

CHEMICAL COMMUNICATION

CHEMICAL CHARACTERIZATION OF VOLATILE CONSTITUENTS
OF URINE OF THE SOUTHERN AFRICAN CHEETAH,
ACINONYX JUBATUS JUBATUS, USING HEADSPACE SAMPLING
AND GC-MS

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entire or in part been submitted at any other university for a degree.

R.C. Visser

SUMMARY

The cheetah, *Acinonyx jubatus*, sometimes referred to as “the greyhound of the cats”, is probably the most elegant member of the cat family. Formerly widespread in southern Africa it is now threatened with extinction. It occurs in open savanna and light woodland, but also hilly country on occasion. The cheetah is a predator that can reach speeds of more than 110 km/h in short bursts. With a mass of between only 40 to 60 kg, it is not very powerful and cannot defend itself very effectively against carnivores such as the lion and hyaena, for example. This might be the reason why, in order not to advertise its presence, the urine of this animal is practically odourless. In turn, this might explain why no research has so far been devoted to the urine of this animal. In contrast extensive work has been done on the chemical characterisation of the urine of many other carnivores, notably the lion.

Extraction of the urine with dichloromethane gave too little material for GC-MS. SPME sampling of the urine itself gave insufficient sample enrichment. Better results were obtained when an SPME-type of headspace sampling, using a larger mass of polydimethylsiloxane, was employed although many of the constituents of the urine could still barely be detected. The compounds identified include a large number of ketones, aldehydes, cyclic and acyclic ethers, carboxylic acids, amides two sulphur compounds in barely detectable quantities, and elemental sulphur. Perhaps the most remarkable result of this thesis is that it was found that the urine of the cheetah, although it is a carnivore, does not contain several sulphur compounds in relatively high concentrations. However, it does contain elemental sulphur. Perhaps a mechanism exists by which the cheetah can convert strongly odorous sulphur containing compounds into the less strongly smelling sulphur.

OPSOMMING

Die jagluiperd, *Acinonyx jubatus*, 'n roofdier wat tydens 'n jagtog vir kort tye snelhede van meer as 110 km/h kan bereik, is waarskynlik die elegantste lid van die katterfamilie. Met sy vaartbelynde liggaam weeg die dier egter slegs tussen 40 en 60 kg kan hy homself nie doeltreffend teen groter roofdiere verdedig nie. Chemiese verbindings, sogenaamde feromone, wat onder andere in die uriene uitgeskei word, word dikwels deur diere gebruik vir gebiedsafbakening en om met ander lede van die spesie te kommunikeer. Die uriene van die jagluiperd is egter feitlik reukloos. Tot dusver is die chemiese samestelling van die uriene van die jagluiperd nog nie ondersoek nie.

Ekstraksie van die uriene met dichlorometaan het te min materiaal vir gaschromatografies-massaspektrometriese analise opgelewer, maar bevredigende resultate is verkry deur van 'n verbeterde monsternemingsmetode gebruik te maak. 'n Groot aantal organiese verbindings en twee swavelbevattende verbindings in skaars waarneembare hoeveelhede is in die uriene geïdentifiseer. Daar is egter 'n aansienlike hoeveelheid van die element swavel in die uriene gevind. Laasgenoemde ontdekking, is die mees verbasende resultaat van hierdie navorsing. Die uitskeiding van swavel in uriene is, sover bekend, eenmalig in die soogdierwêreld. Dit is moontlik dat die jagluiperd oor 'n meganisme beskik om swavelverbindings wat baie sterk ruik om te sit na swavel wat nie so sterk ruik nie. Hierdie meganisme sal die dier se kanse op oorlewing verbeter deur van die swavelbevattende verbindings in sy dieet ontslae te raak sonder om sy teenwoordigheid in die omgewing te adverteer.

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CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1 GENERAL INTRODUCTION

The core idea of ecological sustainable development as a moral and political concept is environmental protection and respect for life, a concern about ecological limits and the manner in which we impact on natural systems sustaining our lives. This can be extended to respect for life in general. Article 24 of the constitution of South Africa states the following [1]:

“Everyone has the right to an environment that is not harmful to their health or well-being and to have the environment protected for the benefit of present and future generations, through reasonable legislative and other measures that promote conservation and secure ecologically sustainable development and use of natural resources while promoting justifiable economic and social development.” [1]

To appreciate the influence of the above-mentioned environment on every living creature, it is important to define the nature of such an environment. Within the two major environments – the land and sea – and in almost every corner of the planet, from the highest to the lowest, the warmest to the coldest, above the water and below, the biological conditions have gradually altered as evolution acquired its unique biotic communities [2]. The biotic community is a dynamic system of interacting plant and animal populations [3]. As the growing human population’s economical and social demands and developments rapidly increase, sustainable interaction with the natural environment becomes crucial. As a result of these transformations, few species are free from some kind of pressure, whether it is from other species in the form of predation, competition for the same resources, parasitism or from human governance. Nature’s way to deal with these pressures was, to a great extent, through the process of natural selection [4]. Evolutionary adaptations have enabled living organisms to prevail over the ever-present problem of survival, creating a competitive environment for the “survival of the fittest”.

The whole process of communication is subject to the pressure of natural selection. Organisms must be able to communicate with their environment in order to maintain their place in an ecosystem. Their senses are designed for the maintenance of close contact between both the biotic and abiotic environment. They must be able to sense physical and chemical stimuli from the environment and respond to them [3].

"Infrasound" and ultrasound arbitrarily denote those frequencies of sound waves beyond the range that humans are able to hear. Likewise, the narrow "visible" (to Homo sapiens) section of the electromagnetic spectrum is bounded by "infrared" and "ultraviolet" that, for instance, snakes and honeybees respectively are able to perceive. For communication in the animal kingdom, the majority of organisms depend more on olfaction than on vision or hearing and are mostly richly endowed with scent-producing organs and chemoreceptors. Odour perception of animals is so alien to microsmatic man that we cannot even guess how many compounds there are that provide a clear signal to animals at concentrations that are not detectable by humans. The primary role of olfaction is to make the detection of food and predators possible. Its use in communication is, in evolutionary terms, a secondary development although it is of fundamental importance in the creation and maintenance of social organisation of animals and in the control of many facets of behaviour [5]. In mammals and especially in insects, the olfactory sense is extremely sensitive and appears to be highly developed in almost all terrestrial orders [6].

Chemical ecology is the study of the interactions of organisms within their environment mediated by the chemicals they produce [7].

1.2 CHEMICAL COMMUNICATIONS BETWEEN MAMMALS

1.2.1 Mammalian semiochemistry and the pheromone concept

Studies of mammalian chemical communication have increased dramatically since 1959. New terminology of the various concepts was coined over the years as knowledge in this field expanded.

The term **semiochemical**, derived from the Greek *semeion* (a signal or mark) and *chemeceia* (alchemic) was proposed by Law and Regnier in 1971 as the general term for all chemicals carrying messages between organisms [8], subdivided into intra- and interspecific chemical signals (Figure 1.1). Semiochemicals that operate in an intraspecific manner (between members of the same species) have commonly been labelled **pheromones** [6]. The name pheromone is derived from the Greek *pherein* which means to transfer and *hormon* which means to excite [9]. These are chemicals emitted by one member of a species which, when detected by another member, result in behavioural or physiological changes that are likely to benefit both individuals. Pheromones are divided into two main categories according to their mode of action, *viz.* *signalling* (releasing) and *primer* pheromones. Signalling pheromones are stimuli that enable an individual to discriminate between different classes of individuals such as familiar *versus* strange, dominant *versus* subordinate, male *versus* female, oestrous *versus* non-oestrous or own *versus* different species. Signal pheromones also set in motion the appropriate behaviour, such as sexual attraction, aggressive behaviour or avoiding a territory. By contrast, primer pheromones set in motion endocrine responses that show their effects much later. The best known primer pheromones are those that affect sexual maturation and the oestrous cycle. An example of these pheromones is found in the urine of house mice, *Mus musculus* [10].

Semiochemicals that provide signals between species (interspecific) are termed **allelochemicals**. If such an allelochemical is primarily of benefit to the emitter, as in the defence secretion of skunks (odour of butyl mercaptan) [11], or in venomous animals, it is designated an **allomone**, while **kairomones** are of adaptive value to

the receiving organism [12]. The sex pheromone of the green stinkbug, *Nezara viridula*, used by a parasite, the tachinid fly, *Trichopoda pennipes*, to locate its host is an example of a kairomone [13].

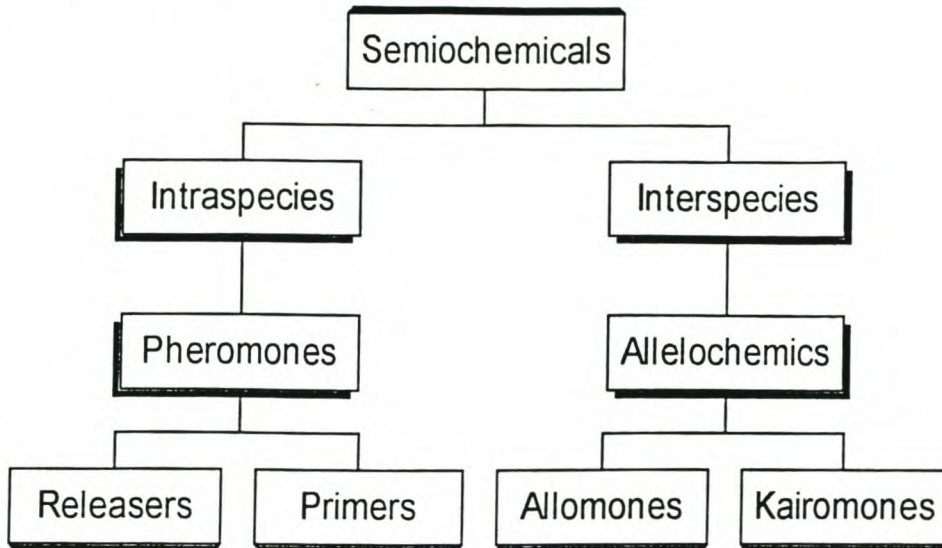


Fig. 1.1: The pheromone concept

Unlike insects, mammals are not known for communicating with single species-specific compounds that by themselves or in combination with only a few other compounds have unique behavioural effects. Instead of using complex molecule structures, mammals most often achieve complexity by blending many compounds into one functional pheromone [12]. This versatility is of great meaning for the diversity of life [11].

1.2.2 Odour-guided behaviour in mammals

An odour is the sensory stimulation by a group of molecules [5]. Odorous communication between mammals serves many functions [14]. Information known to be transmitted in these chemical signals and the effects of these signals on the receiving or releasing organisms will be briefly discussed.

- *Olfactory recognition of species and subspecies:*

Perhaps the most basic discriminatory task for an animal is to identify members of its own species, using individual or combined signals in all sensory modalities [15]. Species-specific and strain odours, as well as community odours, probably arise from several sources. Firstly, the active components of scent gland secretion or urine and faeces may differ due to genetically controlled metabolic variations for a species or population. There might be differences, not only in the chemical composition of secretions, but also in the ratios of the active components within a substance. Secondly, genetic differences in the production of these chemical substances can also add to community-specific odours. Thirdly, variations may result from environmental factors; populations in different habitats are likely to vary their diet and differences in waste products are to be expected [17].

In rabbits, *Oryctolagus cuniculus*, the dominant male marks group members with glandular secretions and in some rodents the males often urinate on conspecifics, especially females, during social encounters. With one individual marking all the others, some homogeneity of odour may be produced [17]. Laboratory experiments have been carried out with bank voles (*Clethrionomys*) where the males were confronted with odours of females of different species, recognising and preferring the odour of a female of their own species, without exception. On the level of subspecies, the results varied. In one instance the males preferred females of their own subspecies, but in three other subspecies no preferences were observed. Among ungulates, two subspecies of *Odocoileus hemionus*, namely the Rocky Mountain mule deer (*O.h.hemionus*) and the black-tailed deer (*O.h.columbianus*), respond to tarsal scent of their own subspecies rather than to the scent of the other subspecies [15].

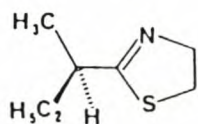
- *Olfactory recognition of sex:*

There are numerous examples of recognition of sex odours among animals. Often only males possess specialised skin glands and consequently a particular odour such as those of the subauricular and dorsal glands in the male pronghorn, while the odour indicating oestrus is confined to the females. In the golden hamster, experimental transfer of vaginal secretions of intact females onto castrated males

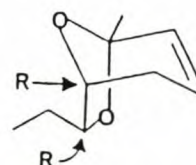
or ovariectomised females caused other males to treat these experimental animals like intact females [16]. Browsers and Alexander [18] have shown that mice can identify the sex of a conspecific by odour. It has also been demonstrated that duikers, *Cephalophus maxwelli*, black-tailed deer, *Odocoileus hemionus columbianus*, the collared peccary, *Dicotyles tajacu*, and the brown bear, *Urses arctos*, respond differently to the odours from glandular secretions depending upon whether the secretion is from the same or the other sex [17].

- *Olfactory recognition of physiological state:*

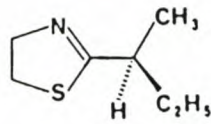
Le Magnen [19] showed that male rats prefer the odour of receptive females to that of non-receptive females. The olfactory discrimination between oestrous and non-oestrous females has been known in dogs for a long time [15]. Odour of male urine in mice accelerates sexual maturity in females but inhibits that of young males. The four major volatile urinary constituents responsible for these processes were identified as (*R*)- and (*S*)-2-sec-butyl-4,5-dihydrothiazole and (*R,R*) and (*S,S*)-3,4-dehydro-*exo*-brevicommin (Figure 1.2). A question of some interest is what is the relation of this type of isomerism to biological activity in mice and to what extent does this stereospecificity affect their chemical signalling. The answer in this specific case is still unknown but it could be a very important question since stereospecificity of various proteins is known, including the ability of humans to distinguish the odour of different enantiomers of some compounds [20]. The urine of female mice has different affects on other mice, depending on the physiological condition of the donor female. If, for example, food is scarce, responses to the different effects appear to be ecological adaptations to environmental conditions and serves as a mechanism to bring the rate of reproduction in balance with available resources [10].



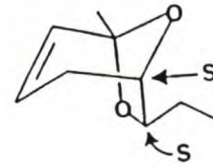
(*R*)-2-sec-butyl-4,5-dihydrothiazole



(*R,R*)-3,4-dehydro-*exo*-brevicommin



(S)-2-sec-butyl-4,5-dihydrothiazole



(S,S)-3,4-dehydro-exo-brevicomine

Figure 1.2: Four major volatile constituents in the urine of male mice.

- *Group odours:*

Mammals that touch each other frequently and/or nest in communal sites, dens etc. invariably exchange odours and thus produce a group odour. In addition to accidental transfers of odoriferous material, stereotyped behaviour patterns ensure the spread of glandular secretions or urine within a population. This behaviour seems to be important for the maintenance of social bonds. Sugar glider males, *Petaurus breviceps papuanus*, rub their frontal glands against the sternal glands of females in their group, producing a clan-specific odour [21]. African dwarf mongooses, *Helogale undulata rufula*, mark one another with their anal glands [22]. Female rabbits will tolerate their own young, harass those of other females in the same colony and kill kittens of another colony. Olfactory cues are most important in this behaviour [23].

- *Recognition of individual odours:*

It appears that every individual animal presents a unique chemical pattern by which it can be identified. Various sensory modalities, such as posture, gait and the combination of smell, will contribute to individual recognition. In a group of black-tailed deer, hostile encounters particularly between strangers, may start with a sniffing of the tarsal organs of another individual [24]. Pronghorn males, *Antilocapra americana*, mark their territories with their subauricular glands and maintain dung sites, where pawing, urinating and defecation take place. They can also clearly distinguish their own dung from that of other males with whom they are familiar [25]. In a number of mammalian species, mothers recognise their young by their olfactory sense. Contact with the young during nursing, frequent

grooming by the mother and transfer of the mother's saliva onto the young, may maintain the similarity in odour between them [26].

There are other functions of olfactory signals in mammals. Examples are odours which trigger avoidance or defensive responses and odours that can lead to the discrimination of age or the identification of mood and social status between members of a population.

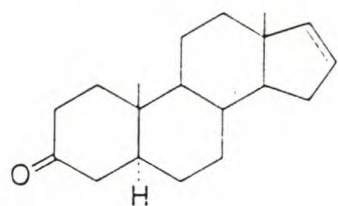
1.2.3 Sources and detection of mammalian chemosignals

The factors that modulate a signal source can be summarised as hormonal, bacterial, genetic, dietary and environmental. The hormonal state of an animal influences not only secretion production, but also its response to semiochemicals [27]. Compounds that serve as signals originate in the following major sources: specialised glands, such as skin glands, salivary glands, accessory glands and anal glands, in urine, faeces, birth fluids or genital secretions and may also be produced by micro-organism [28].

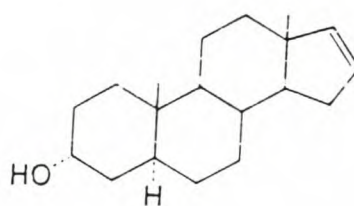
There are two main types of skin glands, flask-shaped sebaceous glands and tubular suboriferous glands. Scent organs containing sebaceous glands produce oily secretions that release volatiles slowly, are long lasting and are used to mark objects and conspecifics. In contrast, suboriferous glands produce watery volatile secretions that are involved in short-term signalling. The most specialised scent-producing organs contain both types of glands [29].

In the secretion of the submaxillary salivary gland of male swine, two steroids, 5α -androst-16-en-3-one and 3α -hydroxy- 5α -androst-16-ene (Figure 1.3), are emitted during courtship and cause the sow to assume the mating stance [10]. These substances also occur in the apocrine sweat glands of the boar's skin [30]. What is interesting, is that these pheromones are also found in large quantities in human armpit secretion and is structurally closely related to testosterone. In the sub-auricular gland of the male pronghorn the components of scent-marking secretions

include isovaleric acid, which occurs with 2-methyl-butyrac acid, 12-methyl-1-tetradecanol, 12-methyl-1-tetradecanol, and the four corresponding esters [31].



5 α -androst-16-en-3-one



3 α -hydroxy-5 α -androst-16-ene

Figure 1.3: Compounds of the secretion of the submaxillary salivary gland of the boar

The potential contribution of bacteria to the generation of mammalian semiochemicals is increasingly being realised. The mammalian body surface provides a variety of habitats within which micro-organisms flourish by utilising and transforming the mammal's own secretions and excretions. Mammalian semiochemicals may in this manner be formed by the microflora which its body supports [27]. The best investigated example is the production of the odours in the anal glands of the red fox, *Vulpes vulpes* [32]. This organ contains high concentrations of odorous volatile fatty acids (C2 to C6), ammonia, trimethylamine, 1,4-diaminobutane and 1,5-diaminopentane, all of which are known to be microbially produced by anaerobes, including *Clostridia* [30]. In rabbits, *Oryctolagus cuniculus*, fatty acids, especially acetic and isovaleric acids, occur in the secretion of the inguinal glands and other rabbits respond to the odour of these acids with increased heart rates [28].

Urine can contain signalling chemicals that stimulate or inhibit aggression and factors that not only signal sex and breeding status but also accelerate and delay sexual maturation in both sexes and influence oestrus cycles and male mating behaviour. The urinary volatiles of tigers and leopards were studied by Brahmachary and Dutta and compounds such as phenylethylamine, cadaverine and putrescine were found in the urinary volatiles of these mammals and suspected to be part of the pheromonal system [33].

Faeces appear to carry chemical signals, some due to diet-dependent metabolites, as in the maternal pheromone of the rat, *Rattus norvegicus*, while others are added to the faeces by the anal glands, as in the "marking pellets" of rabbits [10].

Birth fluids seem to be important in the initial attachment of a mother to her newborn young while she sniffs and licks them. Mammals may also communicate with odours that are derived directly from the environment. Rats can, for example, communicate to colony members the nature of distant food sources with traces of odours that cling to their body [10].

In mammals, chemical stimuli are received by five different "olfactory organs", in addition to the sense of taste [34]. These are the olfactory epithelium, the vomeronasal organ, the trigeminal nerve, the terminal nerve and the organ of Masera. The olfactory epithelium and vomeronasal organ are probably the most important for pheromone communication [6] and are remarkably uniform in the mammalian species [35].

The olfactory epithelium is the layer of cells covering the surface of the nasal cavity, extending up the nasal cavity into the skull area. It is the tissue with which the odour molecules come into contact when air flows through this cavity. It has a geometry which enlarge its surface area (Figure 1.4). The olfactory bulb, a nerve ganglion, is the consecutive component in the system. It is situated in the skull, beyond the olfactory epithelium, and forms part of the limbic system of the brain. Nerve fibres, named axons, from the olfactory epithelium run in bundles to the olfactory bulb where synapses (connection between two nerves where messages are transferred) occur, resulting in axons which then extend deeper into the part of the brain where scent messages can be interpreted [36].

