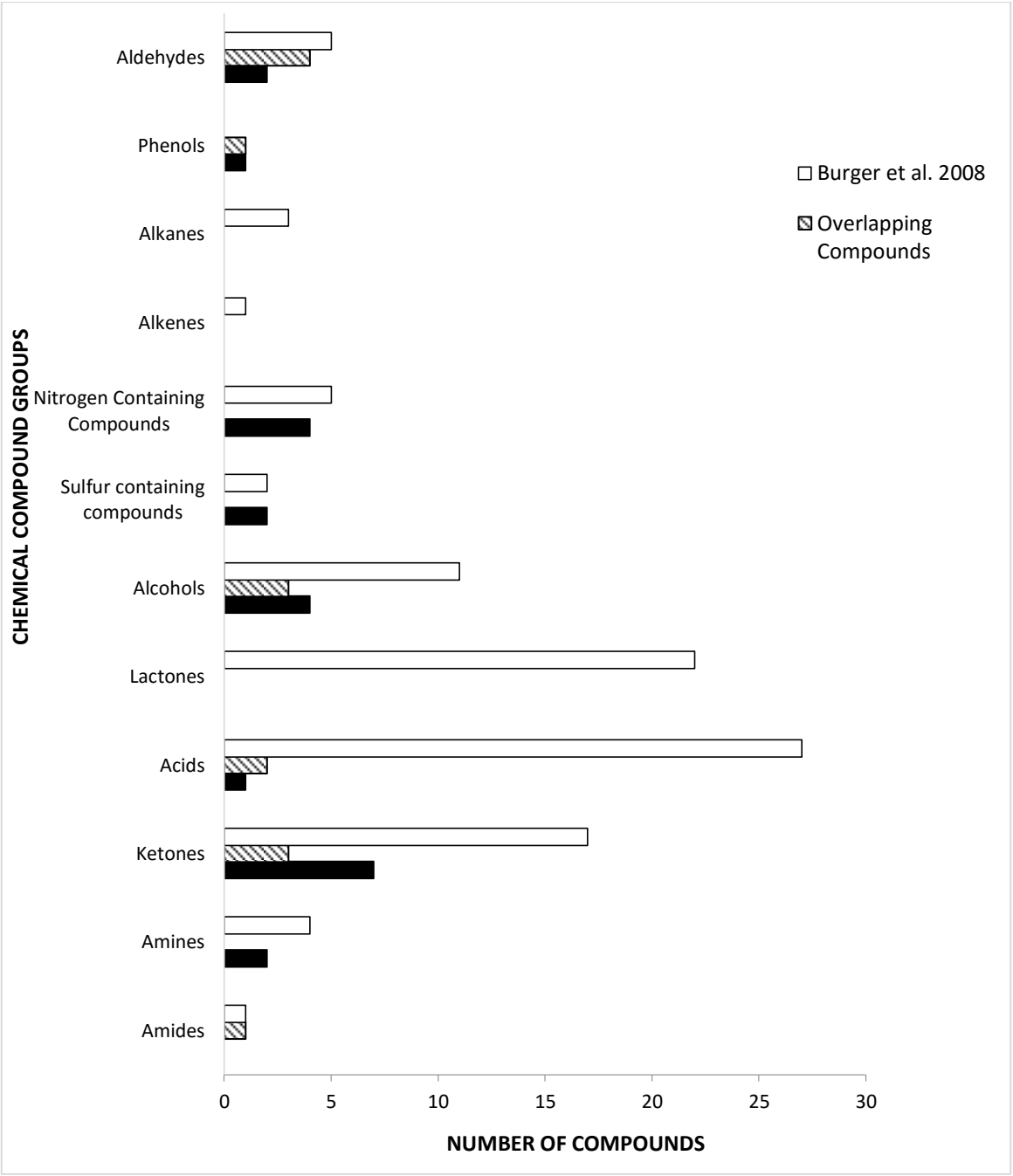

Figure 3. Comparison of chemical compound groups and number of identified with previously published *P. tigris tigris* urine and marking fluid compounds by Burger et al. [11].

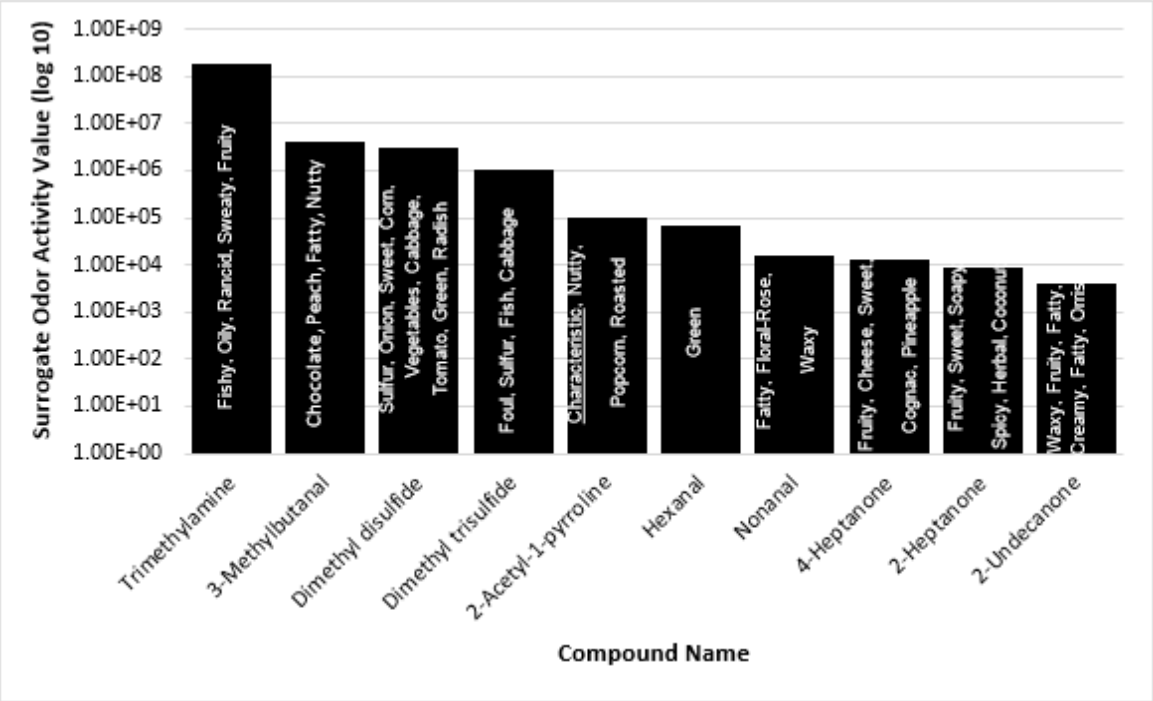

Figure 4. Summary of top 10 compounds, identified with standard chemical confirmation, in *P. tigris altaica* marking fluid with the highest surrogate odor activity values, SOAV (SOAV = odor detection threshold/peak area count) and their odor character descriptors. Confirmation of compounds was performed via chemical standards for all listed compounds with the exception of 2-AP.

Trimethylamine remains the highest ranked odorous compound. In addition, three of the seven compounds that are defined as being characteristic are also amongst the top ten odorous compounds in Siberian tiger MF. 2-AP is ranked 3rd in highest odor intensity among all of the odorous compounds in Siberian tiger MF. 2-AP is considered one of the main characteristic compounds associated with the "nutty" aroma of tiger MF [4]. The majority of the highly odorous compounds fall between the column retention time of 10 min and 17 min. This timeframe had the highest number of organoleptic identified peaks out of the 40 min chromatographic run. Urea and 4-methylphenol are two of the seven highly odorous characteristic compounds responsible for the urinous aroma of Siberian

tiger MF. 4-Methylphenol, another odorous compound, was ranked 6th in odor of highest odor ranking compounds. 4-Methylphenol is a highly odorous compound found in a variety of scent-markings of mammals including lions and swine [26,38]. This could explain its importance in intraspecies communication or evolutionary evolvement. In addition, Figure 5 illustrates the fact that "big peaks" do not necessarily result in detectable odor. Significant odors are sometimes causes by highly potent odorants represented by "small peaks". This highlights the usefulness of simultaneous chemical and sensory analyses.

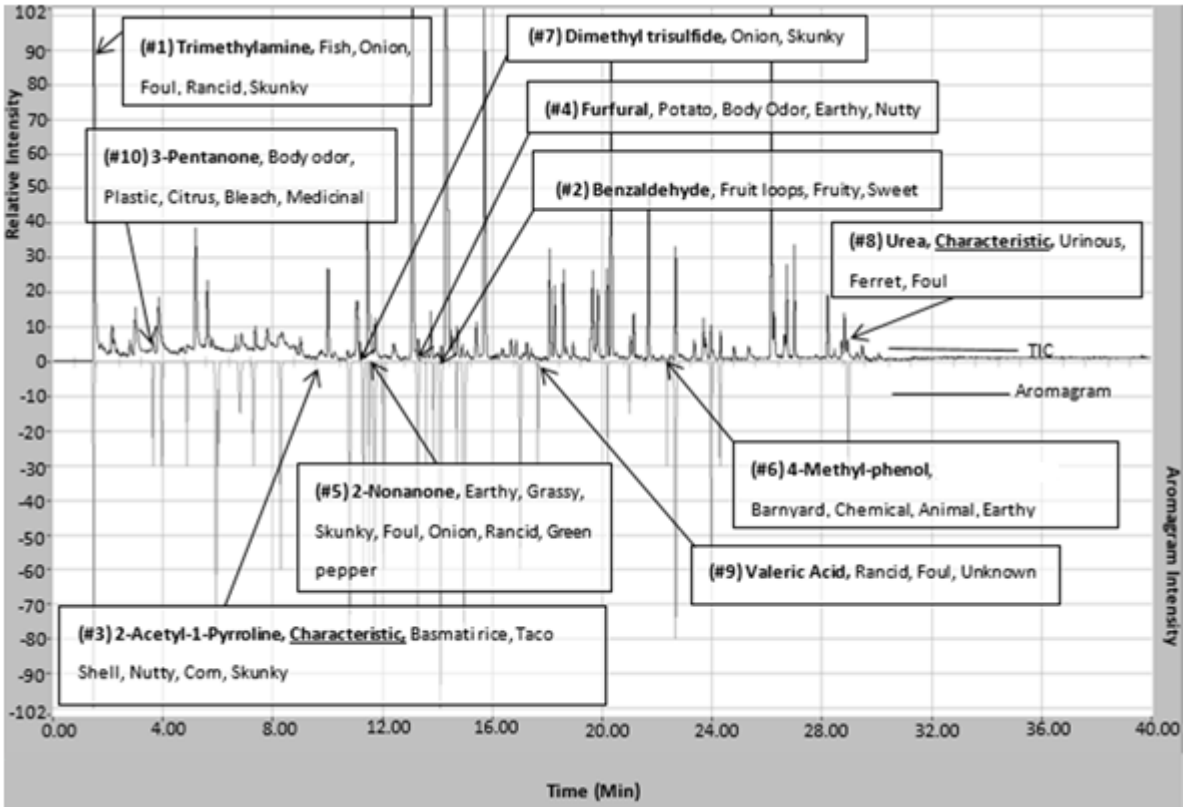

Figure 5. Chromatogram (top) and aromagram (bottom) resulting from simultaneous chemical and sensory analyses highlighting identified compounds in *P. tigris altaica* marking fluid responsible for the highest top 10 measured intense odors. Confirmation of compounds was performed via chemical standards for all listed compounds with the exception of 2-AP. 2-AP was confirmed using top five ions spectral match, retention time, and odor panelist observations. The odor characters listed are based on observed panelists' evaluations.

Determination of Characteristic Compounds from P. tigris altaica Marking Fluid

Amongst the various odors that were observed, seven compounds were responsible for the key characteristic odor of Siberian tiger MF. These compounds include 2-AP, 3-methylbutanamine, (*R*)-3-methylcyclopentanone, propanedioic acid, urea, furfural, and 3-hydroxybutanal. The confirmation of these compounds was essential to prove their existence in tiger MF. The National Institute of Standards and Technology (NIST) mass spectral library was used to confirm the presence of the characteristic compounds along with odor confirmation (S9B Fig). All of the spectral matches for these compounds were above 75%.

2-Acetyl-1-pyrroline is a compound previously identified as the characteristic compound of Bengal tiger MF [4,5,32]. The only method proven to identify this compound was paper chromatography and human organoleptics [4,5]. Burger et al. used SEP-GC-MS analysis and was unable to detect 2-AP [11]. The use of GC-MS-O allowed for a more precise and advanced identification of the 2-AP aroma area so that better software background removal could be done to match (84% spectral match) the compound. Upon refining the analytical technique using more sophisticated instrumentation with high sensitivity and odor capability, we were able to detect 2-AP, contrary to the previous review by Brahmachary and Poddar-Sarkar [5] (Table 3, S10B Fig).

In using SPME, the sample is not altered or subjected to solvent influence and alteration through sample preparation. We have determined that the presence of 2-AP is a natural occurrence and not the result of a Maillard reaction. Previously, the use of GC and GC-MS could not account for the presence of 2-AP in Bengal tiger urine and MF, however through the introduction of SPME-MD-GC-MS-O, 2-AP was identified. An additional reason for the positive identification of 2-AP in Siberian tiger MF could be due to higher concentrations of this compound in Siberian tiger scent-markings. The absence of 2-AP in

the lipid portion of *Panthera tigris tigris* MF may explain that it may reside solely in the urine, however looking at only the lipid fraction or the urinous fraction of MF may result in a lower number of VOCs.

All of the characteristic compounds belong to one of five groups: amines, aldehydes, ketones, nitrogen-containing compounds, and acids (Figure 6). Ketones have the greatest number of odorous compounds with high intensities amongst all of the nine chemical groups that comprise tiger MF. Aldehydes (5) and nitrogen-containing compounds (4) had the largest number of medium-to-intense odorous compounds. Alcohols and amides had the highest number of undetectable odor compounds (Figure 6).