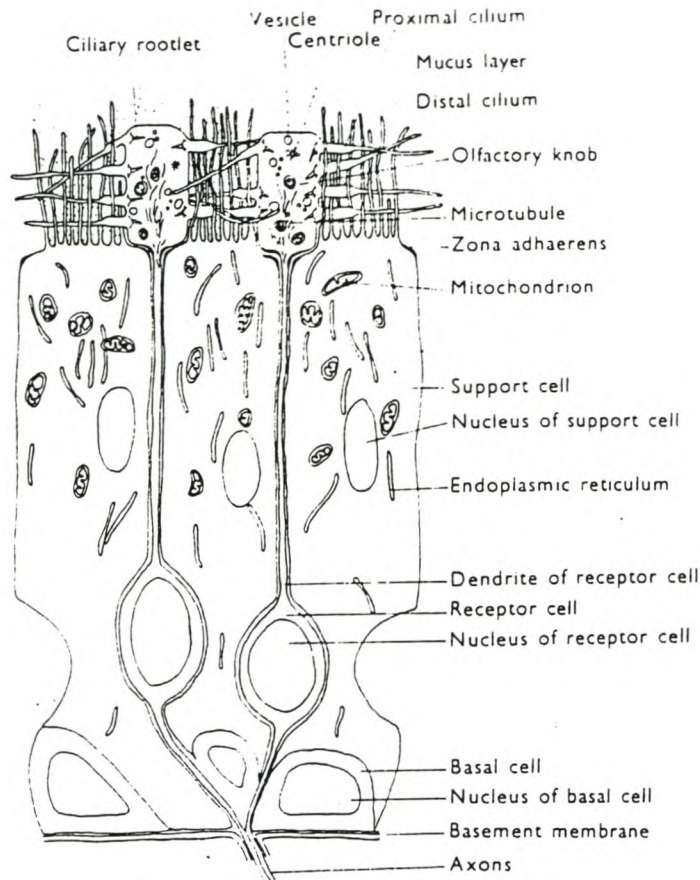


Figure 1.4: Schematic cross-section of the olfactory epithelium, illustrating its main components [36].

The organ of Jacobson (Figure 1.5) is another chemical sensory system found in most terrestrial species, with the exception of bats and certain primates. It is described as a pair of elongated fluid-filled sacs which are lined with receptor cells and which open anteriorly to connect with the palate *via* the nasopalatine duct. This organ is believed to detect chemicals independently of taste and olfaction [36].

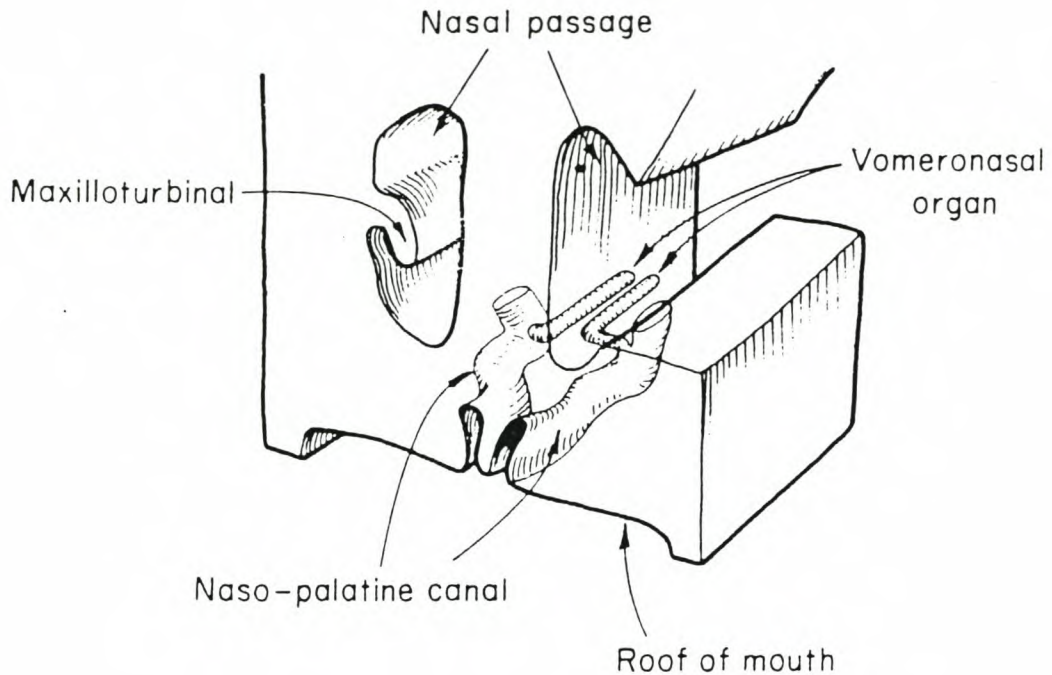


Figure 1.5: Diagram of the organ of Jacobson and the nasal passage of the hedgehog.

The morphology of the mammalian olfactory system is well known, but the manner in which the olfactory system operates, is not fully understood. To quote Moulton, “the molecular bases of our ability to detect and recognise odours are the central problems in olfaction. We have no accepted descriptions of these processes. We lack a full understanding of even the most elementary aspects of receptor function” [37].

1.2.4 Molecular insight into chemical signalling in mammals

Volatile compounds play the most important role in nature as means of guiding olfactory behaviour. It was recognised that *volatility*, hence molecular weight up to around 300-400, is a chief requirement for most airborne mammalian pheromones, even though the required degree of volatility will depend on function and therefore vary considerably [38]. The widespread importance of, for instance, terpenoids as agents of chemical communication both between and within species probably results from their volatility combined with the extraordinary structural diversity [29]. Mammals

also use some higher-molecular compounds as pheromones that are detected at short range by contact or licking [38].

Specificity is very important; a molecule must be of limited distribution or ubiquitous compounds that blend to become very specific chemical signals in the form of odour mixtures. Mammalian pheromones are often found in a *matrix* of fixatives that releases them slowly and thus counteracts their volatility. For example, sebum on the skin or in a scent mark serves that purpose [38].

Whether pheromone molecules are *soluble* in water or in lipids is a critical characteristic for all phases of communication, from the production by the sender to reception by the conspecifics. Compounds that are water soluble are found in apocrine gland secretions and will be captured by mucus in the nasal cavity, while lipid-soluble compounds that are mixed into sebum provide scent marks that have to survive unpredictable environmental conditions [28]. Their release from the mark can be slowed down by controlled release substances, such as squalene which is normally present in sebum in large amounts [32]. For example, the water soluble secretion of the metatarsal glands of the black-tailed deer, *Odocoileus hemionus columbianus*, has an airborne odour that serves as an alerting stimulus to group members. These glands are more developed in arid climates and may be absent under humid conditions. This suggests that the function of its secretion would be de-emphasised in a humid environment [28].

The mammalian olfactory system can recognise and discriminate between a large number of different odourant molecules [39]. These molecules may be considered as molecular *ligands* that bind with specific receptors in olfactory sensory neurons to give a sensory response [40]. In other words, the molecular shape of an odour molecule is highly important and should fit the shape of the receptor [41]. Subtle alterations in the molecular structure of an odourant can lead to profound changes in perceived odour. There are examples where mammals discriminate between *geometric isomers* and between *enantiomers* of pheromone components. In black-tailed deer, *Odocoileus hemionus columbianus*, (*Z*)-4-hydroxy-6-dodecenoic acid lactone from urine is a constituent of the tarsal scent, carried on the hocks by both

sexes and elicits approach when followed by another deer. Black-tailed deer responds to the *Z* isomer, but not to the *E* isomer of this compound [28].

The average human being, it is said, can recognize up to 10 000 separate odours. Amoore has done laboratory experiments on six primary odourants of the human sense of smell, namely isovaleric acid, 1-pyrroline, trimethylamine, isobutyraldehyde, 5 α -androst-16-en-3-one and ω -pentadecalactone. Primary odourants are odours from which all other complex odours could be reproduced by appropriate mixtures. He found significant corresponding primary odours and according to Amoore there is evidence that mammals possess many of these primary odour sensitivities. The results are summarized in Table 1.1 [42].

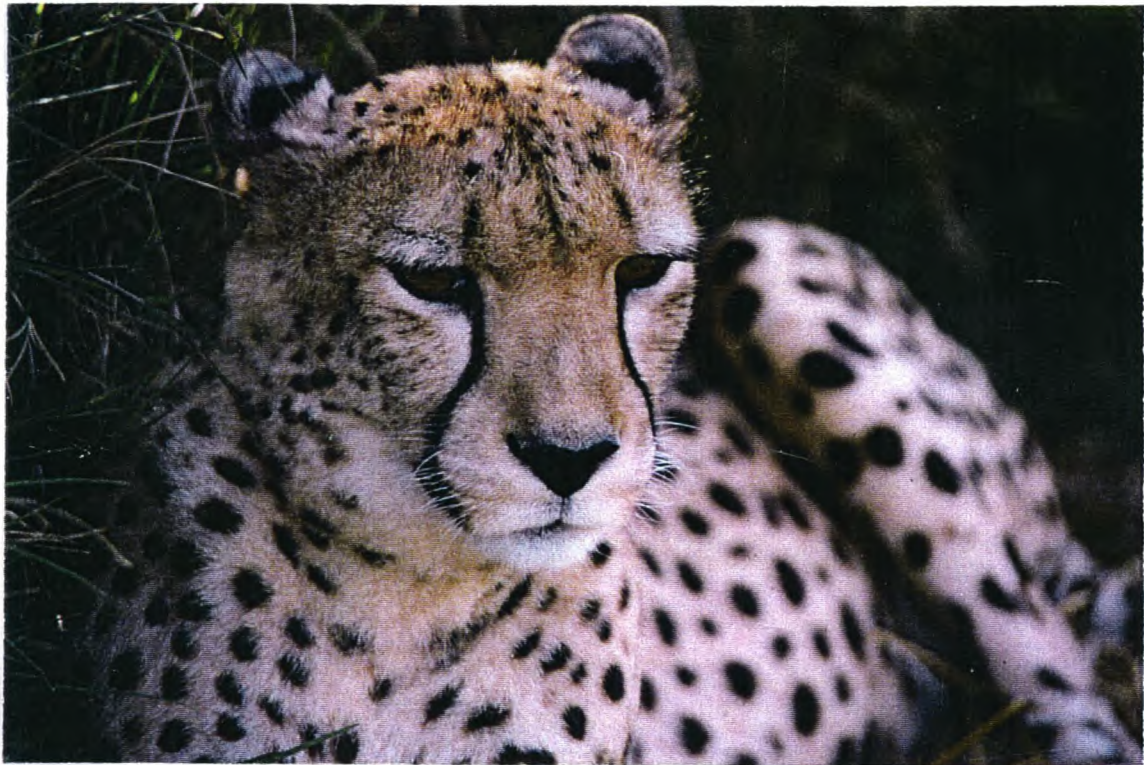
Table 1.1: Olfactometric data on six of the primary odourants [42].

Primary odour	Primary odourant
Sweaty	isovaleric acid
Spermous	1-pyrroline
Fishy	trimethylamine
Malty	isobutyraldehyde
Urinous	5 α -androst-16-en-3-one
Musky	ω -pentadecalactone

We are surrounded by odourant molecules that emanate from trees, flowers, earth, animals, food, industrial activity, bacterial decomposition, other humans and we many times fail to describe that which we smell. In the past we have placed such low value on olfaction that we have never actually developed a proper vocabulary for it. Odours can also not be measured on the kind of linear scale that scientists use to measure the wavelength of light or the frequency of sound. Randall Reed, an HHMI investigator at Johns Hopkins University School of Medicine said that it would be nice if one smell corresponded to a short wavelength and another to a long wavelength, such as rose versus skunk and you could place every smell on this linear scale, but there is no smell scale since odourant molecules vary widely in chemical composition

and three-dimensional shape [43]. And it adds to our appreciation of the spectacular and fascinating world we live in.

1.3 CHEETAH (*Acinonyx jubatus jubatus*)



1.3.1 Description and specialization

The cheetah has a slender body with semi-retractable claws and a non-retractable dew claw with which it trips prey during a hunt. The shoulder height of an adult cheetah is about 73 cm and the average weight about 34-54 kg. The male is slightly larger than the female. Its coat is a tan colour with small round black spots and the fur is short and coarse. The cheetah has a small head with high-set eyes and black "tear marks" which run down from the corner of the eyes. These marks serve as a rifle barrel to keep the sun's reflection from their eyes and aid in hunting. Cheetahs can see up to 5 km in detail.

The cheetah's enlarged heart, increased lung capacity, oversized liver, flexible spine and thin, muscular body make this cat the swiftest hunter of Africa. The cheetah can

reach a speed of 110 km/h in seconds, covering eight meters in a stride and accelerates to 80 km/h in three seconds.

Cheetahs make chirping sounds and can hear over a distance of 3 km. Cheetahs, like pumas, are not anatomically developed to roar but purr very loudly.

1.3.2 Distribution

Once found throughout Africa and Asia, this species is now only scattered in Iran and in small populations in sub-Saharan Africa. Less than 15 000 cheetahs remain in 26 African countries and Namibia has the world's largest numbers of cheetah. Home range for males is on average 800 to 1500 km² and 1500-3000 km² for females.

1.3.3 Habitat

Cheetahs thrive in areas with vast expanses of land where prey is abundant. In southern Africa, cheetahs are found in a variety of habitats including grasslands, savannahs, dense vegetation, mountain terrain. Ninety-five percent of the cheetahs in northern Namibia live on commercial farmlands.

1.3.4 Diet

Evolution has favoured speed and not strength for this species. Therefore they do not hunt prey which are larger or stronger than themselves, but small antelope, young of larger antelope, warthogs, hares and game birds. On rare occasions two males from the same litter will team together and hunt larger antelope.

1.3.5 Reproduction and life expectancy

Sexual maturity occurs at 20-23 months. The gestation period is about 95 days and the average litter size is 4-5 cubs. The female does not have a periodic cycle but decides when she wants to come into season. There are a few factors that determine

this phenomenon; her environment must be safe, there has to be a sufficient food supply and the male must be genetically fit to produce healthy offspring. Because they breed poorly, cheetahs suffer from a lack of genetic diversity making them more susceptible to disease and decreasing reproduction.

Studies have not been conducted on longevity in the wild. In captivity the average life expectancy is 8-12 years. Cub mortality is high for both wild and captive cheetahs and on average, 30% of all cubs born in captivity die within the first month after birth. In the wild, due to competition with larger predators, 90% of cubs die before reaching three months of age.

1.3.6 Behaviour and scent marking

Cheetahs have a unique, well-structured social order. They are territorial and mainly solitary animals. Females live alone except when they are raising their cubs for a minimal period of 18 months. During this time the female teaches the young all the hunting and survival skills. At 18 months the female leaves the cubs, who then form a sibling group which stays together for another 6 months. At about two years of age, the female siblings leave the group. Males can live alone or in coalitions made up of brothers from the same litter which aid in hunting and breeding.

Cheetahs are diurnal, hunting during cool mornings and early evenings. They approach their prey by stalking it until it is within reach of 10-30 metres and only then the chase begins. Cheetahs run their prey to exhaustion, trips it with their dew claw and suffocates the prey by biting the underside of the throat. Cheetahs normally hunt twice a week.

Cheetahs have several means of demarcating their environment. Males usually spray urine onto objects, leaving a scent of their presence. It has also been observed that territorial males excrete a white mucoid fluid, often been termed anal gland secretion, but it is in fact, released through the urinary channel, while the anal gland is likely to "mark" faeces only [45].

Defecation also occurs, but it was found not to be for territorial purposes. Cheetahs have a tendency to rub the sides of their faces (facial glands) as well as their body against objects, leaving a trace of body-odour. This is indeed a very catlike behaviour. In southern Africa, cheetahs exhibit a strong drive to visit playtrees or newspaper trees (usually Camelthorn trees) regularly and often leave urine and faeces on them as markers [44]. Research into this unique behaviour has indicated that cheetahs probably leave messages related to territorial marking and reproductive status in the form of scent on these sloping trunks for other cheetah to interpret (see picture below).



A cheetah in a playtree at CCF (Cheetah Conservation Fund), Otjiwarongo, Namibia

1.3.7 Conservation issues

The cheetah is Africa's most endangered wildcat (endangered under the United States Endangered Species Act, listed on CITES Appendix 1). Almost thirty years ago there were approximately 100 000 cheetahs, free-ranging and captive, across the world. Today only 12 400 cheetahs remain in 26 African countries and 200 cats have survived in Iran. Namibia has the world's largest number of cheetahs, yet only 2400 remain in the wild. This tremendous decrease can primarily be ascribed to the following two reasons:

- invasion of the cheetah's natural habitat by farmlands and agricultural cultivation
- demarcation of natural ecosystems into reserves which leads to inevitable competition between cheetahs and other larger predators, decline of prey, poaching and shooting of the cheetah as a livestock predator.

This sleek hunter of the African wild could statistically be extinct within the next 10 years.

1.4 OBJECTIVES OF THIS STUDY

Cheetahs are exposed to severe competition for survival. This vulnerability is due to the fact that they lack the physical (muscularity) ability to guard their territory and protect their young against larger predators. However, cheetahs have developed certain strategies to survive over the years. One of the possible strategies could be intraspecific communication through the releasing and detecting of very small quantities of extremely volatile compounds, only detectable by conspecifics, which are difficult to detect or totally undetectable by other predators. This would enable these animals to operate as if "invisible" to the olfactory world of their predators and would to a certain extent ease the game for survival.

R.L. Brahmachary and J. Dutta studied some of the urinary volatiles of tigers, lions and leopards which might be part of their pheromonal system[45]. So far no work has been undertaken on the semiochemical communication in cheetahs.

The main objective of this study was to investigate volatility of components identified in the urine of the cheetah, and characterise these volatile chemical constituents which might form part of *their* pheromonal system. An investigation on the possible bacterial activity in the bladder of the cheetah was also done.

I hope that this study will not only further the understanding of the chemical communication system of these extraordinary species, but also create a sense of appreciation for Africa's most endangered cat species.

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CHAPTER 2

EXPERIMENTAL

2.1 GENERAL

All Pyrex glassware used in the handling and preparation of biological material and extracts were heated to 500°C in an annealing oven to remove any traces of organic material. Aluminum foil was heated to 200°C for purification. Syringes, tweezers, *etc.* were cleaned with dichloromethane (Residue Analysis Grade, Merck).

2.2 GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC ANALYSIS

A Carlo Erba QMD 1000 GC-MS system with VG Analytical Lab-Base software was used for the recording of electron impact (EI) mass spectra (70 eV). This system consists of a Carlo Erba 5300 gas chromatograph, coupled to a VG Analytical TRIO 1 quadropole mass spectrometer. A glass capillary column (40 m x 0,25 mm), with a 0,25 µm film of apolar stationary phase PS-089-OH, which is a silanol-terminated (95%)-dimethyl-(5%)-diphenylsiloxane copolymer, was used throughout this study. This glass column was manufactured by the Laboratory for Ecological Chemistry University of Stellenbosch. Cryotrapping of volatile analytes on the capillary column was used in all cases where samples were introduced into the injector of the GC or GC-MS system in a solventless manner. Helium was used as the carrier gas at a linear velocity of 38 cm/sec at a column temperature of 40°C. The temperature of the interface (line of sight) and the ion source were maintained at 250°C and 150°C respectively. The injector was used at 220°C and samples were injected at an oven temperature of approximately 30°C. The oven was heated ballistically to 40°C and programmed from 40°C to 280°C at 2°/min. A scan rate of 0,9 scan/sec, with an interval of 0,1 seconds between scans, was employed. All commercially available compounds were diluted with dichloromethane (less than 1 µl/ml solvent) for GC-MS analyses.

2.3 SAMPLE COLLECTION AND PREPARATION

Cheetah urine was collected from three male and two female animals (in captivity) at Cheetah Outreach at Spier Wine Estate near Stellenbosch. Urine samples from the males were collected in the mornings after they had eaten or when they were taken into another enclosure, which included marking of their new territory. A 500 ml pyrex glass beaker was used to catch the urine as they sprayed. The sample was immediately transferred to a 50 ml pyrex glass bottle, closed with a Teflon-lined screw cap and stored at a temperature of -30°C until used for analyses. Female urine was collected only once during an operation for artificial insemination by using a catheter. These samples were also stored in glass bottles with Teflon-lined screw caps at the same temperature. Equipment for the collection of urine from free-ranging cheetahs was sent to Hoedspruit (Mpumalanga, South Africa) but as a result of ecological factors the sampling was unsuccessful. Urine was also collected from four wild male cheetah at Cheetah Conservation Fund near Otjiwarongo in Namibia. A sample was taken from each cheetah during an operation by using a catheter while the cheetah was under an anaesthetic. These samples were stored under the same conditions as the samples from the captive cheetahs until used for analysis.