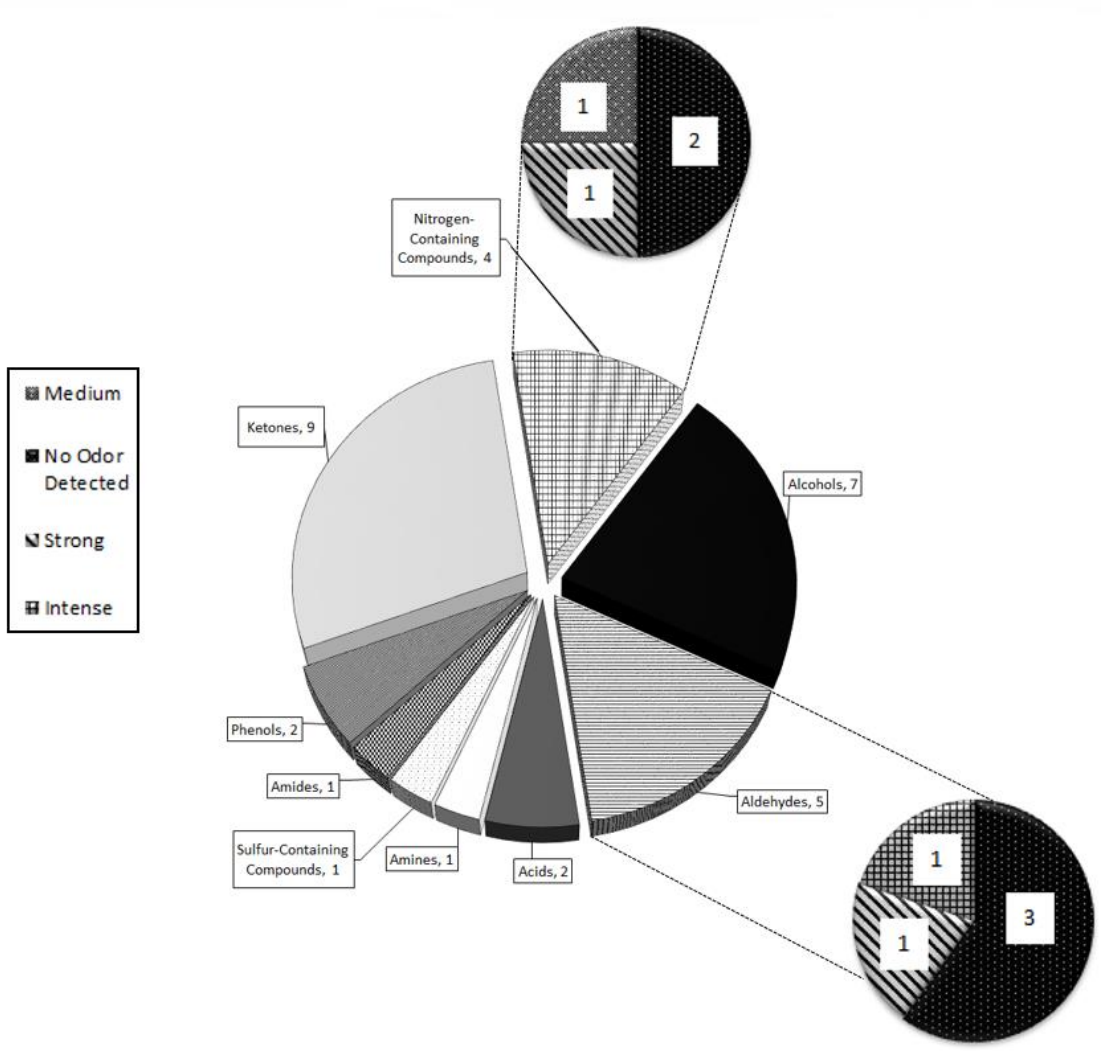

Figure 6. Total number of compounds responsible for the chemical and odor composition of *P. tigris altaica* marking fluid. The chemical groups having the highest

number of intense, medium, and strong odor compounds were the ketones, aldehydes, acids, and nitrogen-containing compounds.

Conclusions

Thirty-two compounds were identified in the MF of Siberian tigers through the development of a novel sample preparation and analysis technique. Fourteen of these were identified through olfactometry analysis. These compounds consisted of ketones, nitrogen-containing compounds, sulfur-containing compounds, alcohols, acids, aldehydes, phenols, amines, and amides. Panelists determined seven compounds as possessing the characteristic 'nutty', 'urinous', and/or 'corn-like' aroma of Siberian tiger MF. 2-Acetyl-1-pyrroline, 3-methylbutanamine, (*R*)-3-methylcyclopentanone, propanedioic acid, urea, furfural, and 3-hydroxybutanal were characterized as contributing to the overall characteristic odor of Siberian tiger marking fluid. Five of these compounds (2-Acetyl-1-pyrroline, (*R*)-3-methyIbutanamine, 3-hydroxybutanal, propanedionic acid, and (*R*)-3-methylcyclopentanone) were identified through spectral matches with the top five ions, odor panelists' detection, and published odor descriptors. This study is the first to identify 2-AP through separation and spectral/sensory match on a mdGC-MS-O and extractions with SPME in tiger marking fluid. It is the first study to analyze tiger MF in its totality, giving rise to a new chemical previously unidentified in other tiger subspecies. Simultaneous chemical and sensory analysis made it possible to identify compounds that otherwise may have been overlooked and continued to be undetected. This research can lead to collaborations amongst various facilities and conservation parks. Knowledge gained from this work could proliferate the species and reduce the human-wildlife conflict occurring in various countries. The approach used on this research can be used as a model for aiding conservation of other globally endangered species.

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CHAPTER V

GENERAL CONCLUSIONS

General Discussion

Chemical and sensory analyses of semiochemicals can potentially aid wildlife conservation. These volatile compounds are essential to the comprehension of animal communication. Scent-markings of mammals, in general, are relatively rarely investigated in comparison to insects and domesticated animals (e.g., cattle, horses, rodents). The field of conservation biology and animal ecology could greatly improve animal welfare practices, reproductive success in captivity and the wild, behavior modification, and enrichment for wildlife by understanding the roles of chemical constituents in these chemical signals. Great cats, specifically, are on the brink of extinction and could benefit from alternative and improved conservation approaches. Understanding of scent-marking constituency aids in the identification of key chemical markers responsible for behavior associated with mating, territoriality, health, and resource management. In order to properly study the impact of these scent-markings sensory analysis is essential. The use of animals, human olfaction, and simple GC analysis in the determination of odor composition is limiting at best. A novel and efficient method of scent-marking sample preparation and analyses was needed in order to unlock the mystery behind the odor and constituency of scent-markings of great cats.

This study developed a novel method for the simultaneous chemical and odor identification of lion and tiger MF. The implementation of mdGC-MS-O, helped to bridge

the knowledge gap about total odor composition of scent marks and the characteristic odorants of lion and tiger marking fluid. Combining chemical and sensory analysis allowed for the identification of lion MF volatiles that would otherwise be difficult to isolate using a typical GC-MS and GC-FID instrumentation.

Chapter 2 reviewed present sample preparation methods, analytical, and sensory tools used to characterize and identify volatile organic compounds. This chapter gives an in depth look into the approaches used to analyze the scent markings of large mammals. Frequently the use of solvent-based extraction practices are used in conjunction with gas chromatography or liquid chromatography. In terms of sensory analyses there has been very limited research performed on these large mammals' scent-markings.

Chapter 3 used SPME and mdGC-MS-O to investigate the total composition and odor of lion marking fluid as well as the characteristic odorants. This novel method was able to determine that lion MF is potentially composed of 81 volatile organic compounds, 45 odorous compounds, and 3 characteristic compounds. This study identified the following chemical compound groups in lion MF: ketones (39.29%), aldehydes (25%), alcohols (7.14%), aromatics (7.14%), phenols (7.14%), amines (3.57%), sulfur containing compounds (3.57%), acids (3.57%), and phenyls (3.57%). Chapter 3 also explored the relationship between the VOCs identified within lion MF with known pheromonal compounds identified in other animal species. This aided in a better understanding of the roles that these VOCs could have on the behavior of lions. The VOCs identified in lion MF play a role in sexual reproduction, sexuality, gender and age differentiation, aggression,

attraction, anti-attraction, defense, and locomotion in a variety of species. The top 3 compounds with the highest surrogate odor activity values in lion MF (4-methylphenol, nonanal, and dimethyl disulfide) were also the compounds with most researched olfactory functions and animal behavioral studies. The interest in studying these highly odorous compounds could be due to their pungent smell and ubiquitous nature. Their high odor intensity in lion MF could be revealing their importance in lion communication.

The use of solid phase microextraction and mdGC-MS-O with standard compounds allowed for the additional identification of phenols, phenyls, and acids. These chemical groups were previously unidentified. The identification of these compounds in the African lion MF could indicate that urine and marking fluid contain different volatile compounds.

The characteristic odor of lion MF was defined using organoleptics. The characteristic 'sour', 'urinous', 'animal' aroma of lion MF is primarily due to three key compounds. The three characteristic odorants are 4-methyl phenol, 2,5-dimethylpyrazine, and 3-methylcyclopentanone. Previous studies, Andersen and Vulpius (1999), were unable to detect and identify these three characteristic compounds in lion urine alone. This could be indicative of the difference in urine and marking fluid composition. The use of selective heart-cutting with cryotrap allowed for the probable identification of 3-methylcyclopentanone. Selective heart-cutting created more defined peaks for 3-MCP and 2,5-DMP improving their spectral matches, 84% and 97% respectively.

Chapter 4 focused on the identification of chemical and odor composition of Siberian tiger MF. Thirty-two compounds were identified in the MF of Siberian tigers through the development of a novel sample preparation and analysis technique. Fourteen of

these were identified through olfactometry analysis. These compounds consisted of ketones, nitrogen-containing compounds, sulfur-containing compounds, alcohols, acids, aldehydes, phenols, amines, and amides. Panelists determined seven compounds as possessing the characteristic ‘nutty’, ‘urinous’, and/or ‘corn-like’ aroma of Siberian tiger MF. 2-Acetyl-1-pyrroline, 3-methylbutanamine, (*R*)-3-methylcyclopentanone, propanedioic acid, urea, furfural, and 3-hydroxybutanal were characterized as contributing to the overall characteristic odor of Siberian tiger marking fluid. Five of these compounds (2-Acetyl-1-pyrroline, (*R*)-3-methyIbutanamine, 3-hydroxybutanal, propanedionic acid, and (*R*)-3-methylcyclopentanone) were identified through spectral matches with the top five ions, odor panelists’ detection, and published odor descriptors. This study is the first to identify 2-AP through separation and spectral/sensory match on a mdGC-MS-O and extractions with SPME in tiger marking fluid. It is the first study to analyze tiger MF in its totality, giving rise to a new chemical previously unidentified in other tiger subspecies. Simultaneous chemical and sensory analysis made it possible to identify compounds that otherwise may have been overlooked and continued to be undetected. This research can lead to collaborations amongst various facilities and conservation parks.