The following methods for the preparation of the sample for GC-MS analyses were used:

- Solid Phase Micro Extraction (SPME)

The headspace of male cheetah urine was sampled by SPME (100- μl PDMS) at 40°C for 12 hours. The volatile material was desorbed from the SPME fiber at 220°C for two minutes and analyzed by GC-MS.

- Sample enrichment device

A device consisting of polydimethylsiloxane (PDMS) tubing (Silastic[®], Dow Corning) (120 mg), coiled on a length of 0,35 mm I.D. fused silica tubing, was made to collect headspace volatiles from cheetah urine. The device was then suspended in the headspace of a sample of ca. 25 ml of urine in a 100-ml bottle closed with a PTFE (Teflon[®])-lined screw cap for periods of 25 to 50 hours for the

male, and 69 hours for the female urine. The collector, suspended in an injector liner, was introduced into the cold injector of the GC-MS system. The volatile material was desorbed from the rubber tubing by heating the injector ballistically to 220°C and was cryotrapped on the capillary column and analyzed by GC-MS. The enrichment device was removed from the injector before the analysis was started. This method gave the best results and was used throughout the study.

- Extraction of organic volatiles from urine saturated with sodium chloride

A sample, containing the urine from three captive male cheetah (*ca* 25 ml), was saturated with an excess of sodium chloride. The organic volatiles were extracted from the urine with the minimum volume (*ca.* 1 ml) of dichloromethane and centrifuged at 2500 r.p.m. for 5 minutes in order to separate the mixture into a light-yellow organic extract and the yellow-brown aqueous phase. The bottom organic phase was transferred with a 100- μ l syringe to a small vial with a teflon-lined screw cap. The extract was concentrated for further work by slow evaporation of the dichloromethane from the uncapped vial and the residual extract analyzed by GC-MS.

Another three urine samples from wild male cheetah were extracted. These samples were not saturated with sodium chloride. The organic compounds were extracted from the urine (*ca* 25 ml) with volumes of about 1 ml of dichloromethane and centrifuged at 2500 r.p.m. for 10 minutes. This resulted in the separation of clear layers of the dichloromethane extract and the urine. However, a substantial emulsion layer that could not be cleared by prolonged centrifuging, remained at the interphase. This problem was circumvented by freezing the sample containing all three layers at -30°C, centrifuging the material for 15 minutes or longer until the ice had melted, and repeating the freeze-thaw cycles, with centrifuging until two clear layers were obtained. The lower organic phase was transferred with a 100- μ l syringe to a small vial. The extract was concentrated by evaporating the dichloromethane and analyzed by GC-MS.

The identification of the compounds in the urine of cheetah is based on the computerised comparison of the mass spectra of the natural compounds and the

spectra in the NBS library containing about 54 000 spectra. Further diagnostic information was obtained by the GC-MS comparison of the natural compounds with commercially available compounds and compounds synthesised in the laboratory, using, *inter alia*, co-injection techniques.

2.4 REFERENCE COMPOUNDS

Most of the chemicals used for synthetic work were obtained from Aldrich Chemical Co., Merck and Lancaster Co.

2.4.1 Preparation of a mixture of butyl propyl ether and di-propyl ether [1]

Sodium (9,2 g, 0,4 mol) was cut into small pieces and placed in a 500 ml flask with an efficient double surface condenser. Propanol (48 g; 0,8 mol) was added to the sodium through a dropping funnel at a rate of 1 drop per second, heated and stirred until all the sodium had been consumed. A mixture of 1-bromobutane (13,7 g; 0,1 mol) and 1-bromopropane (12,3 g; 0,1 mol) was added dropwise to the propoxide solution, the reaction mixture was refluxed for 3 hours, cold water was carefully added to the reaction mixture to hydrolyze the excess sodium propoxide, and the resulting reaction mixture was poured into cold water. The mixture of organic products was purified by washing with water until neutral. The organic material containing some propanol, di-propyl ether and butyl propyl ether was dried over anhydrous magnesium sulphate and used without further purification for comparison with constituents of the urine extracts.

2.4.2 Preparation of a mixture of butyl ethyl ether and amyl ethyl ether [1]

A mixture of butyl ethyl ether and ethyl pentyl ether was prepared as described above from ethoxide (36,8 g; 0,8 mol ethanol) and a mixture of 1-bromobutane (13,7 g; 0,1 mol) and 1-bromopentane (15,1 g; 0,1 mol).

2.4.3 Preparation of acetaldehyde diethyl acetal [2]

A 250-ml round-bottomed Pyrex flask, fitted with a calcium chloride guard tube, was charged with ethanol (36,8 g; 0,8 mol), acetaldehyde (17,6 g; 0,4 mol) and a cation exchange resin (2,00 g Amberlyst 15). The reaction mixture was stirred for 15 hours, the resin filtered off and the organic material distilled over a short Vigreux column. The distillate, collected at a boiling point of 76-78°C, contained acetaldehyde diethyl acetal and unreacted ethanol (GC-MS)

2.5 EXPERIMENTS TO ASCERTAIN A MICROBIOLOGICAL PRESENE

2.5.1 DNA Extraction

One male and two female urine samples were collected for microbial experiments. Two procedures were followed to obtain the necessary material for subsequent DNA extraction procedures. In the first procedure, 1 ml of urine was aliquoted directly from the urine samples and in the second, 100 µl was cultured in 10% Tryptic soy broth overnight at 37°C. A small number of the originally aliquoted urine samples (1 ml) were transferred to 1,5 ml Eppendorf tubes and centrifuged at 3000 G for 3 minutes. The overnight urine samples were centrifuged in 10 to 15 ml clean polypropylene tubes at 10000 G for 10 minutes and pelleted. This was performed in order to remove any excess media from the urine sample. The supernatant was removed and the pellet resuspended in 10 ml of Sterile Ringer's solution. One ml of the resuspended pellet was aliquoted into 1,5 Eppendorf tubes and as the originally aliquoted samples, centrifuged at 3000 G for 3 minutes. The supernatant was removed and the pellet resuspended in 1 ml DNA extraction buffer. The Eppendorf tubes containing the resuspended solutions were exposed to 3 freeze-thaw cycles (-70°C, 65°C) to break gram-positive cells. Each freeze-thaw cycle comprises the exposure of the cells to 15 minutes in the -70°C freezer, immediately followed by shocking these cells for 2 minutes in a water bath (65°C). To the freeze-thaw samples, 5 µl of proteinase K (20 mg/ml) was added, mixed well and incubated at 37°C for 30 minutes in a shaking incubator. After incubation, 150 µl of 20% SDS was added, the sample incubated at 65° C for 2 hours and mixed gently every 20 minutes. Upon completion of the

incubation period, the samples were centrifuged at 6000 G for 10 minutes. The subsequent supernatant was then transferred to a 10-15 ml polypropylene clean tube (chloroform resistant). To these supernatants an equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed well. After centrifuging the mixture at 3000 G for 3 minutes, the aqueous layer was transferred to clean Eppendorf tubes. A small volume of cold 2-propanol was added to the material in the tubes, and the contents of the tubes were mixed well and left overnight in the -20°C freezer. The tubes were centrifuged at 16000 G for 20 minutes and the 2-propanol decanted. The pellets were washed with cold (0°C) aqueous ethanol (70%, 1 ml), centrifuged at 3000 G for 2 minutes, the aqueous ethanol decanted and the pellets allowed to dry completely. The dried pellets were dissolved in 150 µl of TE (10 nM Tris, 0,1 nM EDTA). The DNA sample (6 µl) was run on a 0,8% molecular grade agarose #D1-LE gel obtained from Whitehead Scientific to isolate any traces of DNA.

4.5.2 DNA Purification

Purification consisted of the use of MicrospinTM S-300 HR columns DNA purification kit obtained from Amersham Pharmacia Biotech Inc. Of the subsequently purified DNA 6 µl was run on a 0,8% molecular grade agarose #D1-LE gel. A polymerase chain reaction (PCR) was then performed.

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CHAPTER 3

DISCUSSION OF ANALYTICAL PROCEDURES

3.1 SAMPLE COLLECTION AND PREPARATION

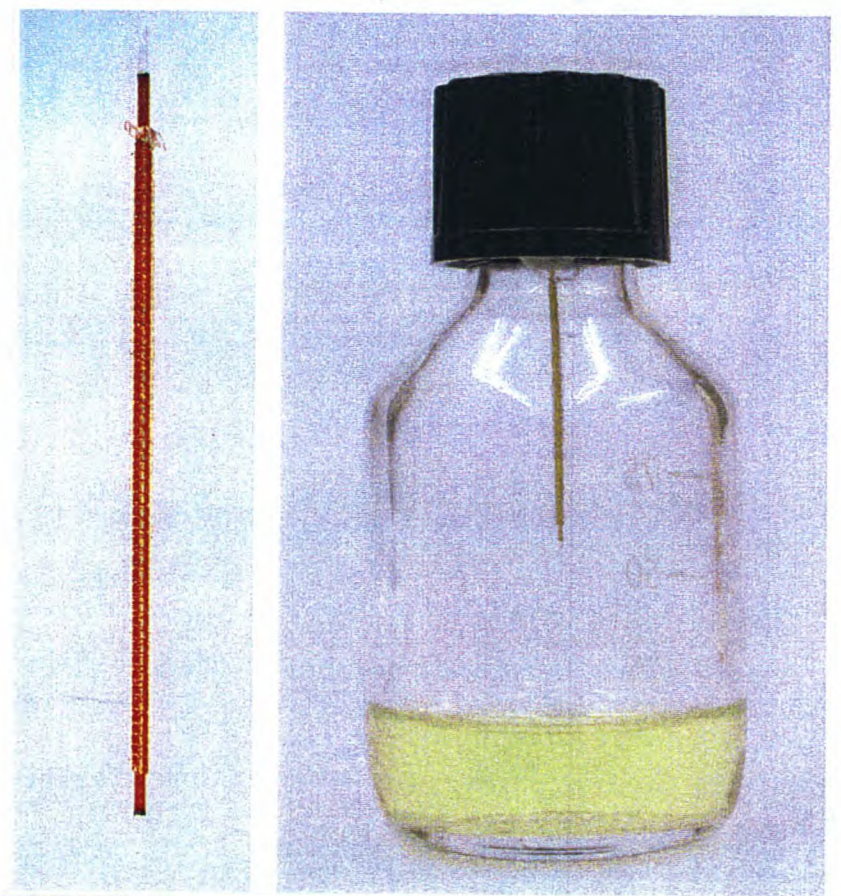
The urine samples used in this study were collected from male captive cheetah at the Cheetah Outreach facilities at Spier Wine Estate near Stellenbosch throughout the year and on one occasion from wild male cheetah at the research station of Cheetah Conservation Fund (CCF) near Otjiwarongo in Namibia. Samples were also obtained on two occasions from anaesthetised females at Spier.

The first GC-MS analysis of headspace of urine from a captive male cheetah, sampled with SPME (100- μ l PDMS) at 40°C for 12 hours, gave a very weak total ion chromatogram (TIC) (Figure 3.1) in which only a few components were observed. Urine from the same male cheetah was collected and the SPME sampling time was increased to 48 hours. The GC-MS analysis gave a stronger TIC (Figure 3.2) but components in the lower retention time range were still visible at only relatively low concentrations. Urine samples of two different captive male cheetah were collected using the same method. The GC-MS analysis of both these samples gave a very weak TIC (Figure 3.3, 3.4). In the TIC of both these samples a prominent peak, tentatively ascribed to the presence of urea, was observed.

The three samples were subsequently combined, saturated with sodium chloride in order to increase the polarity of the solution and extracted with dichloromethane. The extract was concentrated by evaporating the dichloromethane. The GC-MS analysis of the resulting sample gave a TIC (Figure 3.5) with barely visible peaks in the lower retention time range. In this TIC the urea peak was absent due to the extremely high solubility of urea in water and its relatively low solubility in dichloromethane.

The unsatisfactory results obtained from conventional extraction as well as headspace analytical methods appeared to be due to the low concentration of volatile organic compounds present in the cheetah urine. The challenge was therefore to find a method with which the volatile organic constituents of the urine could be sufficiently

concentrated for GC-MS analysis. One possibility would have been to use the so-called stir bar technique recently developed by Baltussen *et al.* [1]. However, since this technique requires the use of a Gerstel thermal desorption system which was not available for this research, it was decided to employ an adopted SPME device having substantially more polydimethylsiloxane rubber tubing than the normal SPME sampling device [2]. The simplest way to construct such a device was to coil Silastic® medical-grade polydimethylsiloxane rubber tubing (Dow Corning) on an unbreakable and inert support, such as a piece of fused silica tubing. The silastic® tubing is normally used in microsurgery and is known to be thermally stable and excellently suitable for the enrichment of volatile organic compounds [3]. The only disadvantage of this device is that it had to be introduced into a cold injector which was then heated ballistically. This necessitated cryotrapping of the volatiles and the removal of the device from the injector after completion of the desorption stage.



Device for extraction of volatile organic compounds from the headspace of cheetah urine

Using this device, GC-MS gave a relatively strong TIC (Figure 3.6). The presence of the urea can clearly be seen at 49,2 minutes. However, to improve the enrichment even further, urine samples from wild male cheetah were collected and a double layer of tubing (147,6 mg) was used. In the resulting TIC (Figure 3.7) the volatile constituents are well separated and components present in the retention time range between 10-60 minutes are present in satisfactory quantities.

The enrichment of the volatile constituents of the urine for GC-MS analysis according to this method gave the best results and was used in this study.

3.2 MICROBIOLOGICAL EXPERIMENTS

In the PCR (polymerase chain reaction) experiment no bacteria were found in the urine of both male and female cheetah and according to the DNA extraction the absence of bacteria was confirmed. No further selective experiments were done to find thio-bacteria which could be present and involved in possible reactions with sulphur-containing compounds.

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3. B.V. Burger, M. le Roux and W.J.C. Burger, *J. High Resolution Chromatography*, **13**, 777, (1990)

CHAPTER 4

CHEMICAL CHARACTERIZATION OF THE URINE OF THE SOUTHERN AFRICAN CHEETAH, *ACINONYX JUBATUS JUBATUS*

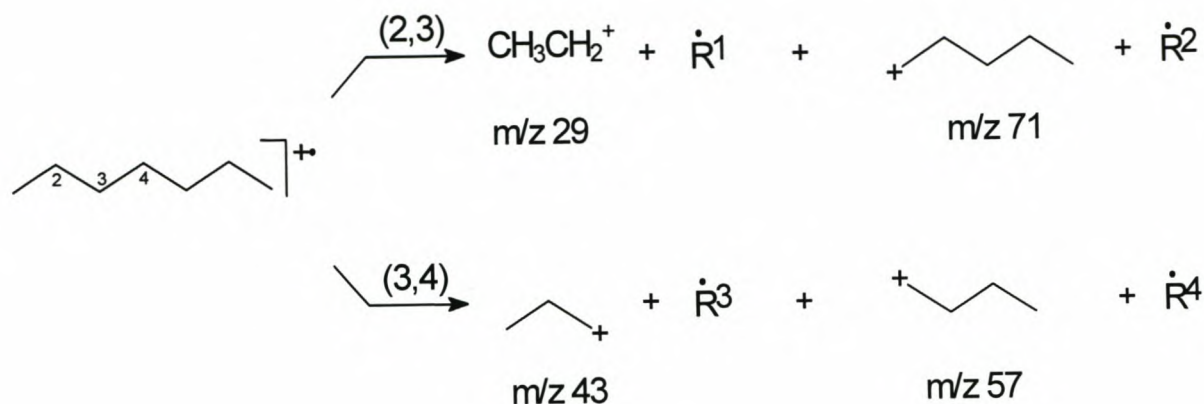
Most of the identified compounds occur in the urine of both sexes, but female urine contained more constituents than male urine. For that reason the gas chromatogram (Figure 3.6) and mass spectra (Figure 4.1 - 4.21) of the urine sample from a female cheetah was used as reference data in the following discussion on the identification of the volatile organic constituents of the urine of the cheetah. The compounds identified in this study are given in Table 4.1.

4.1 STRUCTURAL CHARACTERISATION OF THE COMPONENTS IN THE URINE OF THE CHEETAH

4.1 Hydrocarbons: Aliphatic

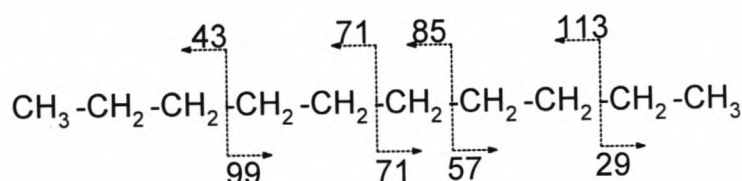
In the total ion chromatogram (Figure 3.6) of the female cheetah, the molecular ion in the mass spectrum of component 442* (Figure 4.1) appears at m/z 100. The characteristic fragmentation pattern of alkanes is observed, namely groups of peaks occurring with gradually decreasing abundance at intervals of 14 mass units [1]. Each of the groups of peaks has as the most abundant species an ion corresponding to the general formula C_nH_{2n+1} , resulting in a species of ions at m/z 71, 57, 43 and 29. The formation of these ions can be rationalised as follows [2]:

* Due to restricted space all the peaks in the total ion chromatogram (TIC) (Figure 3.6) are not numbered. The position of peaks can be found by converting the retention time (in minutes) to seconds, i.e. component 442 appears at 7,37 minutes.

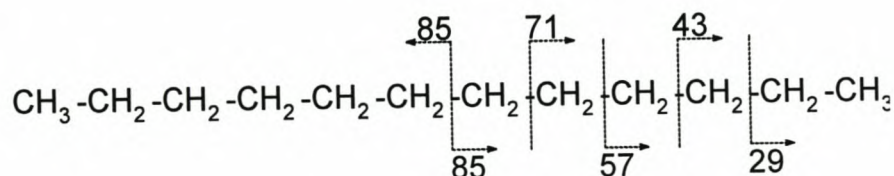


Based on the above evidence, component 442 was identified as heptane. The identification was confirmed by the co-elution of commercial heptane with the natural product.

The presence of molecular ions at m/z 142 and 170 in the mass spectra of components 1767 (Figure 4.2) and 2760 (Figure 4.3), indicated that these are C_{10} and C_{12} compounds respectively. In the spectrum of component 1767 the loss of an ethyl group gives an ion at m/z 113 ($\text{M}^+ - 29$) and each further loss of 14 mass units results in ions at m/z 99, 85, 71, 57 and 43. An analogous fragmentation pattern is observed in the spectrum of component 2760:



Component 1767



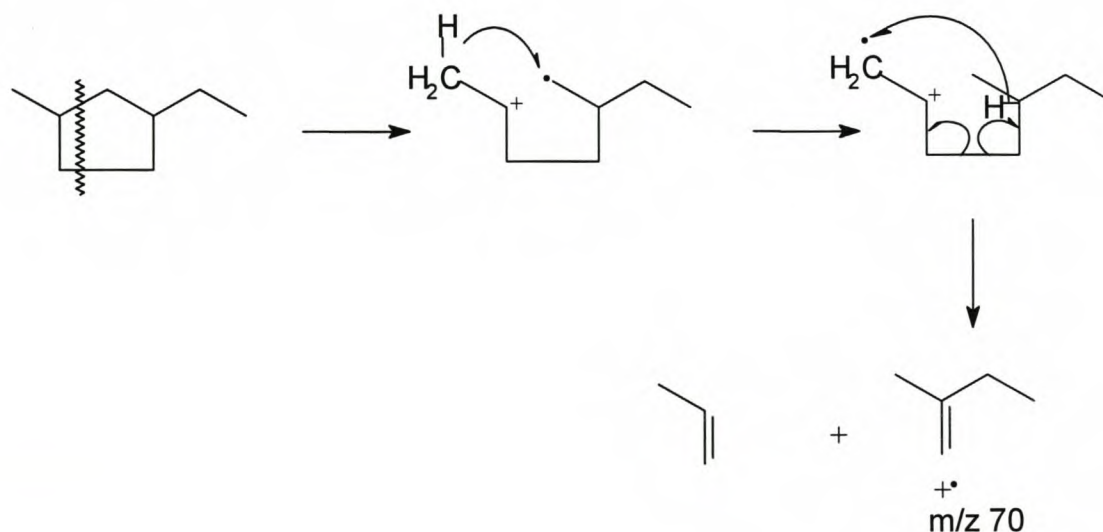
Component 2760

Based on the above evidence, it was accepted that the mass spectra of components 1767 and 2760 are those of decane and dodecane respectively. This identification was confirmed by the co-elution of the commercial compounds with the natural compounds.

4.2 Hydrocarbons: Cyclic

A computerized library search identified component 1469 (Figure 4.4) as *trans*-1-ethyl-3-methyl-cyclopentane. The possibility that this component could also be the *cis*-isomer was investigated. However, the relative intensities of the ion at m/z 69 and the base peak at m/z 55 in the mass spectrum of *cis*-1-ethyl-2-methyl-cyclopentane did not correlate with the intensities of the corresponding ions in the mass spectrum of component 1469. Therefore, only *trans*-1-ethyl-3-methyl-cyclopentane was accepted as the working hypothesis.