Recommendations for Future Research

Future research should focus on performing animal behavior studies to test the effects of these volatile organic compounds on the eliciting of specific behaviors. A clear understanding of sensory processing in great cats has not been studied at length. Berns et al. 2015, utilized functional magnetic resonance imaging (fMRI) to study how the canine brain responded to specific scents. This type of research should be further explored to understand how the brain triggers in response to smell. The simultaneous chemical and

sensory analyses using mdGC-MS-O method can be potentially useful for identification of odor-causing components in scent-markings of other animals. The use of SPME to collect samples in the field and captivity can also be explored. This unique and novel methodology combining SPME and mdGC-MS-O could be used to further understand the way animals perceive scent-markings and potentially prevent the eradication of many large endangered species.

Future studies could test the standard of 3-methylcyclopentanone to determine its presence within lion MF. Chemical confirmation of 3-methylbutanamine, 3-hydroxybutanal, (R)-3-methylcyclopentanone and propanedioic acid, the key characteristic compounds in Siberian tiger MF, could also be tested with standards to further solidify their presence in MF. This study did not focus on quantifiably measuring the concentrations of the chemical components of lion MF, therefore future studies could be performed to determine the exact concentrations of these VOCs. This would aid in understanding at what concentrations the signals are being detected by lions and potential differences among sexes, reproductive status, and animal individuality among others.

APPENDIX A

SUPPLEMENTARY INFORMATION AND FIGURES FOR CHAPTER III

Supplemental Information S1A. Interfacing SPME and multidimensional chromatography with olfactometry provides a unique opportunity to address these knowledge gaps. The emissions of volatiles from MF (defined here as a simultaneous and mixed secretion of MF and urine) was analyzed in totality, MF was not separated from urine, in order to improve understanding of the perceived odor of gases emitted from lion MF. We did not analyze fecal excretions, a common form of scent-marking, because in lions defecation can be done at random [1]. This is indicating its potentially lower order in the hierarchy of scent-markings. Although the scope of this study was limited to lion's MF, the same approach could be used for other species. Once odor and odor-causing compounds in territorial markings are known, this knowledge can be exploited to determine the effects of specific compounds on animal behavioral and/or chemical responses. Future studies can develop behavioral assays and perform chemical analyses of the responses to the introduction of the odorous compounds identified in this study to lions. This unique and novel methodology combining SPME and MDCG-MS-O could be used to further understand the way animals perceive scent-markings and potentially prevent the eradication of many endangered species.

Solid phase-microextraction (SPME) is a solvent-free, one step sampling/sample preparation technique that has been limitedly used in the sample preparation of mammalian scent-markings [2, 3]. Since its conception in the late 1980s, it has proven to be one of the superior sample preparation techniques available for analytical work in the area of fundamental analytical chemistry, environmental analysis, pharmaceutical, food and forensic analyses [2, 4-8]. SPME is a reusable technique that combines sampling and sample preparation and is suitable for laboratory and field environmental work [9]. The SPME process is facilitated on a polymeric coating that has a high affinity for organic compounds. SPME has been used for sampling of volatile compounds in air [10], livestock odor, breath of animals [11], volatiles inside rumen [12], volatiles emitted by decaying animal mortalities [13], and insect-induced plant volatiles [14]. Enrichment associated with SPME often leads to significantly improved method detection limits and elimination of artifacts from solvents compared with other sampling and preparation methods [15].

Multidimensional-GC-MS-O is one of the most advanced methods for simultaneous chemical and sensory analysis, enabling volatile organic compound speciation and isolation of odor-active compounds. Precise and advanced capabilities to detect trace levels of components is due to its multi-column system which allows for a better separation and identification of volatiles [16] many of which are odorous [17-19]. The olfactometry is enabled by a sniff port which gives odor panelists an opportunity to characterize each separated compound as it is being eluted through one of the selected GC columns. This feature allows for the determination and verification of compounds through chemical (GC column retention times, MS spectral matches) and, simultaneous odor matching

confirmation using trained tiger odor panelists and published scent-to-compounds link libraries [20]. There is limited working knowledge of how mammals process odor signals [21,22]. Therefore, the human nose is considered ideal in understanding odor perception in animals because the human sense of smell is capable of distinguishing and recognizing a diverse range of characteristics of volatile compounds [23]. A few studies have indicated that odorous markers can be an identifier in human disease and therefore GC-MS-O has been previously utilized to perform human studies [24-26]. The research from this study could be comparatively studied with humans in order to understand semiochemicals as indicators of health and reproductive status. Headspace-SPME and MDGC-MS-O was used in the identification of VOCs from *Panthera tigris altaica* MF [27]. This use of SPME in conjunction with MD-GC-MS-O allowed for aroma recognition and chemical confirmation of 2-AP, which was previously considered one of the characteristic odor compounds of *P. tigris tigris* MF, but could not be identified previously using solely chemical analysis with GC-FID and GC-MS [27]. The objectives of this study were to: 1) develop a novel method for the simultaneous chemical and scent identification of lion MF in its totality, 2) identify the characteristic odorants responsible for the overall scent of lion MF as perceived by human panelists, and 3) compare the existing library of known odorous compounds characterized as eliciting behaviors in animals in order to understand their functionality in lion behavior.

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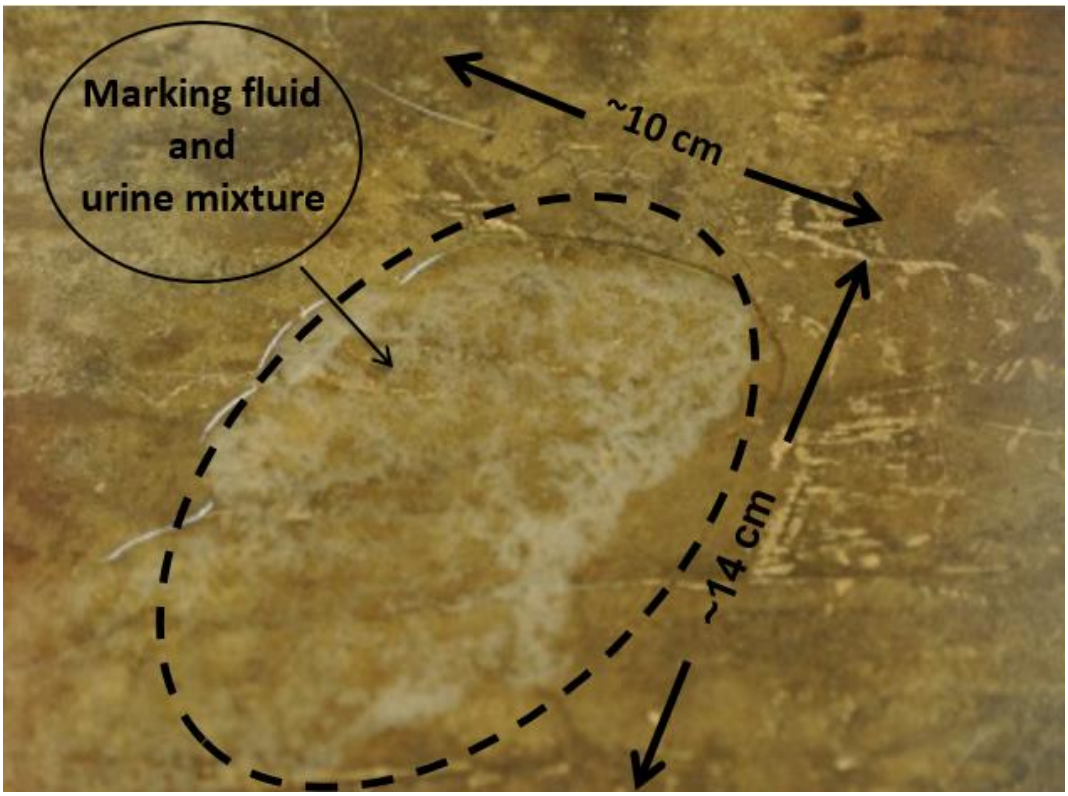

Figure S1A. Lion Marking Fluid. Marking fluid and urine mixture released unto the floor of the indoor enclosure by a male in a squatting downward position. The urine appeared to be yellow in color and the marking fluid had a whitish coloring.

Table S1A. SPME fiber type selection. Fiber types tested for extraction efficiency of characteristic *P. leo* scent marking odor compounds.