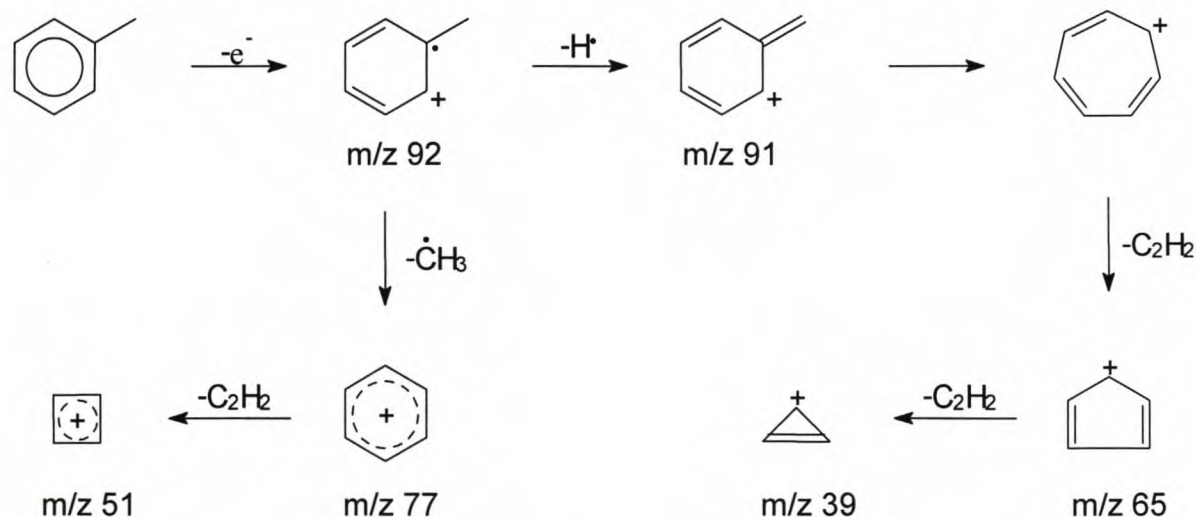
The molecular ion is present at m/z 112 and the base peak at m/z 83 is due to the loss of an ethyl group from the molecular ion. According to an observation by Djerassi [3], the double ring cleavage with apparent hydrogen rearrangement becomes a notable decomposition. This proposed mechanism could possibly explain the presence of the ion at m/z 70 [3]:



This compound was not available for retention-time comparison and it remains unidentified.

4.3 Hydrocarbons: Aromatic

In the mass spectrum of component 644 (Figure 4.5) the prominent base peak at m/z 91 was ascribed to the familiar tropylium ion $[C_7H_7]^+$. The less abundant ion at m/z 92 was presumed to be the molecular ion which loses a hydrogen atom by α -cleavage to give the tropylium ion. Toluene and 1,3,5-cycloheptatriene are two possible candidate structures. In agreement with the mass spectral data of the commercial toluene and its co-elution with the natural substance, this constituent was identified as toluene. The fragmentations of toluene can be formulated as follows [4]:



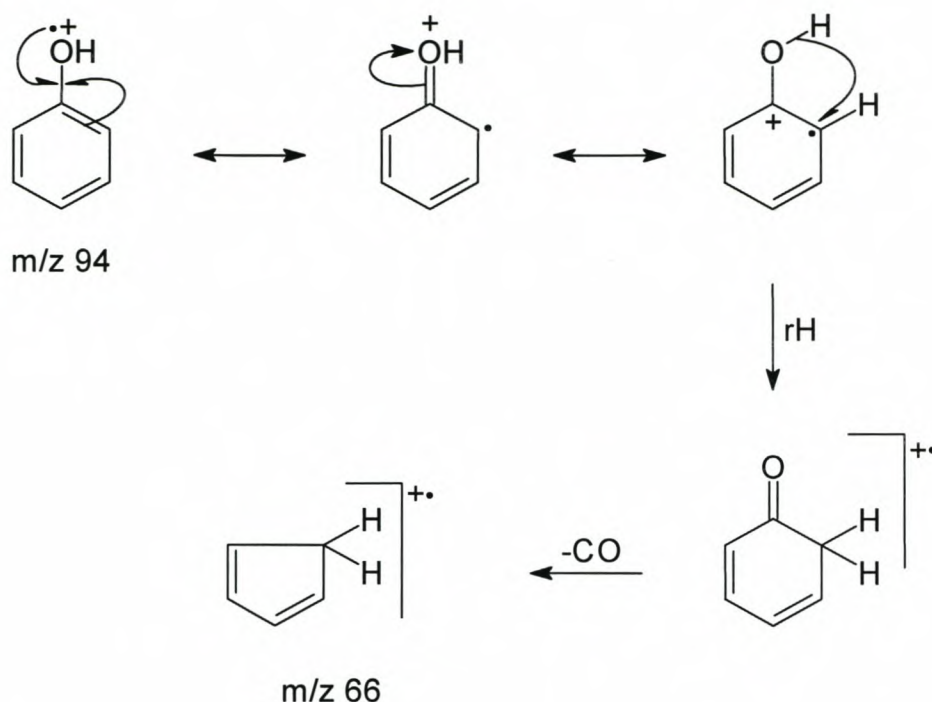
The mass spectra of components 1043 (Figure 4.6) and 1189 (Figure 4.7) are quite similar and could both be compared to the above-mentioned mass spectrum of toluene [5]. If the ion at m/z 106 is presumed to be the molecular ion, the base peak is due to the loss of 15 atomic mass units, representing a methyl group. This means that these components could be homologues of toluene with one of the aromatic hydrogen atoms or one of the alkyl hydrogen atoms substituted by a methyl group; these components could be either ethylbenzene or one of the xylenes. The co-injection of firstly *ortho*-, *meta*- and *para*-xylene and then ethylbenzene with the natural sample, a comparison of their retention times and a study of their mass

spectra, confirmed components 1043 and 1189 to be *m*-xylene and *p*-xylene respectively [6].

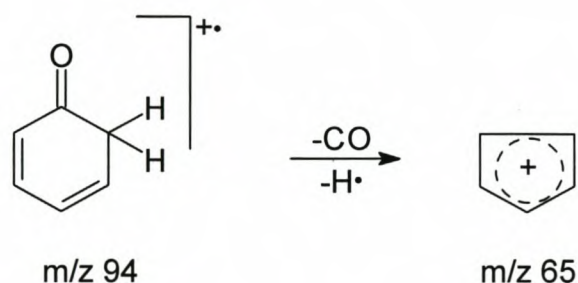
In the mass spectrum of component 1699 (Figure 4.8) the molecular ion appears to be at m/z 120 and the base peak at m/z 105, representing the $[M-CH_3]^+$ ion. Although of low intensity, two peaks are present at m/z 91 and m/z 77 which are indicative of aromatic hydrocarbon compounds. Using these ions and their relative abundance in a computer search yielded 1,3,5-trimethylbenzene and 1,2,3-trimethylbenzene as possibilities. Co-injection of the commercially available compounds with the natural material proved component 1699 to be 1,3,5-trimethylbenzene (mesitylene).

4.4 Phenol

The base peak in the mass spectrum of component 1622 (Figure 4.9) appears at m/z 94, which indicates that the unknown could be phenol [7]. This is in agreement with the results of a computerized library search as well as the mass spectrum of commercial phenol. Characteristic to phenol, the molecular ion (m/z 94) is accompanied by a weak $(M-1)^+$ ion due to loss of hydrogen. The significant fragment in the spectrum is the $(M-28)^+$ ion at m/z 66. Accurate mass measurements as well as deuterium labelling has demonstrated the expulsion of CO *via* a cyclohexadienone intermediate [8]:



This fragment is accompanied by a (M-29) species at $m/z\ 65$ of only slightly reduced abundance, produced by the loss of the elements of CHO from the molecular ion. Deuterium labelling of the phenolic hydrogen atom revealed that only 33% of the hydrogen which is expelled has its origin from this source and 67% evidently arises by random abstraction from the ring. As the true geometry of this species is not known, Budzikiewicz *et al.* represented the resulting species as a cyclopentadienyl cation "for the sake of convenience" [9].

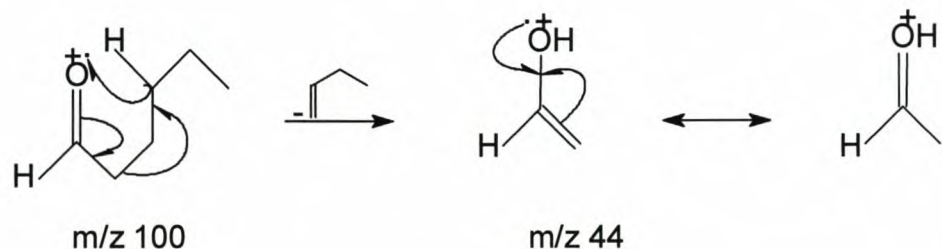


Based on the above evidence, it was accepted that component 1622 is phenol and the identification was confirmed by co-elution of commercially available phenol with the natural material, resulting in co-elution of component 1622 and synthetic phenol.

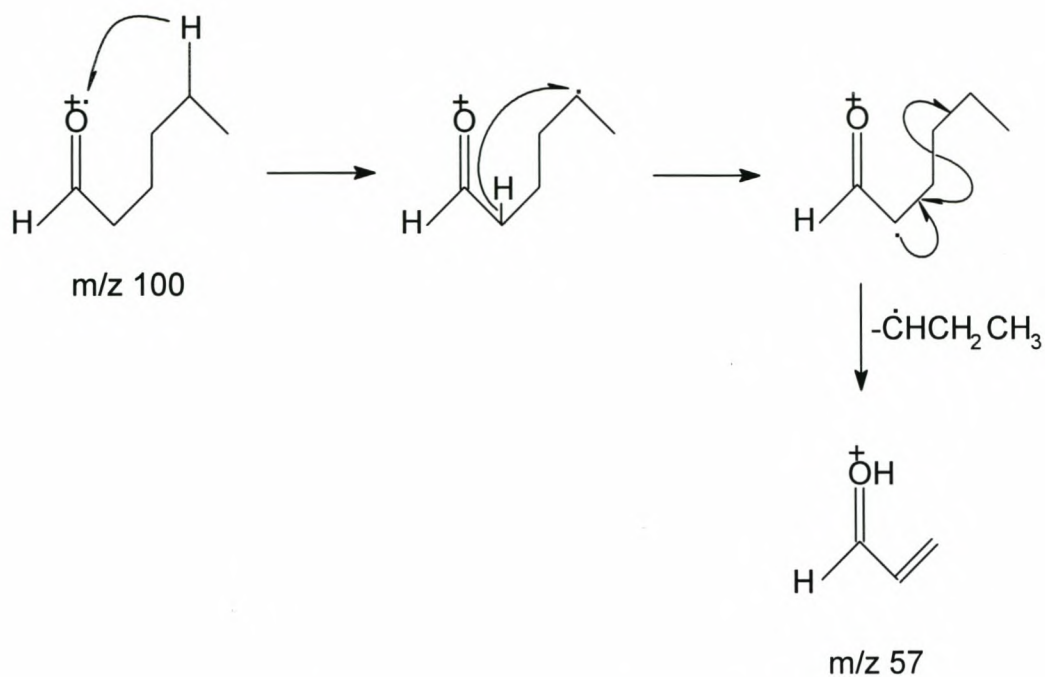
4.5 Aldehydes: Aliphatic (Saturated)

The mass spectra of components 755 (Figure 4.10), 1725 (Figure 4.11) and 2239 (Figure 4.12) exhibit the typical fragmentation patterns of saturated aliphatic aldehydes containing eight or less carbon atoms [10]. The base peak at m/z 44 in the above-mentioned mass spectra confirms this statement. The fragmentation patterns of these aldehydes are uncomplicated and easily recognizable.

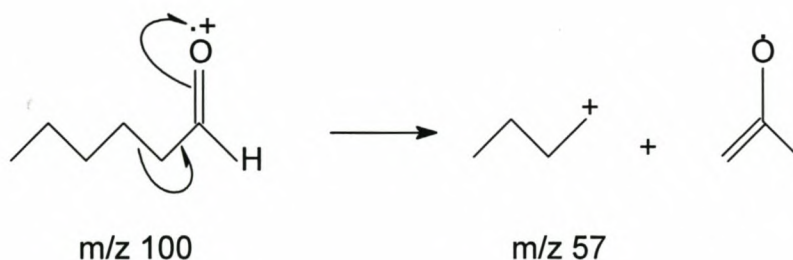
If the ion at m/z 100 in the spectrum of component 755 is assumed to be the molecular ion, the unknown could be presumed to be hexanal [10]. The base peak at m/z 44 must be a rearrangement peak as it occurs at an even mass and can be attributed to the characteristic McLafferty rearrangement, resulting in the elimination of an olefin. In the case of hexanal, the reaction can be formulated as follows [11]:



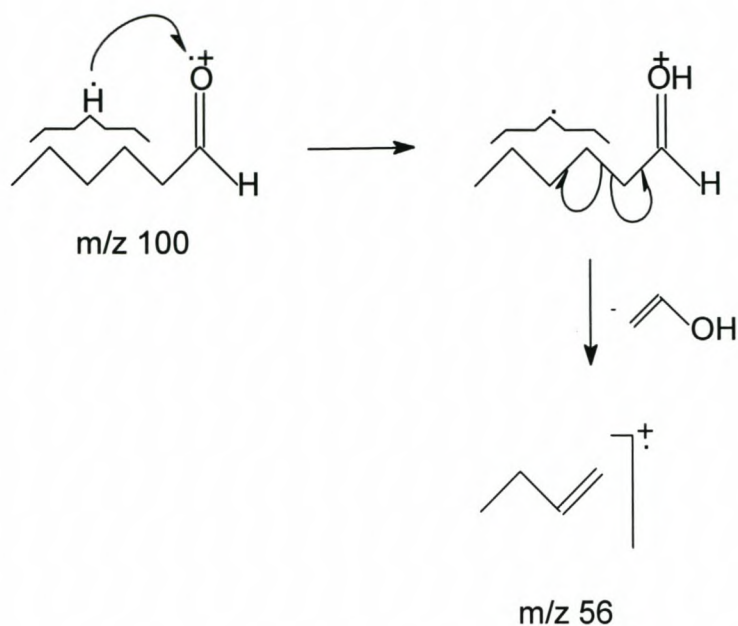
The McLafferty rearrangement is usually accompanied by the formation of another ion, the (McLafferty + 13)⁺ ion. This ion is formed by the removal of a hydrogen atom from the C-atom situated in the δ -position relative to the carbonyl group or from a position further away from the carbonyl group. The radical site of the resulting intermediate is in close proximity to the C-H bond adjacent to the carbonyl carbon atom. Consequently, the α -hydrogen atom is removed, creating a new radical that is stabilized by expulsion of an alkyl radical. The resulting ion at m/z 57 is seen in the spectrum of this aldehyde [11]:



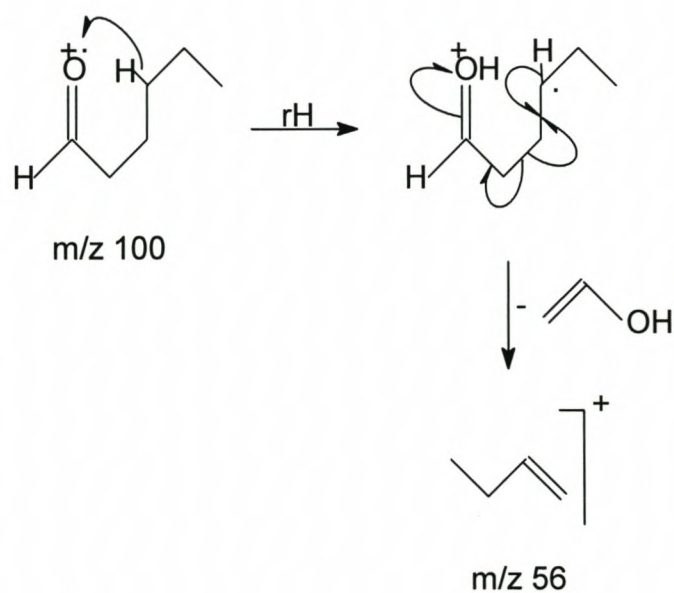
Another type of β -cleavage can also occur. No hydrogen transfer takes place and the positive charge is retained on the alkyl fragment. The contribution of this $m/z\ 57$ ion in hexanal, formed by this fragmentation, is approximately 33% to the general abundance of this mass in the spectrum while the (McLafferty + 13)⁺ ion contributes 67% [12]:



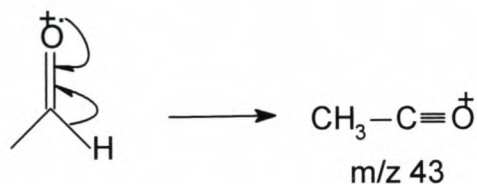
The presence of an (M-44)⁺ ion, or in this case, at $m/z\ 56$, is another characteristic feature of aldehydes which implies β -cleavage with hydrogen transfer as in a McLafferty rearrangement, but with the charge-retention on the alkene fragment [11]:



Labelling experiments with deuterium demonstrates that this process is in fact not of the site-specific McLafferty rearrangement type and that 81% of the total transfer of hydrogen atoms originate from the β -, γ - or δ -positions. The following representation is therefore more realistic [12]:

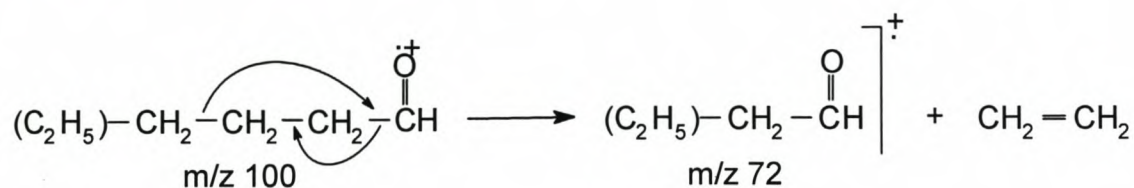


In these spectra another prominent ion at m/z 43 has been shown to be an oxygen-containing fragment with an elemental composition of $C_2H_3O^+$, formed as a result of the loss of a hydrogen atom from the m/z 44 ion [13]:

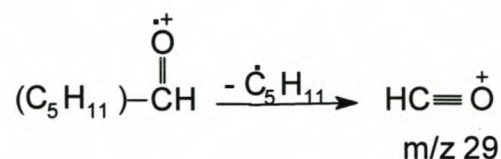


Two minor peaks occur at m/z 67 and 82 and are attributable to the elimination of water from the molecular ion and subsequent loss of a methyl radical [12]. Deuterium-labelling experiments have shown that the major site of transfer for the hydrogen atoms in the elimination of water, is the C-3 carbon atom. The exact mechanism involved in the subsequent expulsion of the methyl radical is still unknown [11].

A minor peak occurs in the higher mass range at m/z 72 and high resolution mass measurements have indicated that this ion corresponds to the loss of an ethylene molecule from the molecular ion [12]. Deuterium experiments show that the C-2 and C-3 atoms are eliminated as a unit [11]:



The formation of the ions at m/z 29 and m/z 99 can be illustrated as follows [11]:



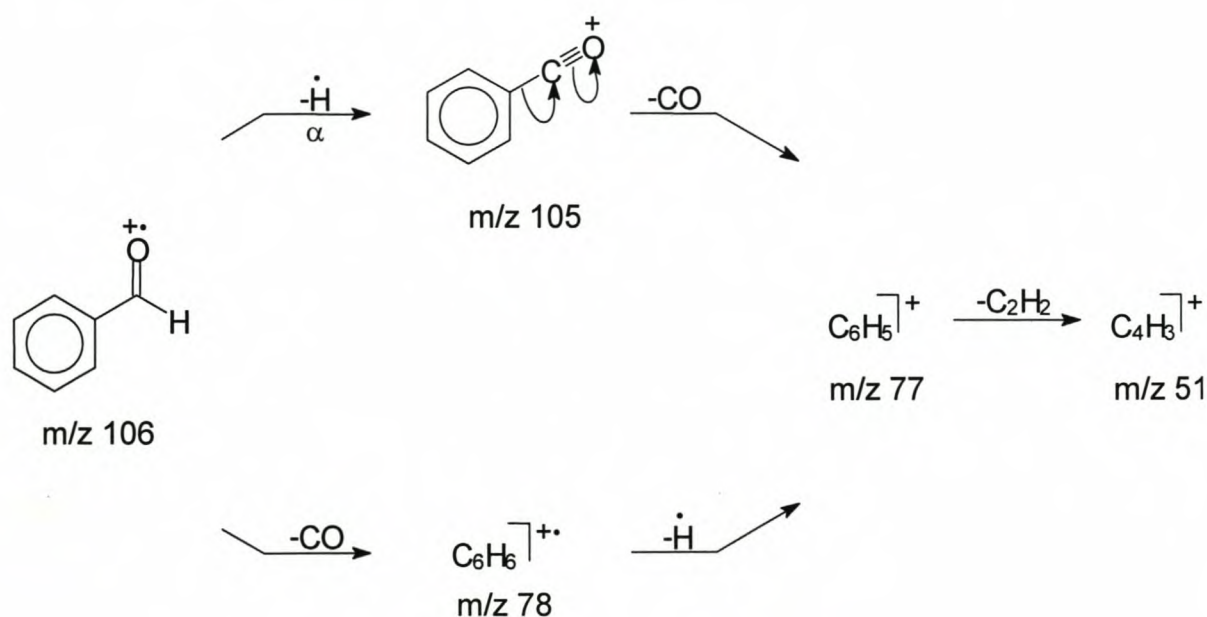
The above evidence proves that component 755 is hexanal and final confirmation was obtained by the co-elution of the commercial compound and the natural material.