Fiber Type	**Size (diameter x length)**	**Target Analyte Description**
**50/30 µm Divinylbenzene/Carboxen/ Polydimethylsiloxane	23 gauge x 2 cm	Broad range of analytes; Flavor compounds; Volatiles and Semi-volatiles; C3-C20 (MW 40-275)
50/30 µm Divinylbenzene/Carboxen/ Polydimethylsiloxane	24 gauge x 1cm	Broad range of analytes; Flavor compounds; Volatiles and Semi-volatiles, C3-C20 (MW 40-275)
65 µm Polydimethylsiloxane/ Divinylbenzene	24 gauge x 1cm	Volatiles; Amines; Nitro-aromatic compounds (MW 50-300)
75 µm Carboxen/ Polydimethylsiloxane	24 gauge x 1cm	Volatile/ low molar mass analytes; Biogenic volatile organic compounds (MW 30-225)

**Fiber type selected for the rest of the study

Table S2A. Effects of extraction sampling time on number of odorous compounds detected. Effect of extraction time (1 min, 1 h, and 24 h) on the number of odorous compounds able to be detected using NHC and HC modes.

MDGC-MS-O Mode	Extraction Time	Mean # of Odorous Compounds Identified	STDDEV	RSD%
No Heart-Cut	1 min	0	0	0
	1 h	5.33	0.47	8.84
	24 h	17.3	1.25	7.2
Heart-Cut	1 min	1	0	0
	1 h	10	0.82	4.41
	24 h	24	0.82	3.4

Table S3A. Summary of all unconfirmed peaks in the chromatogram of *P. leo* MF. Compounds were listed by identifying markers: the top five ions, odor descriptors observed by panelist, and retention time.

No	RT (min)	Top 5 Ions and Their Relative Intensities	Odor Descriptors Observed by Panelists	Measured Odor Intensity
1	2.75	43(99),58(49),41(28),39(24),72(23)		
2	3.18	133(99),73(62),73(32),132(30),59(27)		
3	3.53	57(99),44(33),41(15),58(14),39(11)		
4	3.72	76(99),44(16),32(14),78(7),38(4)		
5	4.13	44(99),56(75),41(60),43(51),57(46)		
6	5.72	30(99),70(5),44(4),41(4),27(3)	Cardboard, medicinal, body odor, rancid, foul	30
7	6.16	43(99),71(67),41(16),114(15),27(11)		
8	6.27	43(99),71(37),41(12),70(8),14(8)	Herbaceous, plastic	80
9	6.57	81(99),80(90),39(22),53(22),42(20)		
10	7.62	43(99),72(81),57(70),41(64),85(29)		
11	7.75	43(99),72(42),41(19),71(15),39(15)		
12	8.03	69(99),55(93),98(68),42(68),56(65)		
13	8.39	81(99),82(26),53(16),138(14),39(7)		
14	8.48	57(99),86(40),71(33),55(26),56(17)		
15	**8.59**	**67(99),54(90),82(90),41(70),81(65)**	**Urinous, sour, animal**	**30**
16	9.20	94(99),67(85),66(20),95(6),68(4)		
17	9.84	41(99),54(68),27(59),55(54	Chemical, cardboard, medicinal, wheat	30
18	10.64	128(99),113(50),99(23),85(13),129(8)		
19	10.79	58(99),135(59),91(49),134(40),196(6)		
20	11.80	122(99),121(82),42(74),39(33),67(23)	Herbaceous, dirt, nutty, earthy	80
21	12.21	73(99),83(26),126(16),111(10),127(1)		
22	12.71	97(99),154(21),98(21),45(5),99(7)	Herbaceous, musty, grassy, earthy, dirt	100
23	13.36	58(99),43(73),71(27),59(24),57(14)	Herbaceous	30
24	13.46	43(99),41(99),57(79),55(55),44(54)		
25	14.03	77(99),106(95),105(95),70(76),202(1)		
26	14.19	83(99),55(73),98(34),139(16),140(2)	Citrus, lemon, fruity	80
27	14.24	55(99),83(88),43(87),29(48),98(46)		
28	14.71	95(99),81(43),124(24),79(20),55(15)		
29	14.79	43(99),56(78),41(61),29(57),57(50)		
30	14.99	58(99),41(5),59(4),43(3),42(3)	Herbaceous, cucumber	60
30	15.46			
31	15.61	73(99),58(79),74(5),59(3),60(1)	Foul, burnt	15
32	16.15	43(99),55(99),41(94),56(82),69(73)		
33	16.25	59(99),31(42),41(42),27(18),29(18)	Herbaceous, potato, nutty,	30

Table S3A continued

34	17.29	43(99),73(33),55(21),41(20),44(20)	Cardboard, green pepper, herbaceous, plastic	30
35	17.70	55(99),41(97),43(84),69(62),57(58)		
36	18.22	58(99),41(5),43(4),59(4),42(3)		
37	18.86	132(99),133(84),118(21),117(17),130(12)	Medicinal, grassy, herbaceous	15
38	19.28	71(99),43(74),56(55),27(54),89(52)		
39	20.01	57(99),41(69),43(58),55(52),67(42)	Waxy, butter	15
40	20.68	55(99),69(82),57(75),83(71),56(67)	Medicinal, chemical	15
41	21.62	96(99),95(88),39(56),38(14),29(14)	Sweet, cinnamon, phenol, meat	15
42	22.06	55(99),69(79),56(69),57(68),83(66)		
43	22.14	43(99),41(83),55(68),67(48),84(45)		
44	22.74	30(99),99(80),42(78),41(72),43(69)		
45	24.51	192(99),91(24),165(22),119(16),65(15)		
46	24.73	83(99),82(28),153(25),55(19),156(19)		
47	25.75	149(99),177(21),76(14),65(12),150(12)	Citrus, lemon	30
48	26.27	135(99),107(38),164(12),136(10),95(10)		
49	26.64	117(99),90(25),89(11),118(94),116(58)		
50	26.96	105(99),77(65),182(48),51(23),181(80)		
51	28.47	170(99),169(60),141(24),115(15),171(13)		
52	28.93	60(99),44(72),17(70),43(26),16(14)		
53	29.08	95(99),67(76),152(54),96(53),55(47)		
54	29.64	114(99),91(53),65(15)		

Abbreviations: No-Number; RT-Retention Time

**Compounds in bold are characteristic compounds

APPENDIX B

SUPPLEMENTARY FIGURES FOR CHAPTER IV

Supplementary Materials: Analysis of Odorants in Marking Fluid of Siberian Tiger (*Panthera tigris altaica*) Using Simultaneous Sensory and Chemical Analysis with Headspace Solid-Phase Microextraction and Multidimensional Gas Chromatography-Mass Spectrometry-Olfactometry

Figure S1B. Prototype of the tiger marking fluid collection system that was attached to the cage of the indoor tiger enclosure areas. (**a**) Exterior portion of the

collection device; (**b**) the lip at the base of the collection device that the marking fluid will drain into (**c**) representative of the placement of the collection system on the case bars; (**d**) side profile of the collection device; (**e**) interior area of the collection device.

Figure S2B. Placement of the tiger marking fluid collection system attached to the cage of the indoor tiger enclosure areas.

Figure S3B. A *Panthera tigris* performing scent-marking behaviors in its outdoor enclosure releasing marking fluid.

Figure S4B. Odor descriptor panel used to characterize the odorous compounds within the tiger markings.

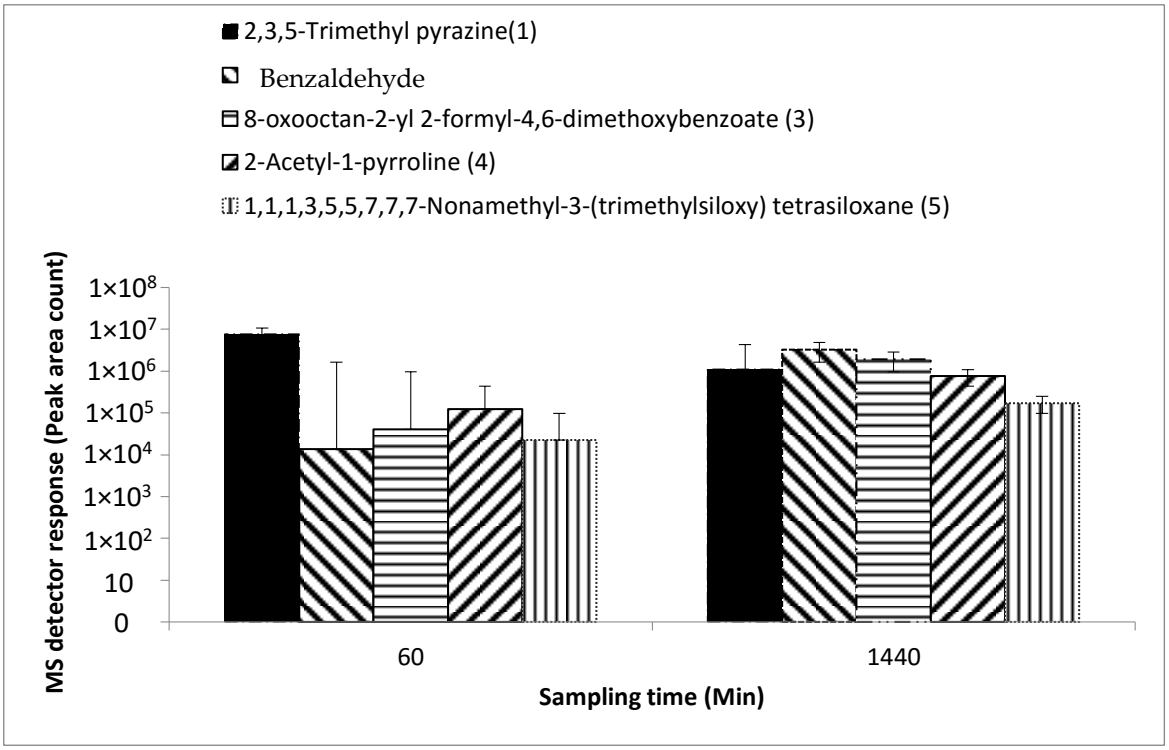

Figure S5B. Effects of SPME extraction time for five odorous compounds released from the marking fluid of *P. tigris altaica* with a 75 µm CAR/PDMS fiber. Extraction time = 60 min, and 1440 min (24 h). Error bars show the standard deviation of the mean (n = 3). Marking fluid (0.25 mL) and a stir bar were inserted into a 2 mL glass vial with a PTFE coated septa for a period of 30 min for equilibration. These compounds were confirmed with the top five ions, odor descriptors observed by panelist not chemical standards.

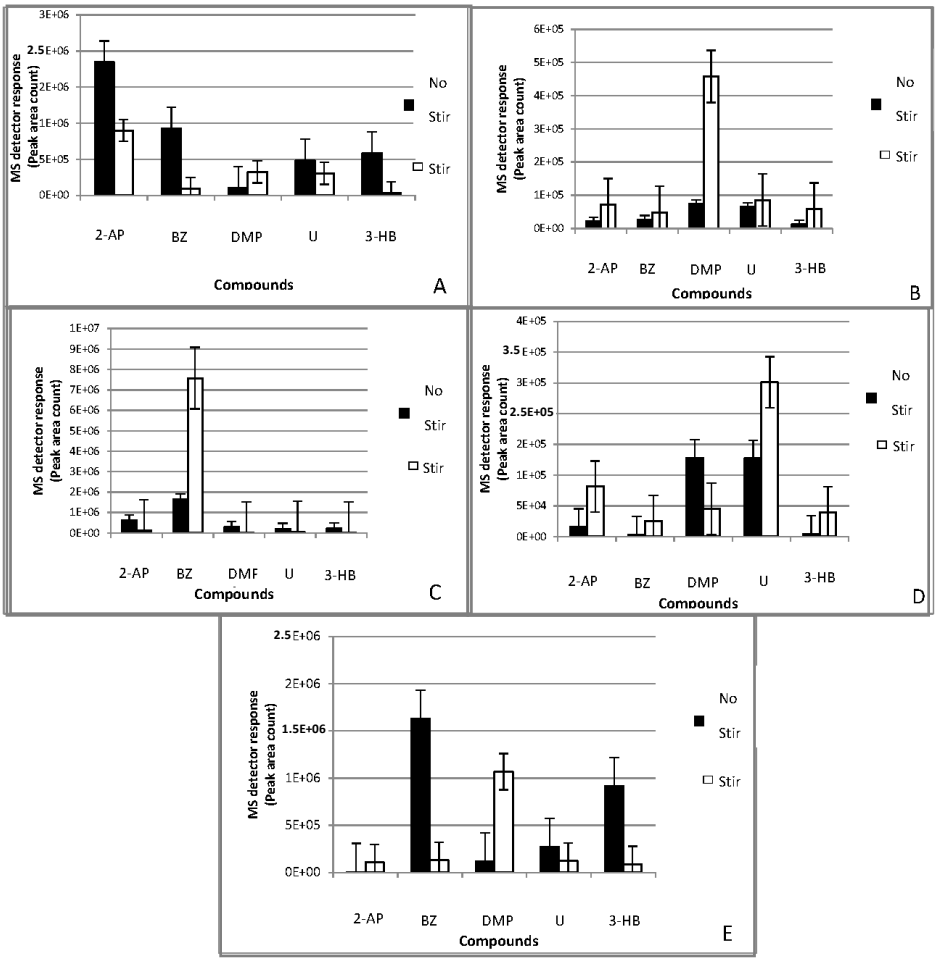

Figure S6B. Effects of agitation 1h extraction, temperature 37 °C, with a 0.25 mL sample. (**A**) 75 µm CAR/PDMS SPME fiber; (**B**) 50/30 µm DVB/Carboxen/PDMS SPME fiber; (**C**) 100 µm PDMS SPME fiber (**D**) 65 µm DVB/Carboxen/PDMS SPME fiber; and (E) 85 µm CAR/PDMS SPME fiber. These compounds were confirmed with the top five ions, odor descriptors observed by panelist not chemical standards. Abbreviations: U = Urea, 2-AP=2-Acetyl-1-pyrroline, 3-HB = 3-Hydroxybutanal, DMP = 2,5-Dimethyl-pyrazine, BZ = Benzaldehyde.

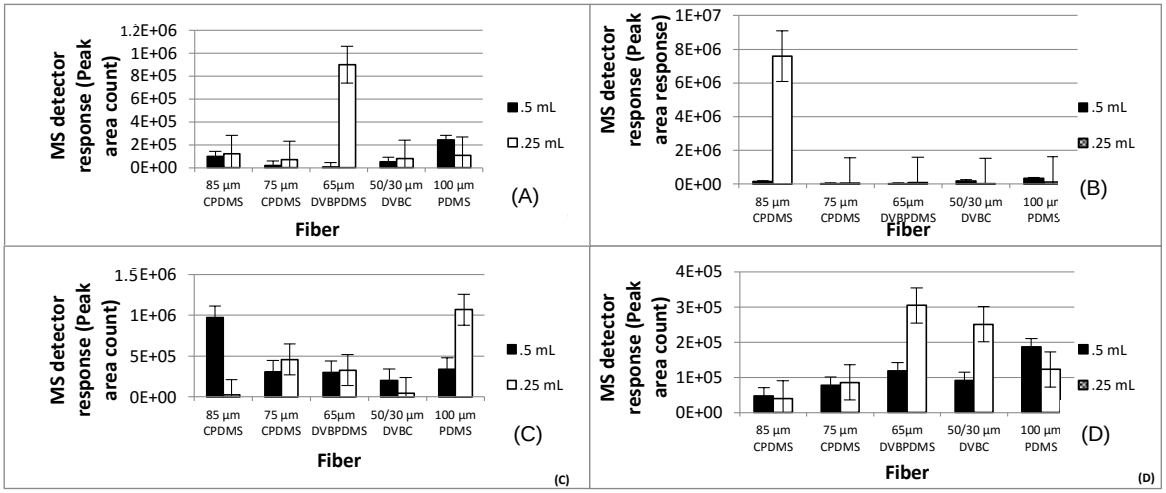

Figure S7B. Effects of sample quantity (0.50 mL and 0.25 mL) for the identification of (A) 2-Acetyl-1-pyrroline, (B) 3-HB = 3-Hydroxybutanal, (C) 2,5-Dimethyl-pyrazine, and (D) Benzaldehyde, key characteristic odor compounds, released from the marking fluid of *P. tigiris altaica*. These compounds were tentatively confirmed with the top five ions and odor descriptors observed by panelist.