The molecular ions of components 1725 and 2239 are not visible in their mass spectra, but they could be identified as octanal and nonanal respectively by mass-spectral and gas-chromatographic comparison of the natural constituents and the commercial compounds.

4.6 Aldehydes: Aromatic

The aromatic aldehydes decompose by relatively few and easily rationalized paths and the fragmentation patterns are significant and uncomplicated [14]. In the mass spectrum of component 1481 (Figure 4.13), the ion at m/z 106 was assumed to be the molecular ion. The loss of the aldehyde hydrogen gives rise to the $(M-1)^+$ ion at m/z 105, which is as abundant as the molecular ion. This fragmentation is indicative of an aromatic aldehyde.

The ion at m/z 77 ($C_6H_5^+$) is formed when the $(M-1)$ species expels CO, after which it decomposes by elimination of acetylene to an ion at m/z 51 ($C_4H_3^+$). These prominent ions can be explained as follows[14]:

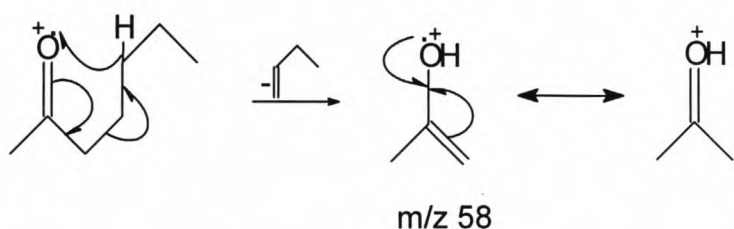


The component was identified as benzaldehyde and the identification was confirmed by the co-elution of commercial benzaldehyde and the natural material.

4.7 Ketones: Aliphatic (Saturated)

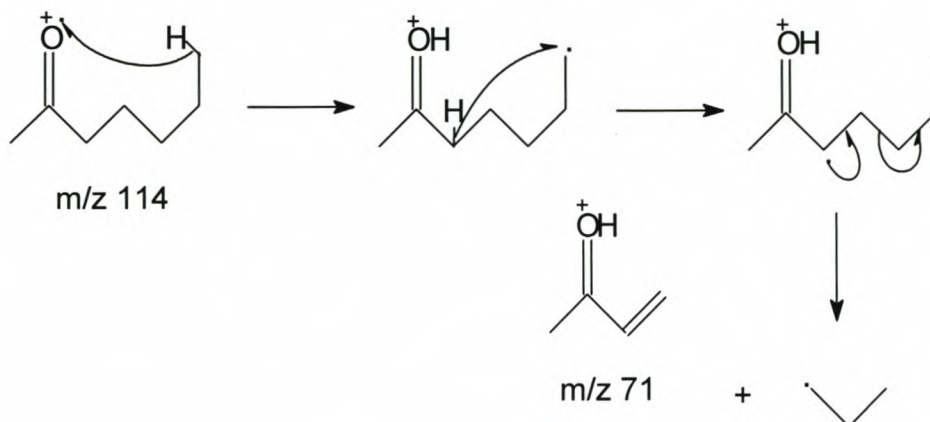
A number of components in the chromatogram of cheetah urine have spectra that are essentially identical in the lower mass range but differ in the higher mass range. The components 206 (Figure 2.14), 383 (Figure 2.15), 715 (Figure 2.16) and 1161 (Figure 2.17) appear to belong to a homologous series of compounds. Constituent 1161 is used as a typical example of this group of compounds in the discussion of their mass spectra.

According to Budzikiewicz it has been shown that m/z 43 is the base peak for at least 10 aliphatic acyclic methyl ketones [15]. The presence of the base peak at m/z 43 can be attributed to α -cleavage, resulting in a $\text{CH}_3\text{C}=\text{O}^+$ fragment. The ion at m/z 99 results from another possible α -cleavage with only the loss of a methyl group. The ion at m/z 58, typical for 2-alkanones, can be explained by the well-known McLafferty rearrangement [16]:

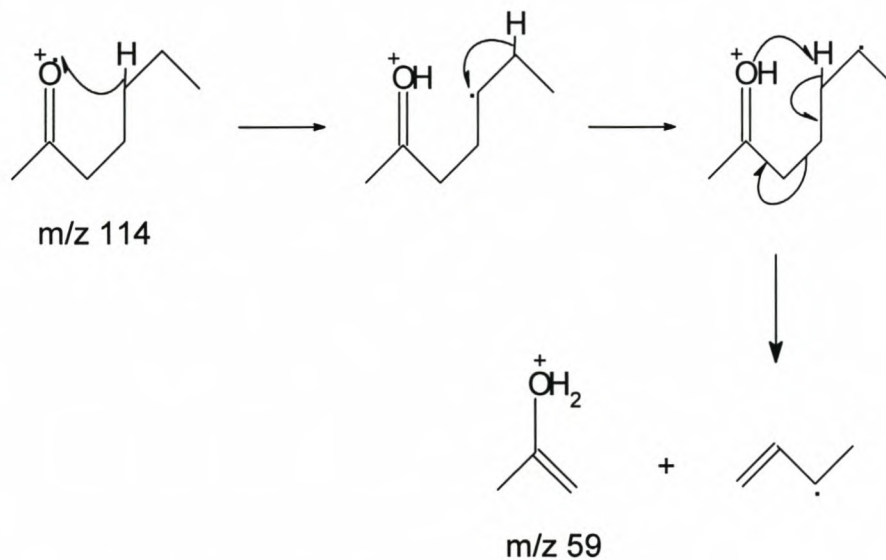


The possibility of this compound being a 2-alkanone was therefore assumed as the working hypothesis and because the molecular ions of these compounds are usually present in their mass spectra, in this case at m/z 114, constituent 1161 was assumed to be 2-heptanone.

The accompanying (McLafferty + 13)⁺ ion at m/z 71 which has been discussed in section 4.2.5, is formed as follows [17]:



The formation of the prominent ion at m/z 59 in the spectrum of this ketone can be explained by a protonated McLafferty rearrangement that occurs by double proton transfer *via* a six-membered transition state [18]:

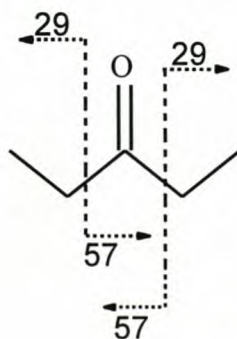


It is known that the relative abundance of the m/z 59 ion is higher in the spectra of the higher homologues in the series and apparently this process is favored by an increase in chain length [11]. It is also noticeable that the m/z 58 ion that represents a McLafferty rearrangement is less abundant in the short-chain components of this series. It is illustrated by comparison of the abundance of these ions in the spectra of components 1161 (Figure 4.17), 1665 (Figure 4.18), 2178 (Figure 4.19) and 3146

(Figure 4.20), which further indicates the presence of a homologous series of ketones. In the mass spectrum of component 206, the ion at m/z 58 is absent and the formation of an ion at m/z 57 is due to the loss of a methyl group.

Using the above-mentioned arguments, based on the data from the mass spectra of components 3146 (Figure 4.20), 2178 (Figure 4.19), 1665 (Figure 4.18), 1161 (Figure 4.17), 715 (Figure 4.16), 383 (Figure 4.15) and 206 (Figure 4.14), the homologous series of compounds was identified as 2-undecanone, 2-nonanone, 2-octanone, 2-heptanone, 2-hexanone, 2-pentanone and 2-butanone respectively. The identification of these components was confirmed by their co-elution with the respective commercial ketones.

In the mass spectra of components 410 (Figure 4.21) and 701 (Figure 4.22) the ion at m/z 57 ($R-CH_2-C=O^+$) and fragmentation patterns are characteristic of hydrocarbon compounds as well as 3-ketones. The mass spectrum of component 701 is similar to that of heptane (Figure 4.1) but the two spectra differ with respect to the relative abundance of the ion at m/z 85. On account of the results of a computerized library search and the co-injection of the commercially available compound with the natural material, the component was identified as 3-hexanone. The ion at m/z 71 is formed by the loss of a methyl group from the molecular ion and the base peak at m/z 43 represents the propyl ion ($CH_3-CH_2-CH_2^+$). In the spectrum of component 410 the ion at m/z 57 constitutes the base peak. Based on the results of the computerized library search and the co-elution of the commercial compound and the natural material, this component was identified as 3-pentanone. The ion at m/z 86 is the molecular ion and two prominent ions at m/z 57 [$M - CH_3-CH_2$] $^+$ and 29 [$M - CH_3-CH_2-C=O$] $^+$ can be explained by α -cleavage. Since this molecule is symmetrical, the α -cleavage could occur on either side of the carbonyl group.



The mass spectrum of component 1082 (Figure 4.23) exhibits prominent ions at m/z 43 and 71, typical for methyl ketones. If the molecular ion is assumed to be at m/z 114, the ion at m/z 71 is possibly due to the loss of a propyl group and the ion at m/z 43 can be attributed to M-71. This formation was construed as evidence in favour of component 1082 being 4-heptanone. For inexplicable reasons the ion at m/z 58, formed by a McLafferty rearrangement, is of quite low abundance. According to the above-mentioned information, 4-heptanone was considered as a candidate structure. A computerized library search and gas-chromatographic co-elution of the natural constituent and the commercial compound confirmed this identification.

4.8 Ketones: Aliphatic (Unsaturated)

The mass spectra of components 1381 (Figure 4.24), 1900 (Figure 4.25) and 2406 (Figure 4.26) seem identical in the lower mass range, all having a base peak at m/z 55 and an ion at m/z 43 of almost equal abundance. Ions at m/z 71 and 97 are also visible in the spectra. Very little structural information could be obtained from the higher mass range. In the gas chromatogram (Figure 3.6), these components elute approximately 8,5 minutes apart and they were considered to be members of the same homologous series. According to a computerized library search, these components could possibly be unsaturated methyl ketones and from the general similarity between their spectra and those of the 2-alkanones, it was deduced that they could be alken-2-ones.

A McLafferty rearrangement requires at least four carbon atoms between the carbonyl group and a double bond. According to Fenselau *et al.* [19], the $[M-58]^+$ ion is more likely to originate from unsaturated ketones with the conventional McLafferty rearrangement product (m/z 58), when there are at least four carbon atoms between the carbonyl group and the double bond. The extremely low abundance of this ion (m/z 58) in the mass spectra of component 1381 and 1900 is an indication that these processes are not favoured. The double bond is therefore more likely to be at C₃, C₄ or C₅ of an alken-2-one.

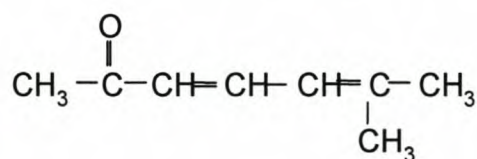
A computerized library search identified components 1381 and 1898 as [*E*]-3-hepten-2-one and [*E*]-3-octen-2-one respectively. This was confirmed by the co-elution of the commercial compounds and the natural material. As may be expected, no conclusion with respect to the double bond stereochemistry could be obtained.

In the mass spectrum of component 2406 the molecular ion is not visible, but on account of a comparison between the mass spectrum of component 2406 and the mass spectra of short-chain alken-2-ones, [*E*]-3-nonen-2-one was the most likely candidate. This identification was confirmed by the co-elution of the commercial compound and the natural material in which case the ions *m/z* 125 and *m/z* 97 could possibly be ascribed to M - CH₃ and M - CH₂CH₂CH₃ processes.

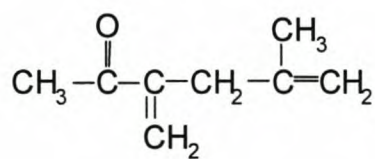
The following conclusions could be drawn from the mass spectrum of component 2224 (Figure 2.27) [20]:

- The *m/z* 124 ion is the molecular ion.
- The *m/z* 109 ion (base peak) is an [M - CH₃]⁺ ion.
- The *m/z* 81 ion (50%) could be an [M - CH₃CO]⁺ and/or [M - CH₃-CO]⁺ ion.
- The *m/z* 43 ion (96%) could be due to the presence of a methyl ketone moiety in the compound [CH₃-CO]⁺.

A computerized library search gave two possible structures, viz 6-methyl-3,5-heptadien-2-one and 5-methyl-3-methylene-5-hexen-2-one.



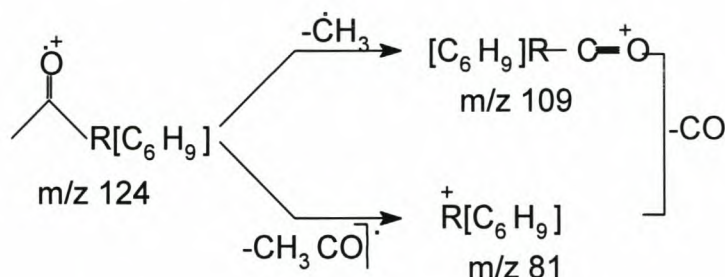
6-methyl-3,5-heptadien-2-one



5-methyl-3-methylene-5-hexen-2-one

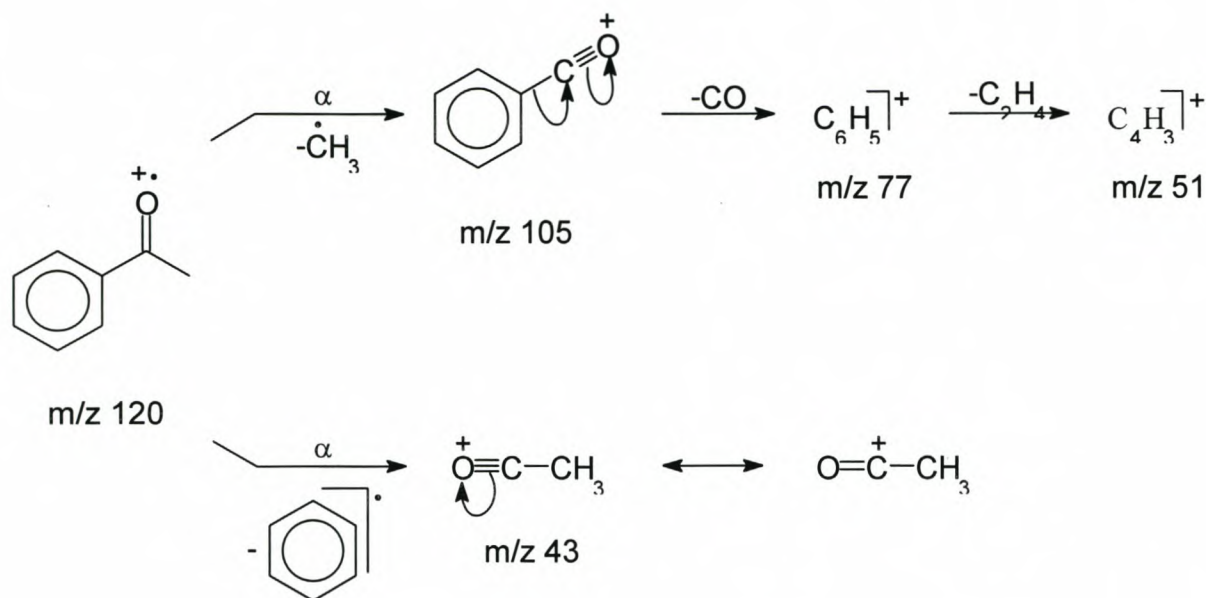
A comparison of the mass spectra of both these structures with the corresponding mass spectra from the Wiley computerized library search yielded 6-methyl-3,5-heptadien-2-one as the only result. This was also confirmed with the co-elution of the commercial *trans*-6-methyl-3,5-heptadien-2-one and the natural material.

The favoured fragmentations in this component are the elimination of a methyl radical (α -scission process), giving the more abundant m/z 109 peak, the formation of the $[M - \text{CH}_3\text{CO}]^+$ ion at m/z 81 and a vinylic cleavage to produce an acyl ion at m/z 43 [21]:

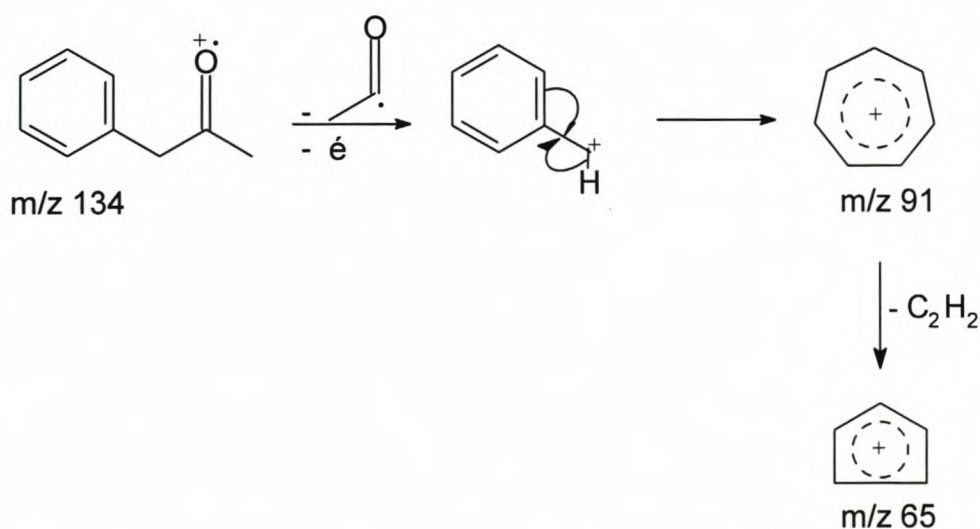


4.9 Ketones: Aromatic

The mass-spectral fragmentation patterns of alkyl aryl ketones are straightforward. The base peak at m/z 77 in the mass spectrum of constituent 2019 (Figure 4.28) is the key to the identification of aromatic ketones and is ascribed to the presence of the phenyl moiety in the molecule. The prominent ions in the mass spectrum of this component at m/z 120, 105, 51 and 43 are similarly prominent in a mass spectrum of acetophenone from the literature [22]. The expulsion of a methyl group from the molecular ion at m/z 120 in the mass spectrum of this constituent gives an ion at m/z 105 which in turn expels carbon monoxide to give the phenyl fragment at m/z 77. This ion decomposes by elimination of acetylene to a $(\text{C}_4\text{H}_3)^+$ ion at m/z 51. The occurrence of a m/z 43 peak can be explained by an alternative α -fission of the phenyl-carbonyl bond [22]. Acetophenone co-eluted with the natural constituent:



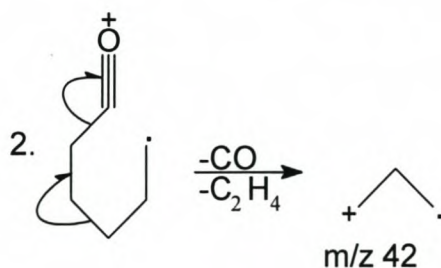
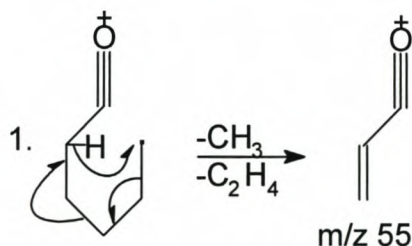
The mass spectrum of component 2328 (Figure 4.29) contains two prominent ions at m/z 43 and 91. The ion at m/z 134 can be assumed to be the molecular ion of this compound. Loss of a benzyl radical gives the well-known acyl ion at m/z 43 and the ion at m/z 91, (C_7H_7^+), can be explained by an α -cleavage with loss of the acetyl group and the formation of a tropylium ion. The formation of the ion at m/z 65 is due to the elimination of C_2H_2 from the ion at m/z 91. Based on this mass spectral data the component 2328 was identified as 1-phenyl-2-propanone. The above fragmentation processes can be formulated as follows:

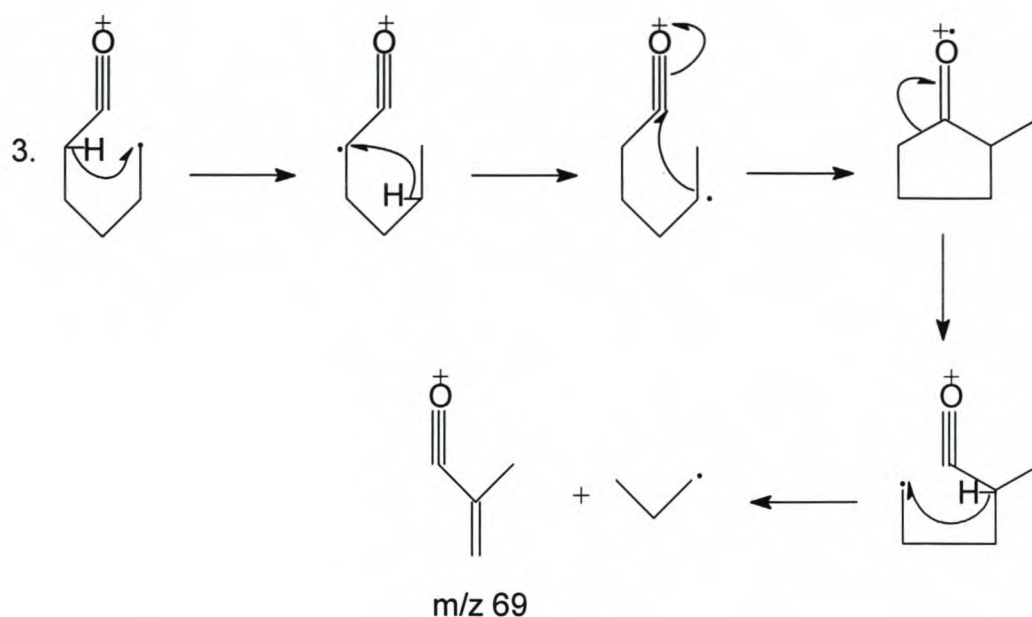


The natural constituent and 1-phenyl-2-propanone co-eluted in a gas chromatographic analysis.

4.10 Ketones: Cyclic

The spectrum of cyclohexanone has been studied by both high resolution [23] and deuterium labelling techniques [24]. The most abundant fragments are products of α -cleavage. The base peak at m/z 55 is formed by an α -cleavage and the elimination of a CH_3 radical and C_2H_4 molecule. Evidence in favor of the existence of the discrete intermediate is found in the occurrence of an $[\text{M} - \text{CH}_3]^+$ ion at m/z 83. The major contributor to the m/z 42 ion is a hydrocarbon fragment (C_3H_6^+), in which C4, C5, C6 are retained. The neutral portion which is lost corresponds to the elements of carbon monoxide and ethylene. The ion at m/z 69 is formed by a mechanism proposed by Seibl and Gaumann [69] in which C2 and C3 are eliminated in a neutral ethyl radical. The sequence proceeds through an ionised methylcyclopentanone that is formed from the molecular ion by hydrogen transfer and ring closure [25].



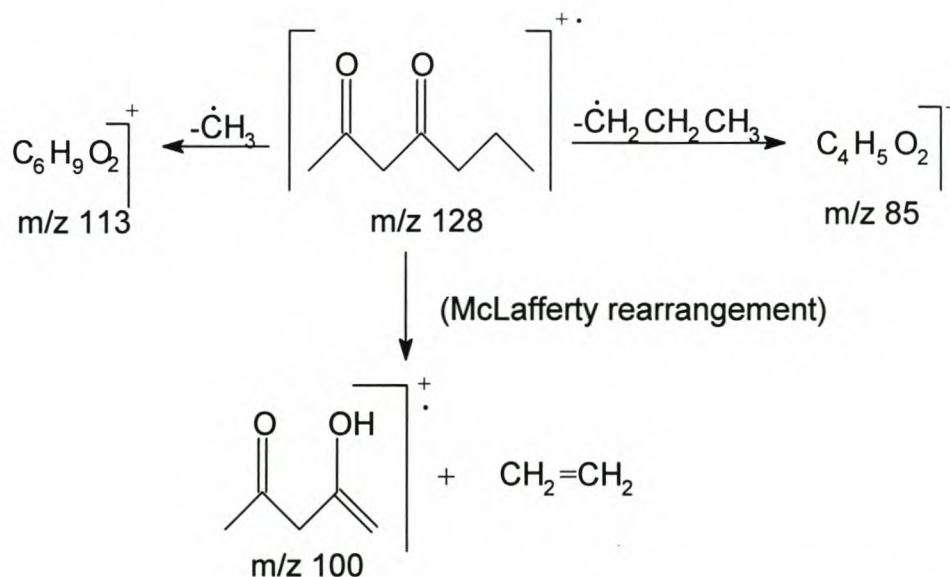


The prominent ions in the mass spectrum of component 1150 (Figure 4.30) can be explained in terms of the above-mentioned mechanisms. However, 2-methylcyclopentanone, was also considered as a possible candidate structure for component 1150. Because the abundance of the molecular ion is higher in the mass spectrum of the natural compound than in that of 2-methylcyclopentanone, it is assumed that this compound is cyclohexanone. This was confirmed by mass-spectral and retention-time comparison of these compounds and the natural material.

Significant similarities are observed between the mass spectra of cyclohexanone and component 938 (Figure 4.31). In the mass spectrum of this component the ions at m/z 42, 55 and 98 are prominent and the base peak is present at m/z 69. A computerized library search suggested 3-methylcyclopentanone as a possibility. Another candidate structure, 2-methylcyclopentanone, was also considered. The high abundance of the m/z 69 ion was accepted as conclusive evidence in favour of 3-methylcyclopentanone as the structure of this constituent because Seibl and Gäumann [24] found a pronounced preference for the 3-4 bond cleavage (yielding m/z 69) in deuterium and ^{18}O labeling studies with this compound. The m/z 69 ion is of quite low abundance in the mass spectrum of 2-methylcyclopentanone. Co-elution of commercial 3-methylcyclopentanone and the natural material confirmed these arguments.

4.11 Diketones

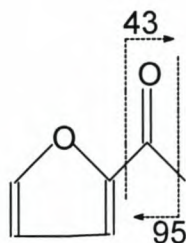
The mass spectrum of component 1564 (Figure 4.32) has two prominent ions at m/z 43 and 85 (base peak) that could possibly be of diagnostic value. A computerised library search gave 2,4-heptanedione, 2-methyl-3-heptanone and 2-methyl-4-heptanone as possible candidates. If it is assumed that the abundant m/z 43 ion is due to an α -cleavage of a methyl ketone giving the acylium ion $[\text{CH}_3\text{CO}]^+$, the possibility of this component being either 2-methyl-3-heptanone or 2-methyl-4-heptanone can be eliminated. Furthermore, 2-methyl-3-heptanone and 2-methyl-4-heptanone both have a prominent ion at m/z 57, which is not present in the mass spectrum of component 1564. If it is assumed that component 1564 is 2,4-heptanedione, the peak at m/z 85 can be explained by another α -cleavage at C4 and the loss of a propyl group. The molecular ion at m/z 128 and the $(M-15)^+$ ion at m/z 113 are in agreement with this assumption. The less abundant McLafferty ion is present at m/z 100. The different fragmentations and McLafferty rearrangement can be illustrated as follows:



2,4-Heptanedione was not commercially available for co-injection with the natural material and this component was not unequivocally characterized.

4.12 Furan

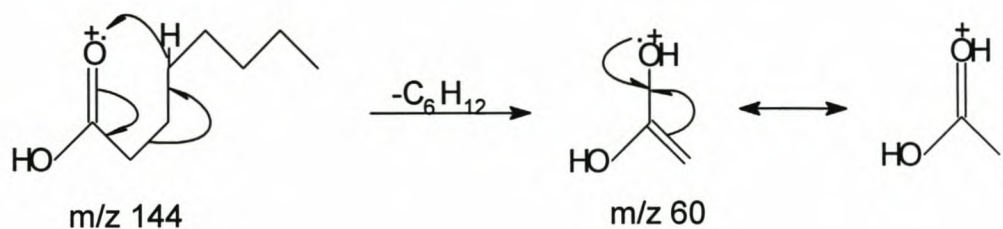
The mass spectrum of component 780 (Figure 4.33) has a base peak at m/z 95, but contains very little diagnostic information. According to a computerized library search the spectrum of this component closely resembles that of 2-acetylfuran [1-(2-furanyl)-ethanone]. It was confirmed that this component is indeed 2-acetylfuran by the co-elution of the commercial compound and the natural material. The molecular ion is seen at m/z 110. Loss of methyl radical (m/z 95) [26] or furanyl radical (m/z 43) is observed:



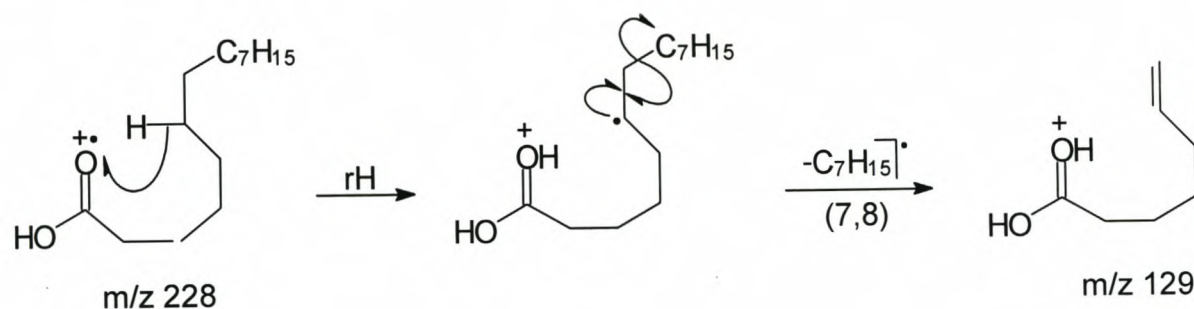
4.13 Acids: Aliphatic

The prominent ions at m/z 60, m/z 73 and m/z 43, characteristic for aliphatic acids, are present in the mass spectra of components 2558 (Figure 4.34) and 5051 (Figure 4.35).

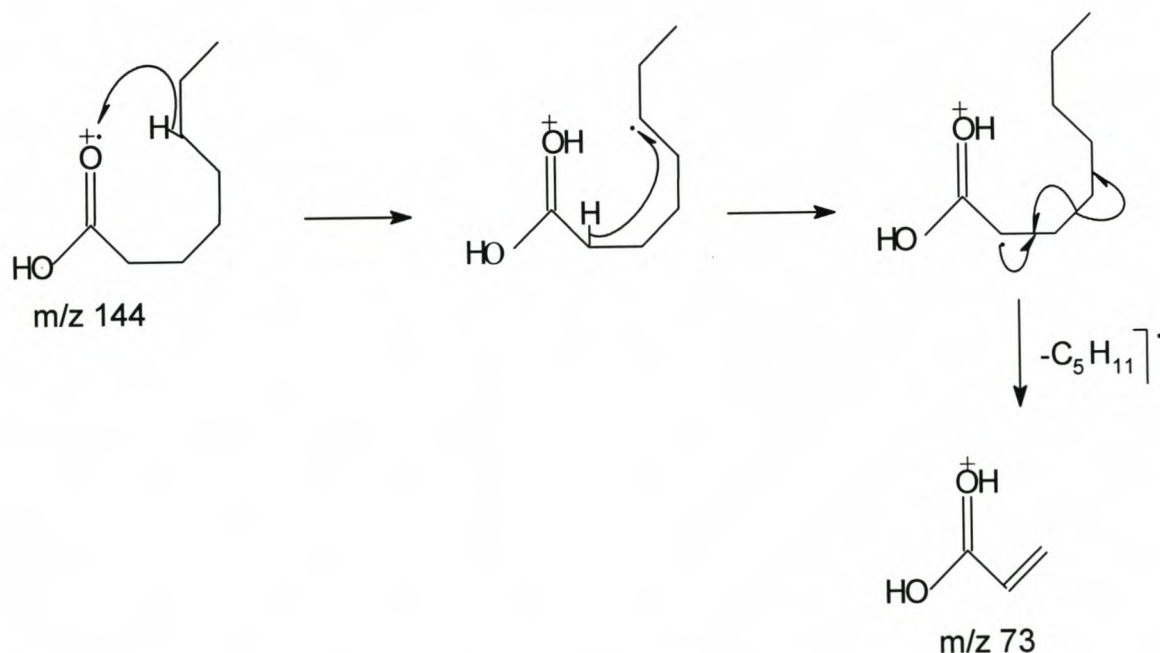
Aliphatic carboxylic acids generally have reasonably prominent molecular ions, the relative abundance of which increase with molecular mass for unbranched aliphatic acids containing more than six carbon atoms [27]. In the mass spectrum of component 5051 the ion at m/z 228 was assumed to be the molecular ion and this component was therefore presumed to be a tetradecanoic acid. The ion at m/z 60 in these spectra is formed by the following McLafferty rearrangement [28]:



A series of ions in this mass spectrum occurs at m/z 73, 115, 129, 143, 157 etc. These ions are formed by hydrogen transfer from carbon atoms along the carbon chain of the acid in conjunction with homolysis of one of the bonds β to these hydrogen-depleted carbon atoms. In the case of the m/z 129 ion, the process can be illustrated as follows [29]:

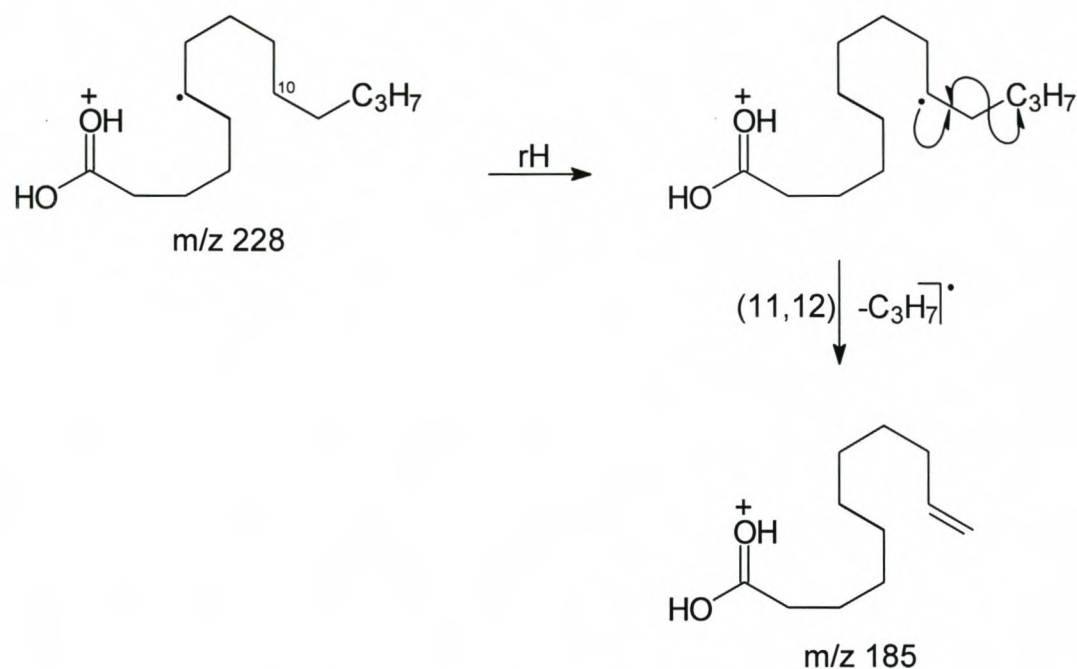


A favoured reaction is the transfer of an H-atom from the α -position relative to the carbonyl group and the subsequent β -cleavage which gives the ion at m/z 73:

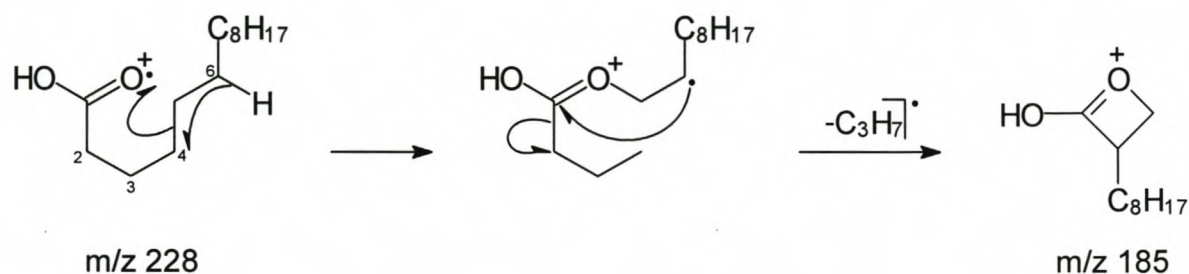


The driving force in this reaction is the high stability of the α,β -unsaturated protonated carbonyl system. The resulting (McLafferty + 13) ion often accompanies the normal McLafferty rearrangement ion [30].

As in the case of the (McLafferty + 13)⁺ rearrangement, hydrogen migration from C10, followed by β -cleavage, results in the formation of the ion at m/z 185 [31]:

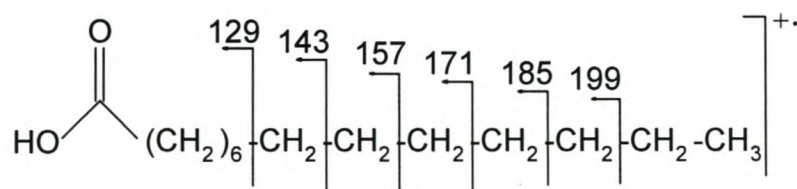


However, this ion can also be formed by the loss of 43 atomic mass units from the molecular ion and can be explained by a rearrangement reaction involving the transfer of a hydrogen atom from C6, resulting in the elimination of the fragment containing carbon atoms C2-C4:



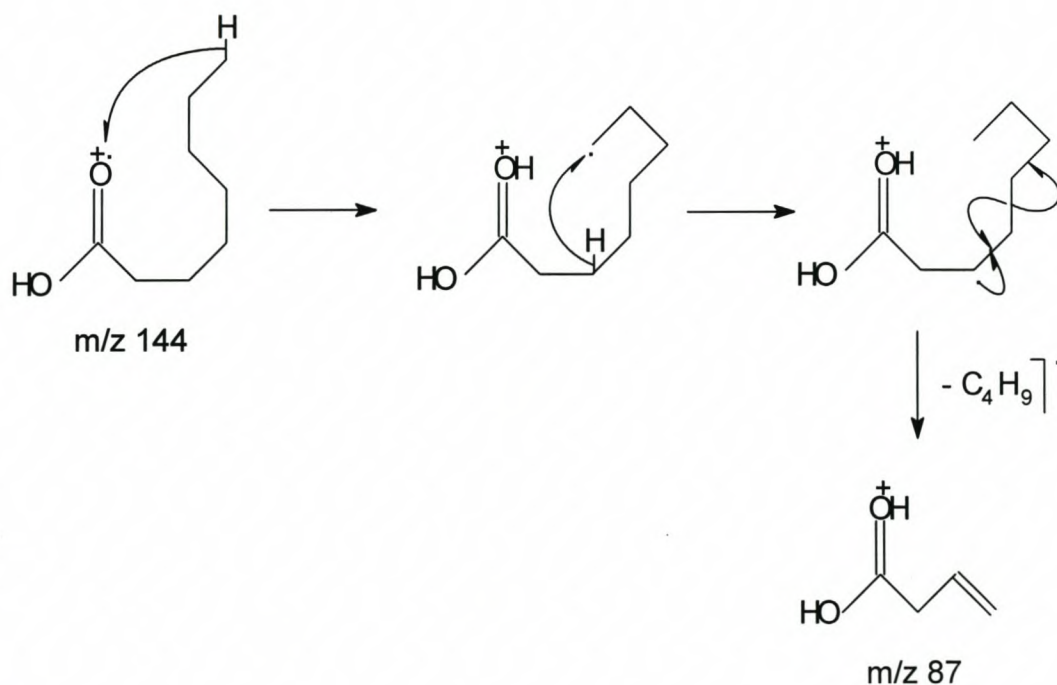
The formation of the series of ions at m/z 157 (M-71), 171 (M-57) and 185 (M-43) can be explained in terms of simple α -cleavage with the charge being retained on the

oxygen-containing fragments. α -Cleavage with charge retention on the alkyl groups accounts for many of the ions in the lower mass range of the spectrum [32]:



Co-injection of a series of synthetic straight-chain aliphatic acids (C4-C14) and the natural material proved component 5051 to be tetradecanoic acid.

The mass spectrum of component 2558 (Figure 4.34) has the typical appearance of that of a straight-chain aliphatic acid with the characteristic McLafferty and $(\text{McLafferty} + 13)^+$ peaks at m/z 60 (base peak) and m/z 73 respectively. The fragment at m/z 87 is formed by a mechanism similar to the formation of the $(\text{McLafferty} + 13)^+$ peak [33]:



The spectrum of this component does not seem to contain a molecular ion. However, co-chromatography of a mixture of synthetic straight-chain aliphatic acids (C₄-C₁₀) and the natural material, proved component 2558 to be octanoic acid.