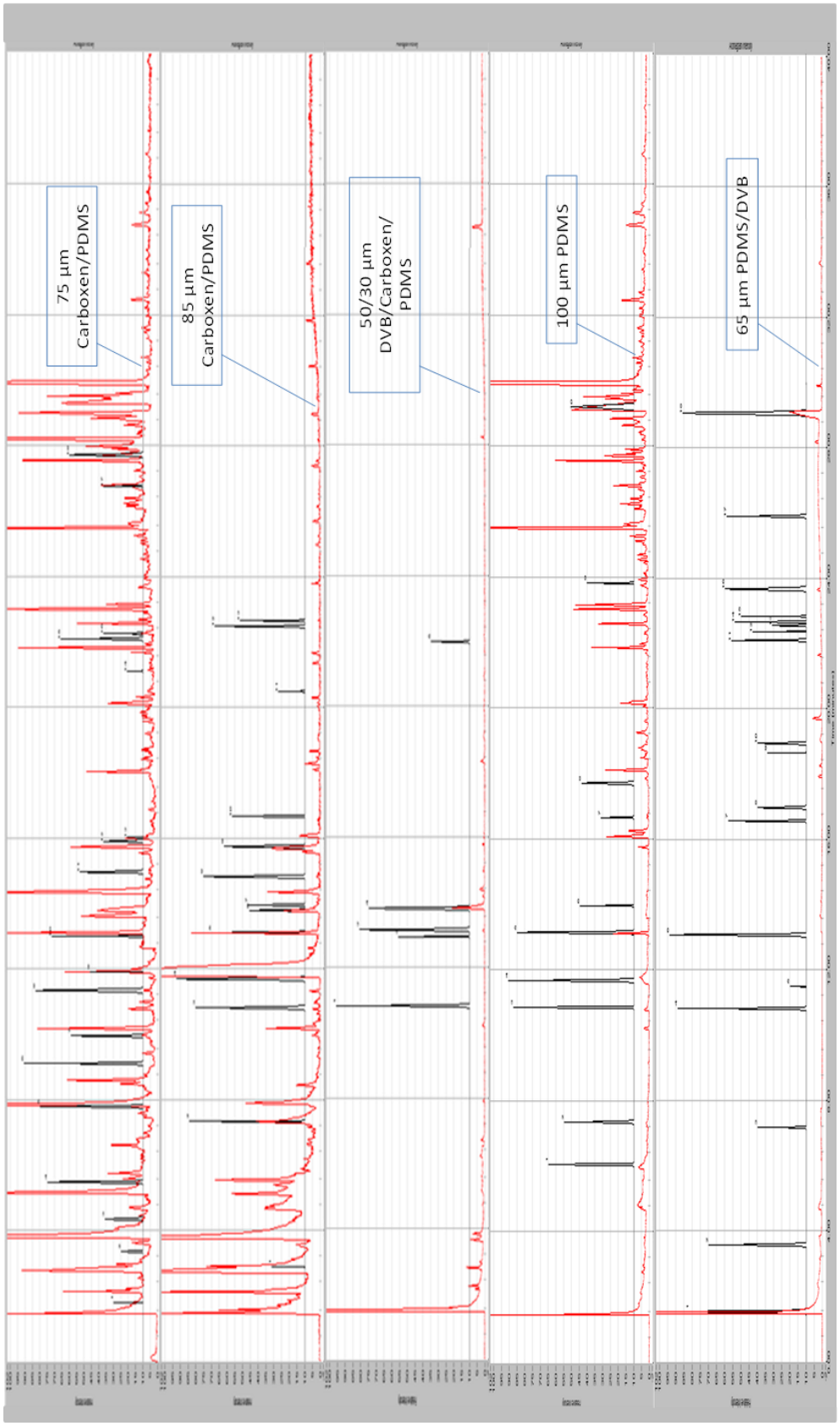

Figure S8B. Comparison of aromagrams and total ion chromatograms of *P. tigris altaica* marking fluid headspace extracted with 85 µm Carboxen/PDMS, 50/30 µm DVB/Carboxen/PDMS, 100 µm PDMS, 65 µm PDMS/DVB coating, and 75 µm Carboxen/PDMS SPME

fibers and analyzed using GC-MS-Olfactometry. Extraction time = 24 h (*n* = 3). Temperature = 37 °C. Samples were analyzed in MS scan mode: total ion scan. Extractions with 75 µm Carboxen/PDMS SPME fiber were associated with the largest number of odorous compounds (14) with a total of 32 odorous events.

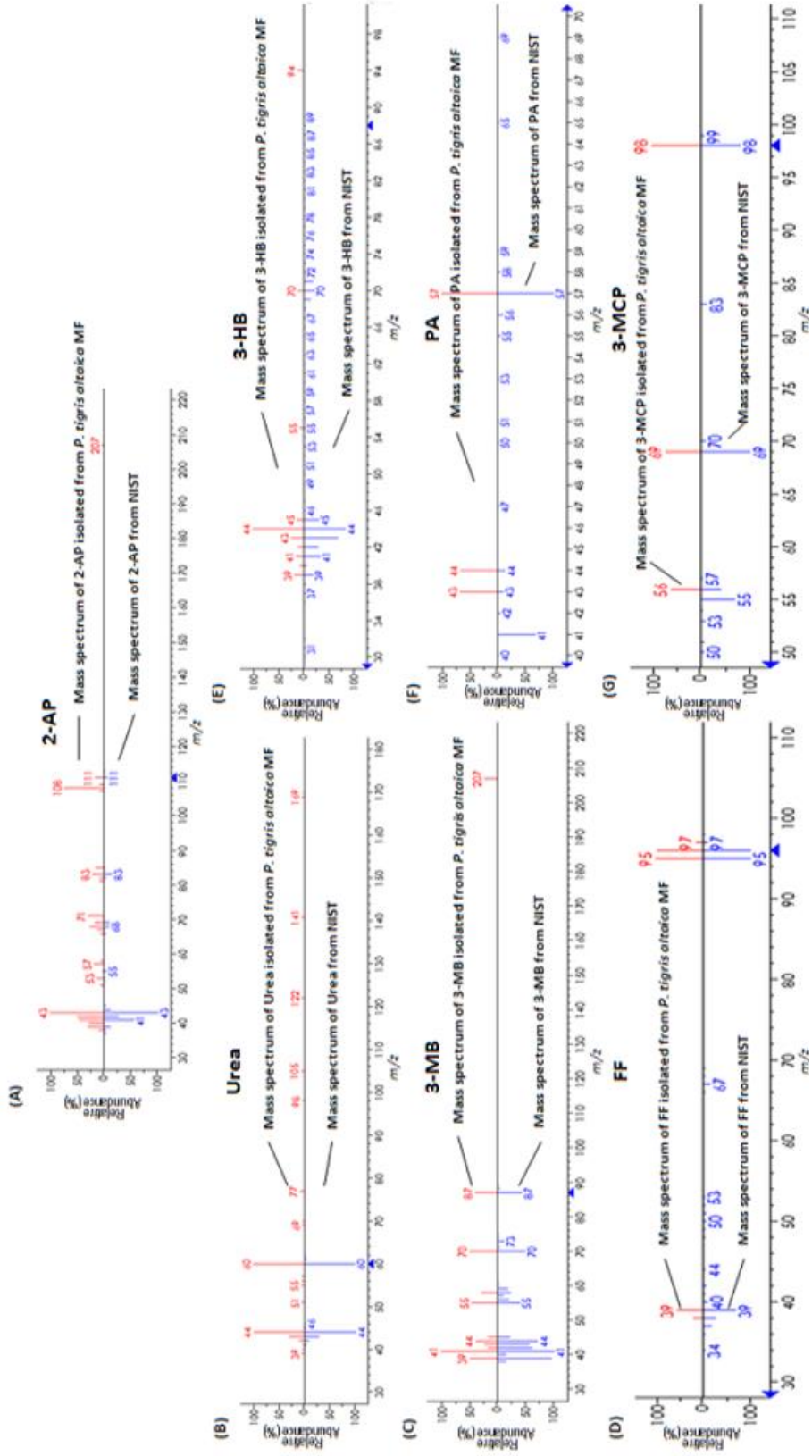

Figure S9B. Identity confirmation of seven compounds responsible for characteristic tiger marking fluid odor through NIST mass spectral library matching ((R)-3-methylbutanamine, 3-hydroxy-butanal, propanedionic acid, and (R)-3-methylcyclopentanone) and chemical standard confirmation (urea, furfural, and 2-acetyl-1-pyrroline). All of the characteristic compounds were selected based on their 'nutty',

‘urinous’, and/or ‘corn-like' aromas. For those compounds not identified with chemical standards, odor panelist and published odor descriptors aided in their suggested identification .(A) 2-Acetyl-1-pyrroline; (B) urea; (C) 3-methylbutanamine; (D) furfural; (E) 3-hydroxybutanal; (F) propanedioic acid; and (G) (R)-3-methylcyclopentanone. Abbreviations: 2-AP = 2-Acetyl-1-pyrroIine, 3-MB = (R)-3-MethyIbutanamine, 3-HB = 3-Hydroxy-butanal, FF = Furfural, PA = Propanedioic Acid, 3-MCP (R)-3-Methylcyclopentanone, m/z = mass-to-charge.