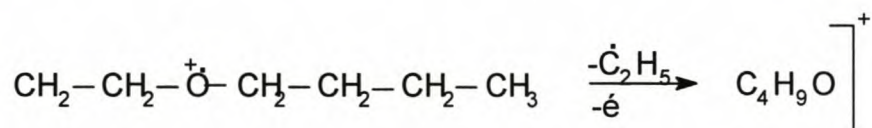
4.14 Ethers: Aliphatic

The similarity between the mass spectra of components 218 (Figure 4.36), 427 (Figure 4.37) and 772 (Figure 4.38) is significant and these components appear to belong to a homologous series of compounds. In addition to the base peak at *m/z* 59, these mass spectra also have ions at *m/z* 31, 41, 43, 60 and 73 in common. According to the results of the computerised library search these components were identified as ethyl propyl ether, butyl ethyl ether and amyl ethyl ether respectively. These ethers were synthesised and co-injected with the natural material to confirm their identification.

It has been shown that principal fragmentation processes of aliphatic ethers can be interpreted by assuming the intermediacy of molecular ions bearing the charge on the oxygen atom [34]. The ether molecular ion undergoes bond fissions similar to those of alcohols [35]. The α -cleavage of the molecular ion of aliphatic ethers is one of the favoured decomposition modes. In an unsymmetrical ether, two ions can arise, depending upon the site of α -bond fission but the most highly substituted fragment is lost preferentially [36].

Butyl ethyl ether is chosen to illustrate the fragmentation patterns of these ethers.

The preferred α -cleavage is as follows:



The abundant ion at *m/z* 73 is a result of the loss of an ethyl group from the ether. The molecular ion is present at *m/z* 102 and the [M-CH₃]⁺ ion is observed at *m/z* 87 in equal abundance. The ion at *m/z* 59 represents a [CH₃CH₂OCH₂]⁺ ion and with

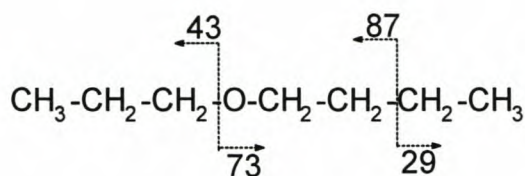
increasing chain length of the alkyl groups, this “hydrocarbon” type of fragmentation will predominate [36].

In butyl ethyl ether, the butyl ion at m/z 57 is produced by carbon-oxygen bond fission. According to McLafferty [36], the rupture of the carbon-oxygen linkage and the increased generation of alkyl ions can be ascribed to the decreased electron density around the oxygen atom and the relative inertness of the carbon-carbon linkages in the alkyl groups. To comply with the octet rule, the charge does not remain with the fragment containing the oxygen. This fragmentation process explains the presence of the ethyl ion at m/z 29. In the mass spectrum of component 218 (Figure 4.36) the prominent peak at m/z 43 represents a propyl ion, formed by this fragmentation pattern. The base peak at m/z 59 in this mass spectrum represents the $[\text{CH}_3\text{CH}_2\text{OCH}_2]^+$ ion and the molecular ion is seen at m/z 88.

There are several fragmentation modes of secondary importance present in the mass spectra of aliphatic ethers, such as, for example, the elimination of an alcohol molecule with the formation of an unsaturated alkyl radical [37]. In the mass spectrum of component 772 the prominent ion at m/z 70 is due to the elimination of ethanol and represents the remaining penten-1-yl ion. The molecular ion, which is of very low abundance, is seen at m/z 116. The base peak at m/z 59 represents the $[\text{CH}_3\text{CH}_2\text{OCH}_2^+]$ ion.

The possibility that the above-mentioned components could possess an α - branched carbon atom is eliminated by the absence of the appropriate peak at m/z 45 for such a compound [36].

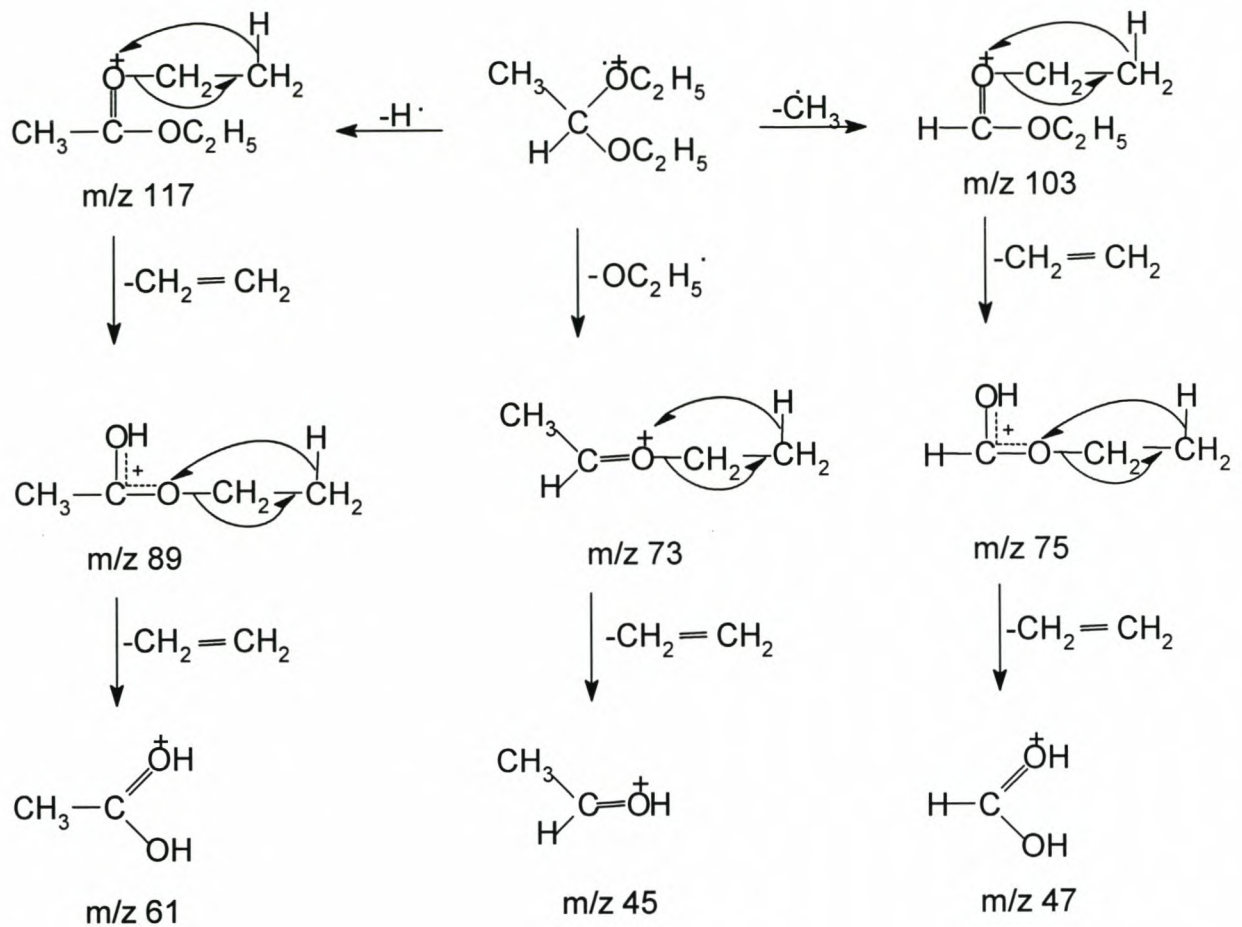
Component 732 (Figure 4.39) was identified as butyl propyl ether through the computerised library search as well as co-elution of the synthetic and the natural material. In the mass spectrum of this constituent the base peak is present at m/z 57 and represents the butyl ion or $[\text{M} - \text{OCH}_2\text{CH}_2\text{CH}_3]^+$ ion. The ion at m/z 43 is ascribed to the formation of the propyl ion. The less intense peak at m/z 73 represents the $[\text{CH}_2\text{OCH}_2\text{CH}_2\text{CH}_3]^+$ ion and is due to the loss of a propyl group from the butyl substituent. The molecular ion is seen at m/z 116 and the peak at m/z 87 is due to the loss of an ethyl group [36].



The component 406 (Figure 4.40) is identified as di-n-propyl ether through the computerised library search as well as co-elution of the synthetic and the natural materials. The prominent peaks represent the molecular ion at m/z 102, the $[\text{M-CH}_2\text{CH}_3]^+$ ion at m/z 73 and the propyl ion at m/z 43.

4.15 Acetaldehyde diethyl acetal

In the mass spectrum of component 529 (Figure 4.41) the base peak is present at m/z 45, two prominent peaks are visible at m/z 73 and 103 and other peaks of small intensity are observed at m/z 47, 61 and 75. A computerised library search gave acetaldehyde diethyl acetal as the most likely candidate compound. According to Budzikiewicz [38], α -cleavage of the molecular ion of this compound can involve at least three different bonds. The least favoured process is the loss of the hydrogen atom ($\text{M}-1$) while the most favoured one involves elimination of an ethoxyl radical to give the ion at m/z 73. The α -cleavage of intermediate abundance involves the elimination of a methyl radical to give the ion at m/z 103. Each of these fragmentations can then be followed by the expulsion of an olefin (ethylene) with concomitant migration of a hydrogen atom. The $(\text{M}-1)^+$ and $[\text{M-CH}_3]^+$ ions can undergo this process twice, while the $[\text{M-C}_2\text{H}_5\text{O}]^+$ ion can sustain such an ethylene loss only once. These processes are illustrated in the following scheme:



Of these, the ions at m/z 45, 73 and 103 are the most prominent in the mass spectrum of acetaldehyde diethyl acetal. This identification was confirmed by the co-elution of the synthetic compound and the natural material.

4.16 Ethyl ester

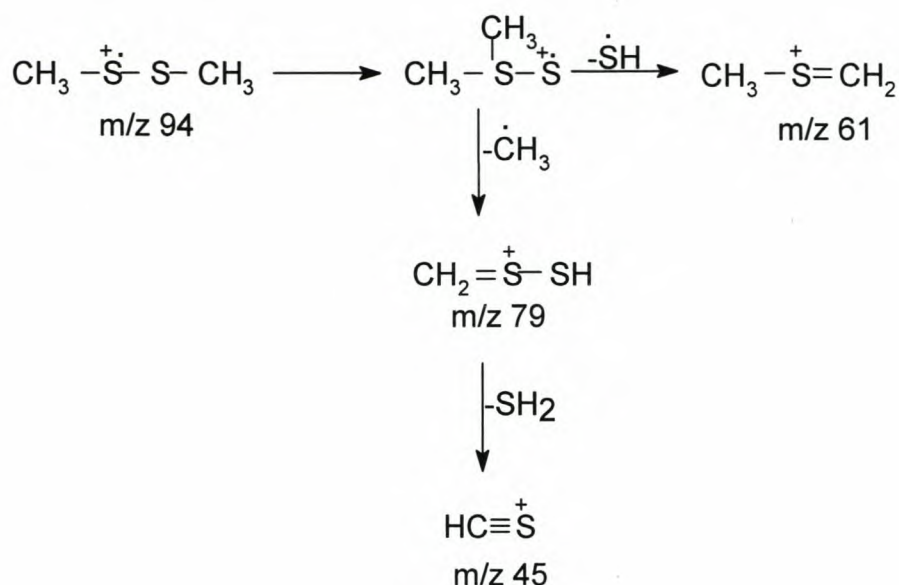
The EI mass spectrum of component 317 (Figure 4.42) contains an ion at m/z 61, which is characteristic of alkyl acetates. If the ion at m/z 88 is assumed to be the molecular ion, component 317 could possibly be ethyl acetate. Co-Injection of the commercially available material with the natural material confirmed this identification.

4.17 Dimethyl sulphone

The molecular ion in the mass spectrum of component 1240 (Figure 4.43) appears at m/z 94 and the base peak at m/z 79, corresponding to the loss of a methyl radical. This correlates with the published mass spectrum of dimethyl sulphone [39]. This compound has already been found in certain moss [40] and plant [41] species, in the exocrine secretions of antelope such as the springbok, *Antidorcas marsupialis* [42] and the black wildebeest, *Connochaetes gnou* [43] and in blood of cows [44]. The identification of this component as dimethyl sulphone was confirmed by co-elution of the commercial compound and the natural substance.

4.18 Dimethyl disulphide

The base peak in the mass spectrum of the component 554 (Figure 4.44) appears at m/z 94. The component was readily identified as dimethyl disulphide by a computerised library search and its co-elution with the synthetic material. The molecular ion of the compound is present in the mass spectrum at m/z 94. The main decomposition mode for higher aliphatic disulphides is the elimination of an olefin molecule but dimethyl disulphide shows exceptional behaviour since the olefin elimination is not possible. The loss of one or two methyl groups to give the ions at m/z 79 (CH_3S_2) and at m/z 64 (S_2) respectively, is not exceptional but the ion at m/z 61 is also produced by the expulsion of a sulfhydryl group. Another important rearrangement ion is formed by the further loss of hydrogen sulphide from the species at m/z 79 to give a peak (50%) at m/z 45 ($\text{HC}=\text{S}^+$). These fragmentations are shown in the following scheme [45]:



4.19 Formanilide

The mass spectrum of component 2743 (Figure 4.45) with its base peak at m/z 93 and a relative abundant molecular ion at m/z 121 suggests that this is most likely an aromatic compound containing an uneven number of nitrogen atoms. According to the computerised library search, formanilide was a possible candidate and co-elution of the synthetic material and the natural material confirmed this identification.

4.20 Elemental sulphur

The mass spectra of components 4005 (Figure 4.47) and 5950 (Figure 4.48) have very similar characteristics containing a base peak at m/z 64 and other peaks at m/z 32, m/z 96, m/z 128, m/z 160, m/z 192 and m/z 224 evenly spaced at intervals of 32 atomic mass units. The mass spectrum of component 5950 also has ions at m/z 224 and 256. The ratios of these ions and their isotope ions at m/z 34, m/z 66, m/z 98, m/z 130, m/z 162, m/z 194 and m/z 258 can be used to calculate the number of sulphur atoms in each of the fragments, from which it became clear that components 4005 and 5950 are the different cyclic sulphur molecules S_6 and S_8 .

In the TIC of the urine volatiles of the cheetah urine, sulphur elutes as a band with a maximum at 99 minutes (238°C). The maximum at this retention time can most likely

be ascribed to S_8 which is the most stable of the molecular structures. The broadness of the elution pattern can be ascribed to the interconversion of the cyclic structures during their passage through the column, a phenomenon similar to the formation of metastable peaks in mass spectrometry.

4.21 Urea

On face value the mass spectrum of component 2952 (Figure 4.49) seemed very similar to the mass spectrum of acetic acid but due to the significant difference in their retention times, comparison with acetic acid was not considered. The base peak at m/z 60 and the other three ions at m/z 44, m/z 43 and m/z 42, together with their relative abundance, were used in the Wiley computer search, which yielded urea with a mass spectrum similar to that of component 2952 as a likely possibility. This compound was expected to be present in the urine of the animal.

3.22 Impurities

Components 127 and 166 in the TIC of male cheetah urine and components 178 and 198 in that of the female cheetah urine were identified as dichloromethane, 2-chloro-3-methylbutane respectively. These compounds are known to be impurities in the dichloromethane used for extraction of the volatiles from the urine.

Components 140, 176, 906 and 1789 present in the TIC of the male and female urine are impurities introduced into the sample by the thermal degradation of the silicone rubber of the enrichment device and were easily identified by their base peaks at m/z 73 as trimethylsilane derivatives.

4.3 CONCLUSION

The urine of the male and female cheetah contains a wide variety of different compound types. Aliphatic and aromatic components include alkanes, aldehydes, saturated and unsaturated ketones, a saturated acetate, an alcohol, several short-chain saturated ethers and two aliphatic acids. Various cyclic components were also found in the urine. Other interesting components were formamide, an alkylfuran, a few sulphur-containing compounds, elemental sulphur and urea. A summary of the identified compounds is given in Table 4.1

Table 4.1: Compounds identified in the urine of the cheetah

§	Compound	El mass spectrum Fig. no.	Female component no.	Male component no.
4.1	Heptane	4.1	442	445
4.1	Decane	4.2	1767	*
4.1	Dodecane	4.3	2760	*
4.2	<i>trans</i> -1-ethyl-3-methyl-cyclopentane	4.4	1469	1481
4.3	Toluene	4.5	644	699
4.3	<i>m</i> -Xylene	4.6	1043	1052
4.3	<i>p</i> -Xylene	4.7	1189	1202
4.3	1.3.5-trimethylbenzene	4.8	1699	1710
4.4	Phenol	4.9	1622	1632
4.5	Hexanal	4.10	755	761
4.5	Octanal	4.11	1725	1730
4.5	Nonanal	4.12	2239	2246
4.6	Benzaldehyde	4.13	1481	1485
4.7	2-Butanone	4.14	206	204
4.7	2-Pentanone	4.15	383	388
4.7	2-Hexanone	4.16	715	724
4.7	2-Heptanone	4.17	1161	1172
4.7	2-Octanone	4.18	1665	*
4.7	2-Nonanone	4.19	2178	*
4.7	2-Undecanone	4.20	3146	*
4.7	3-Pentanone	4.21	410	415

4.7	3-Hexanone	4.22	701	709
4.7	4-Heptanone	4.23	1082	1092
4.8	[E]-3-Hepten-2-one	4.24	1381	*
4.8	[E]-3-Octen-2-one	4.25	1903	*
4.8	[E]-3-Nonen-2-one	4.26	2406	*
4.8	[E]-6-methyl-3,5-heptadien-2-one	4.27	2224	*
4.9	Acetophenone	4.28	2019	2024
4.9	1-phenyl-2-propanone	4.29	2328	2335
4.10	Cyclohexanone	4.30	1150	1163
4.10	3-Methyl-cyclopentanone	4.31	938	949
4.11	2,4-Heptanedione	4.32	1564	*
4.12	2-Acetylfuran	4.33	780	*
4.13	Octanoic acid	4.34	2558	*
4.13	Tetradecanoic acid	4.35	5015	*
4.14	Ethyl propyl ether	4.36	218	214
4.14	Ethyl butyl ether	4.37	427	430
4.14	Ethyl pentyl ether	4.38	529	*
4.14	Butyl propyl ether	4.39	732	739
4.14	Di-n-propyl ether	4.40	772	780
4.15	Acetaldehyde diethyl acetal	4.41	406	408
4.16	Ethyl acetate	4.42	317	317
4.17	Dimethyl sulphone	4.43	1240	1246
4.18	Dimethyl disulphide	4.44	554	559
4.19	Formanilide	4.45	2743	2754
4.20	Elemental sulphur	4.46	4005	4022
		4.47	5950	5962
4.21	Urea	4.48	2952	2926

* Compound not found in the urine of the male cheetah

These compounds were found in the urine of captive cheetah. It is notable that more compounds were found in the urine of female cheetah than in urine of male cheetah. Urine from wild cheetah was used towards the conclusion of this study to verify the presence of compounds found in the urine of the captive cheetah. The urine from wild cheetah contained significantly higher concentrations of the volatile compounds identified in the urine of captive cheetah. This could be due to the effective

communication required for survival in a competitive environment. However, it could also be due to the different diet of the wild cheetah or to the fact that they may not have access to an unlimited supply of water. The bulk of the components found in the urine of both captive and wild cheetah occurs in the high volatility region of the gas chromatogram and suggests that these components, when released by the cheetah, will evaporate quickly and possibly render the animal difficult to detect by other predators.

In contrast to other carnivores, the urine of the cheetah contains only two sulphur compounds, which are present in such low concentrations that they could not be detected by the human nose. Elemental sulphur was also found in the urine of both captive and wild cheetah. The elemental sulphur is of great interest since neither of the other sulphur-containing compounds in the urine is known to give elemental sulphur as a product of a simple chemical reaction. Although unlikely, the possibility that sulphur is produced by microbial activity in the bladder was investigated, but no bacteria were found. Enzymatic activity could be a possible cause but calls for further investigation. According to an article by Pronk *et al.* [46], the spontaneous oxidation of sulphide at low concentrations and in acidic environments may produce a number of products, including elemental sulphur. The question however remains if such a reaction can occur under the conditions in the animal's bladder. Only one example of the production of elemental sulphur by an insect in its secretion was reported by Jerrold Meinwald [47].