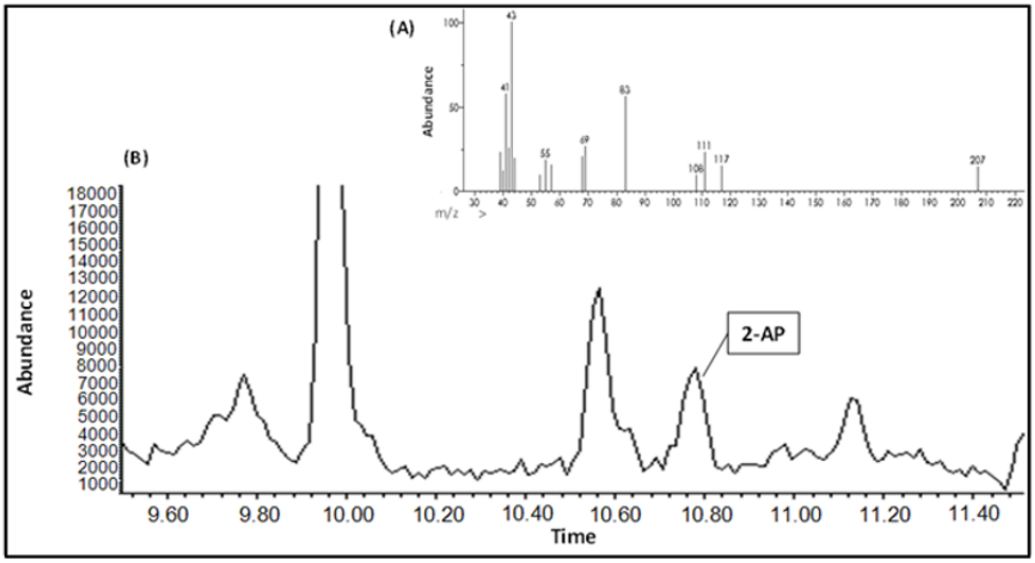

Figure S10B. (**a**) The mass spectrum of 2-AP peak is shown in the upper right comer; (**b**) The mass spectrum of 2- AP isolated from volatiles collected from the headspace of P. *tigris altaica* marking fluid.

Table S1B. Summary of all unconfirmed peaks in the chromatogram of P. *tigris altaica* MF. Compounds were listed by identifying markers: spectral matches with the top five ions, odor descriptors observed by panelist, and retention time. **Bolded entries** are unconfirmed compounds (3-methylbutanamine-RT=7.25 min, (*R*)-3-methylcyclopentanone-RT=8.24 min, propanedioic acid-RT=13.81 min, and 3-hydroxybutanal-RT=5.89 min) that are characteristic odorants of the total aroma of Siberian tiger MF.

No.	RT (min)	Top 5 Ions and Relative Intensities (%)	Aroma Descriptor by Panelist	Measured Odor Intensity (%)
1	4.85	71(99), 43(88), 55(68), 41 (40), 39(30)	Foul, Onion	30
2	**5.89**	**44(99),43(96),41(92),58(82),29(48)**	**Body Odor, Plastic, Urinous, Skunky**	**80**
3	5.98	98(99), 97(92), 71(87), 41 (55), 67(50)	Body Odor, Sour, Skunky	30
4	6.45	267(99), 269(96), 126(63,195(43), 282(37)	Plastic, Smoky	100
5	6.64	43(99), 86(20)41(16), 58(16), 71(16)		
6	6.78	81(99), 80(75), 53(28), 42(23), 39(22)	Foul, Sour	15
7	7.07	43(99), 57(71), 41(41), 71 (38), 85(29)	Grassy, Earthy	80
8	**7.25**	**29(99),44(81),43(66),18 (40),41(32)**	**Skunky, Urinous**	**30**
9	7.48	29(99), 44(65), 15(64), 14(28), 43(18)		
10	**8.24**	**69(99),55(63),42(62),98(62),41(45)**	**Urinous, Foul**	**60**
11	8.99	42(99), 55(94), 41(68), 70(48), 31(36)	Foul	15
12	9.76	55(99), 42(57), 98(56), 41(20), 69(21)		
13	11.21	121(99), 79(23), 120(20), 106(15), 39(5)	Earthy, Grassy, Herbaceous	60
14	11.98	42(99), 122(66), 39(17), 81(16), 40(9)	Herbaceous, Grassy, Earthy, Skunky, Foul, Onion	60
15	12.80	73(99), 281(86), 147(60), 415 (34), 327(33)	Sweet, Fruity	30
16	13.56	57(99), 41(45), 55(36), 43(28), 56(27)	Body Odor, Plastic, Potato, Earthy	30
17	**13.81**	**42(99),45(86),60(77),44 (62),43(62)**	**Skunky, Foul, Urinous, Body Odor**	**30**
18	14.90	57(99),43(72),71(58),85(55),41(28)	Onion, Sulfur	80
19	16.21	30(99), 91(15), 92(12), 121(5), 65(10)		
20	16.58	150(99), 107(75), 108(70), 43(54), 42(52)		

Table S1B Continued

21	16.65	42(99), 28(65), 41(40), 29(49), 27(20)		
22	16.95	136(99), 54(90)	Stale, Sweet, Medicinal, Foul	60
23	18.67	118(99), 91(73), 119(41), 104(31), 132(28)		
24	18.91	207(99), 133(51), 191(85), 177(27), 193(25)	Body odor, Smoky, Unknown	60
25	20.68	57(99), 71(91), 43(78), 85(48), 41(43)		
26	20.93	41(99), 43(90), 29(50), 55(50), 57(48)	Waxy, Sweet	15
27	21.01	94(99), 109(78), 66(54), 39(34), 43(12)		
28	21.52	170(99), 51(52), 77(40), 141(40), 39(30)		
29	21.72	94(99),66(70),39(62),65(50),96(18)		
30	22.36	43(99), 41(95), 39(35), 69(35), 15(30)		
31	22.83	41(99), 55(49), 83(48), 110(47), 43(42)		
32	23.62	121(99),149(58),138(20),196(20)		
33	24.19	120(99), 135(65), 92(54), 65(17), 43(9)		
34	24.23	120(99), 135(45), 92(40), 65(10), 39(2)	Sweet, Fruity, Grape	30
35	24.49	43(99), 58(59), 85(25), 59(27), 41(20)	Sweet, Fruity	15
36	25.86	55(99),70(77),41(61),43(61),29(30)		
37	26.16	43(99), 41(90), 55(85), 57(84), 69(59)		
38	28.34	30(99), 91(36), 43(30), 61(20)40(10)		
39	28.38	91(99), 92(33), 195(24), 194(20), 65(10)		
40	28.70	105(99), 122(95), 77(75), 51(50), 106(15)		
41	28.74	170(99), 169(81), 141(53), 142(18), 115(55)		
42	28.91	31(99), 32(20), 30(10), 29(42), 60(35)	Fruity, Grape, Sweet, Waxy	30
43	29.22	105(99), 77(45), 51(15), 106(5), 50(5)		
44	29.39	91(99), 136(56), 92(30), 65(15), 39(8)		
45	29.53	150(99), 44(58), 166(40), 50(10), 104(8)		
46	29.99	73(99), 60(95), 43(76), 41(50), 57(70)		
47	30.88	44(99), 45(60), 29(22), 52(12), 15(6)		
48	33.79	69(99), 81(55), 41(2), 136(25), 137(24)		

*Abbreviations: No-Number; RT-Retention Time; Bolded lines are tentatively identified characteristic compounds