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ANNEXURE

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 29-Jun-01
 Sample: ACINONYX JUBATUS: MALE A(3) URINE ML SPME P262 40-280 @ 2C/MIN

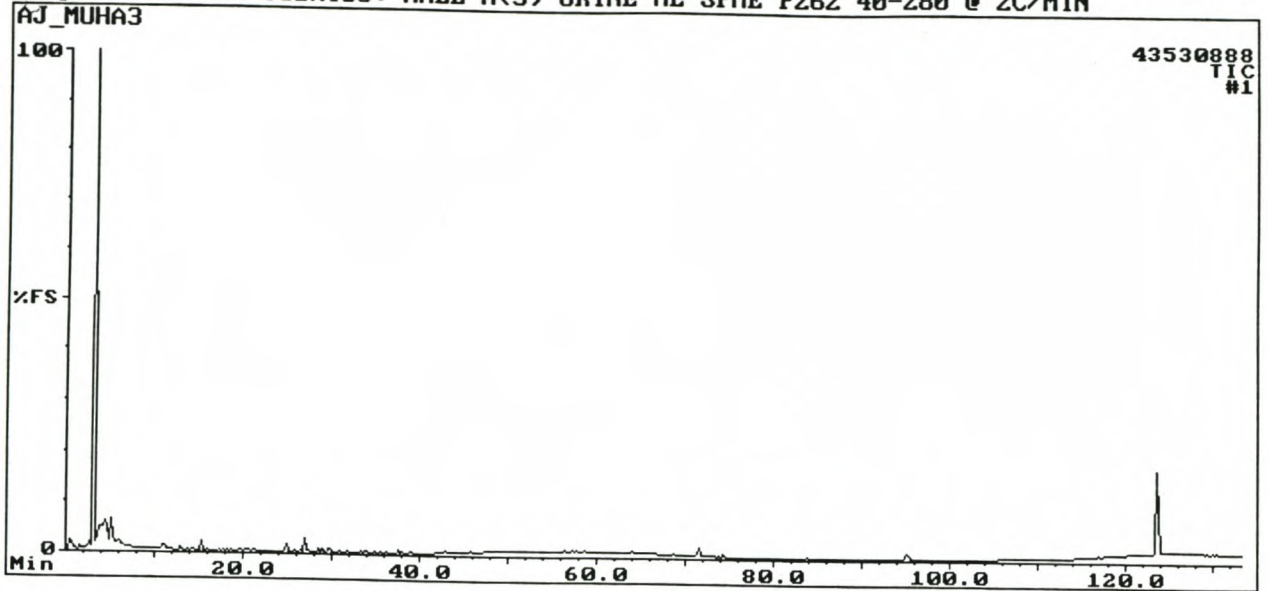


Fig. 3.1: TIC of the organic volatile compounds enriched from urine of male cheetah by SPME. Analytical parameters given in the experimental part, Chapter 3.

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 09-Jul-01
 Sample: ACINONYX JUB:MU HS CRFS 40h, DES 220 5min CRYO P262 40-2280 @ 2C/min

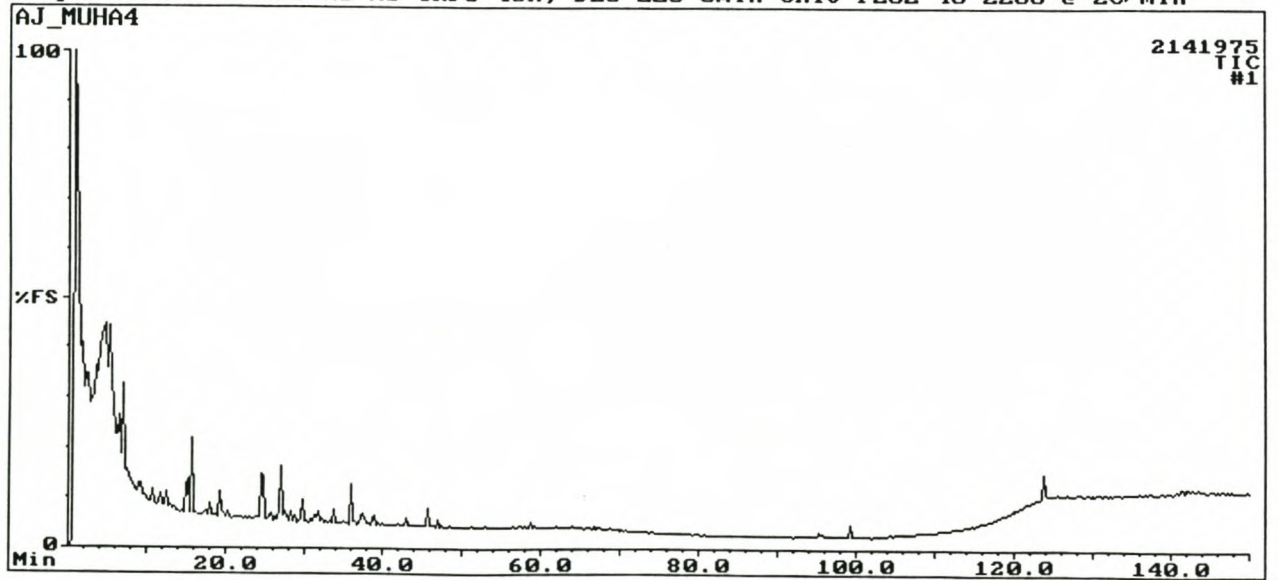


Fig. 3.2: TIC of the organic volatile compounds enriched from urine of captive male cheetah by SPME with increased sampling time. Analytical parameters given in the experimental part, Chapter 3

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 26-Jul-01
Sample:ACINONYX JUB: MCU HS CRFS 50h DES 250 5min CRVO P262 40-280 @ 2C/min

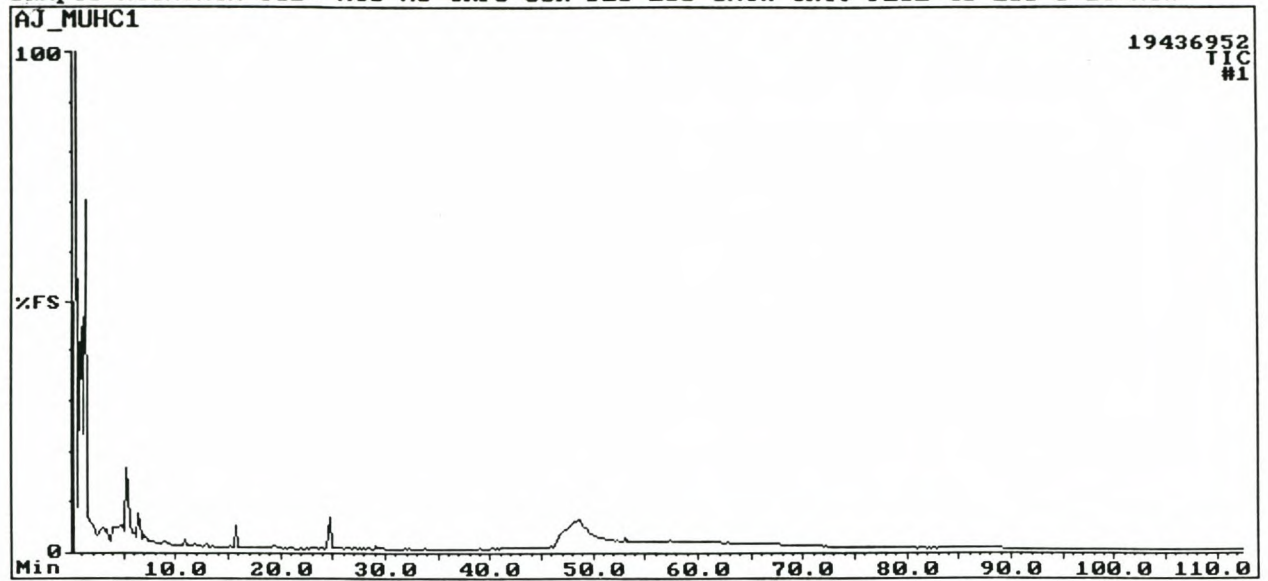


Fig. 3.3: TIC of the organic volatile compounds enriched from urine of another captive male cheetah by SPME. Analytical parameters given in the experimental part, Chapter 3

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 25-Jul-01
Sample:ACINONYX JUB: MBU EX SMALL SPLIT 3min P262 40-280 @ 2C/min

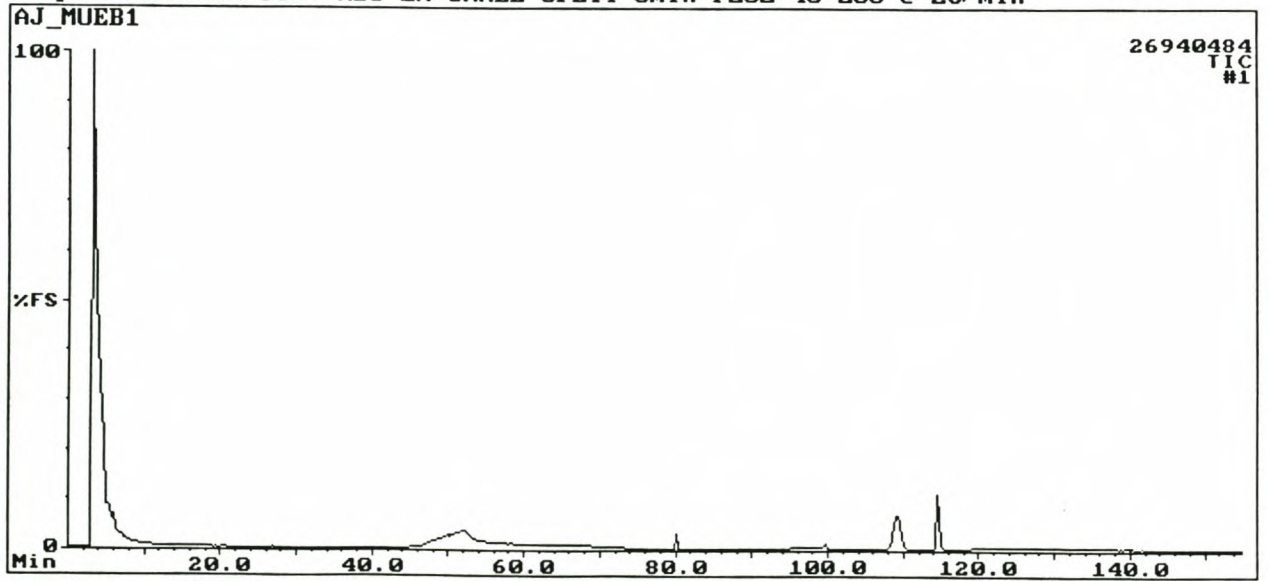


Fig. 3.4: TIC of the organic volatile compounds enriched from urine of another captive male cheetah by SPME. Analytical parameters given in the experimental part, Chapter 3

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 09-Aug-01
 Sample: ACINONYX JUB: MALE URINE COMBINED A-C EX, 40-280 @2 C/min

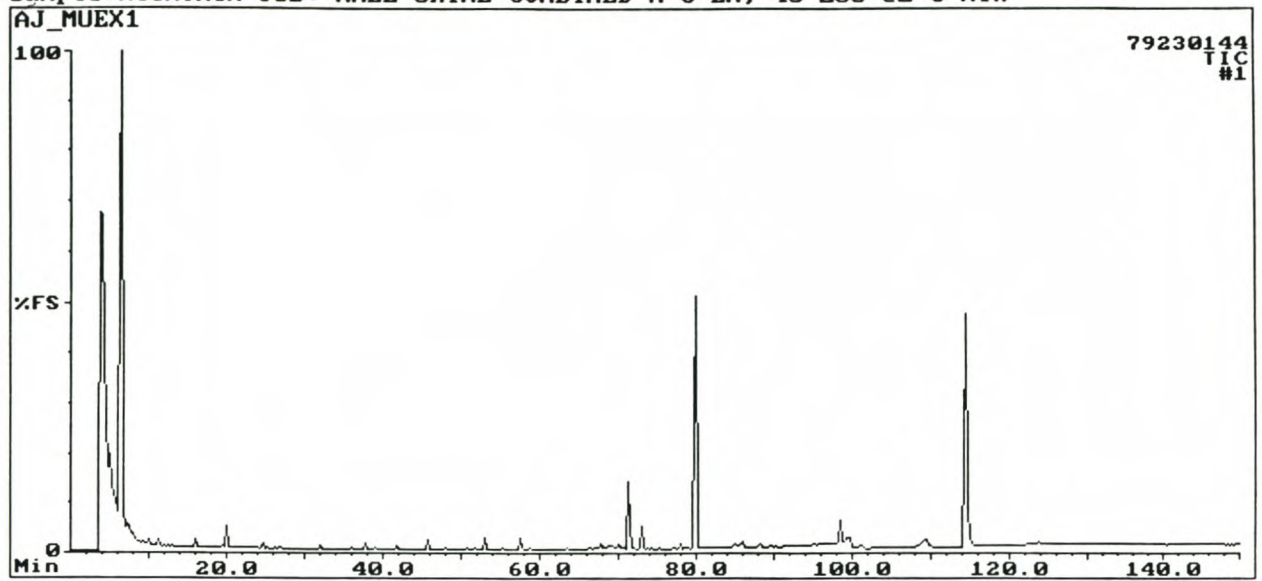


Fig. 3.5: TIC of the organic volatile compounds extracted with dichloromethane from urine of captive male cheetah. Analytical parameters given in the experimental part, Chapter 3

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 26-Aug-01
 Sample: ACINONYX JUB: FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min

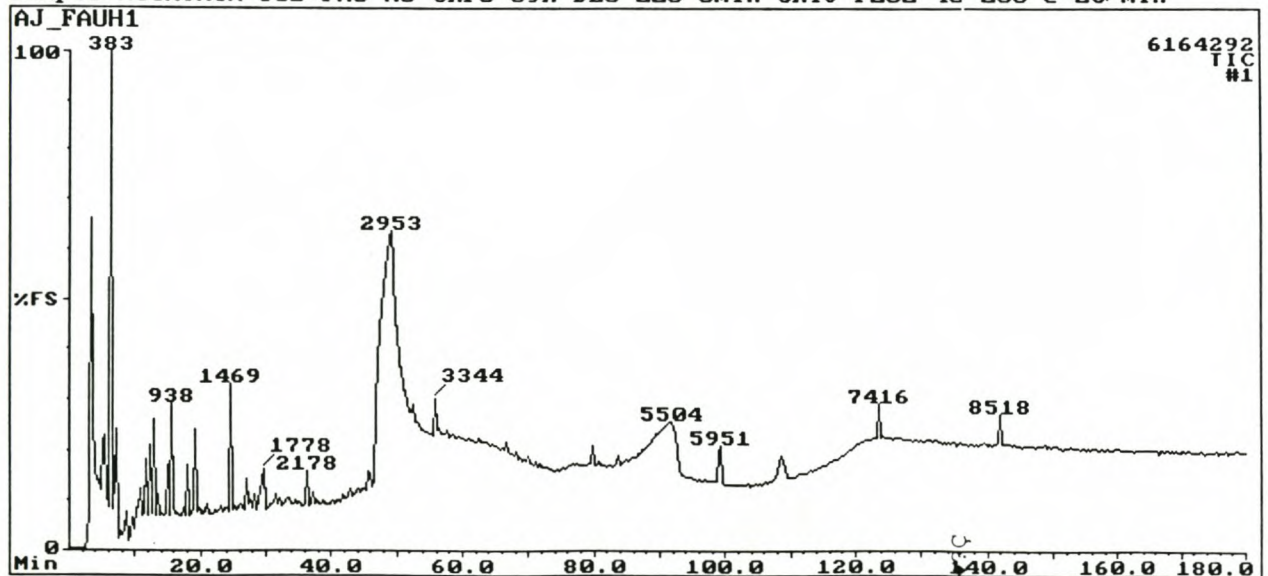


Fig. 3.6: TIC of the organic volatile compounds enriched from urine of captive female cheetah by the SPME enrichment device. Analytical parameters given in the experimental part, Chapter 3

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 17-Feb-02
 Sample: ACINONYX JUBATUS: URINE CHIABAKA RUBHS 22C 25h PS 089, 40-280 @ 2C/min

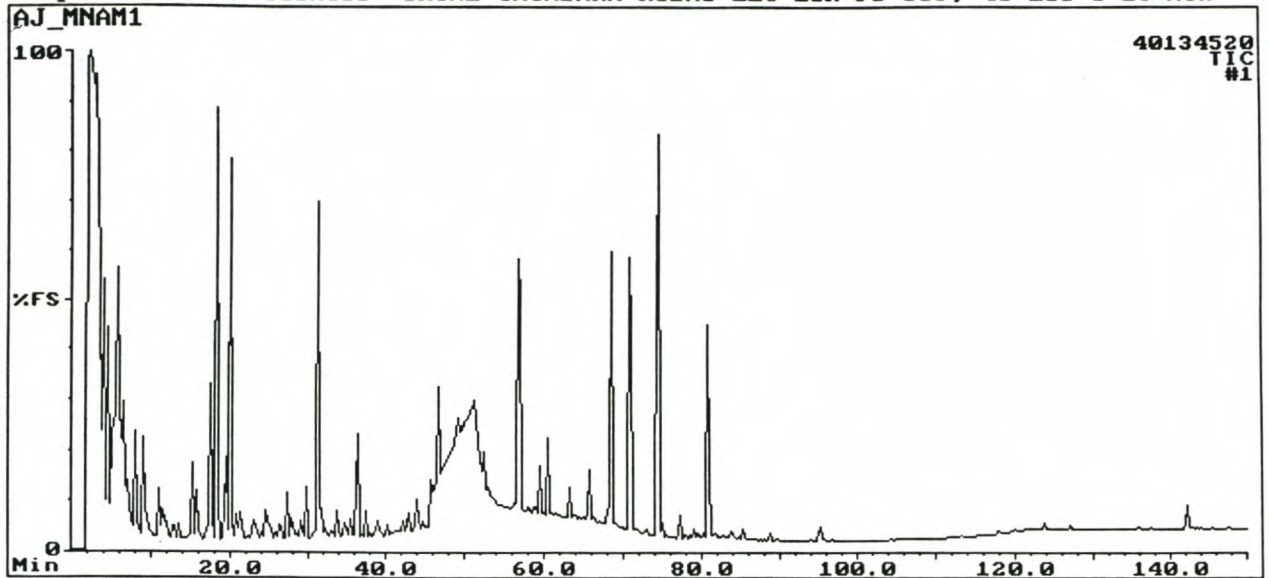


Fig. 3.7: TIC of the organic volatile compounds enriched from urine of wild male cheetah by the SPME enrichment device. Analytical parameters given in the experimental part, Chapter 3

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 26-Aug-01
 Sample: ACINONYX JUB:FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min

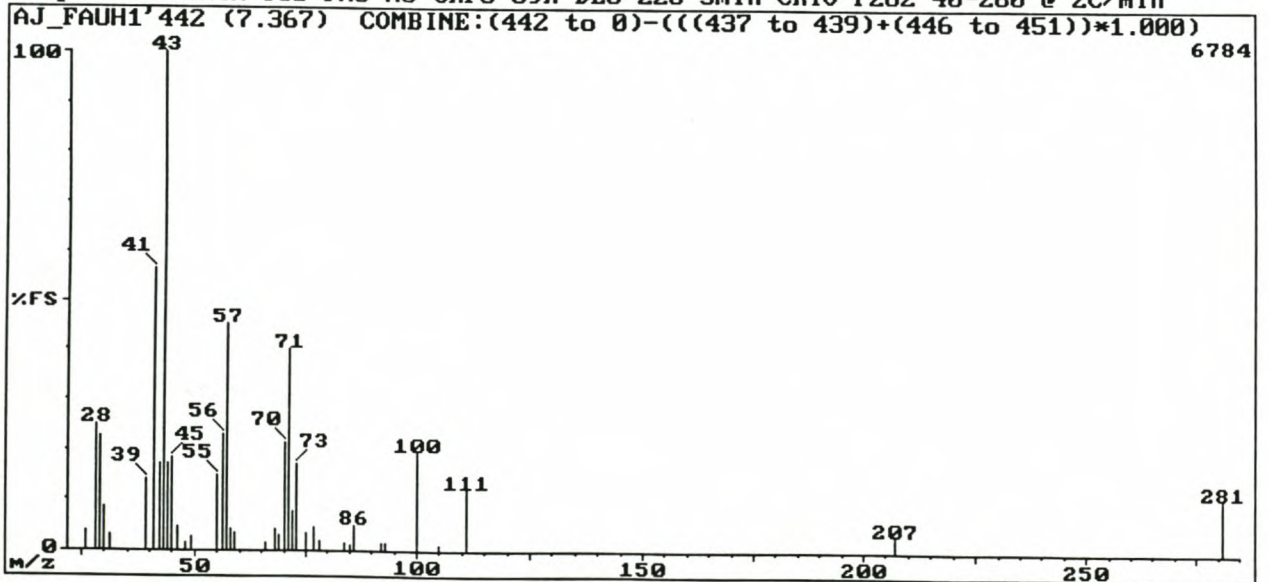


Fig. 4.1: EI mass spectrum of component 442 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

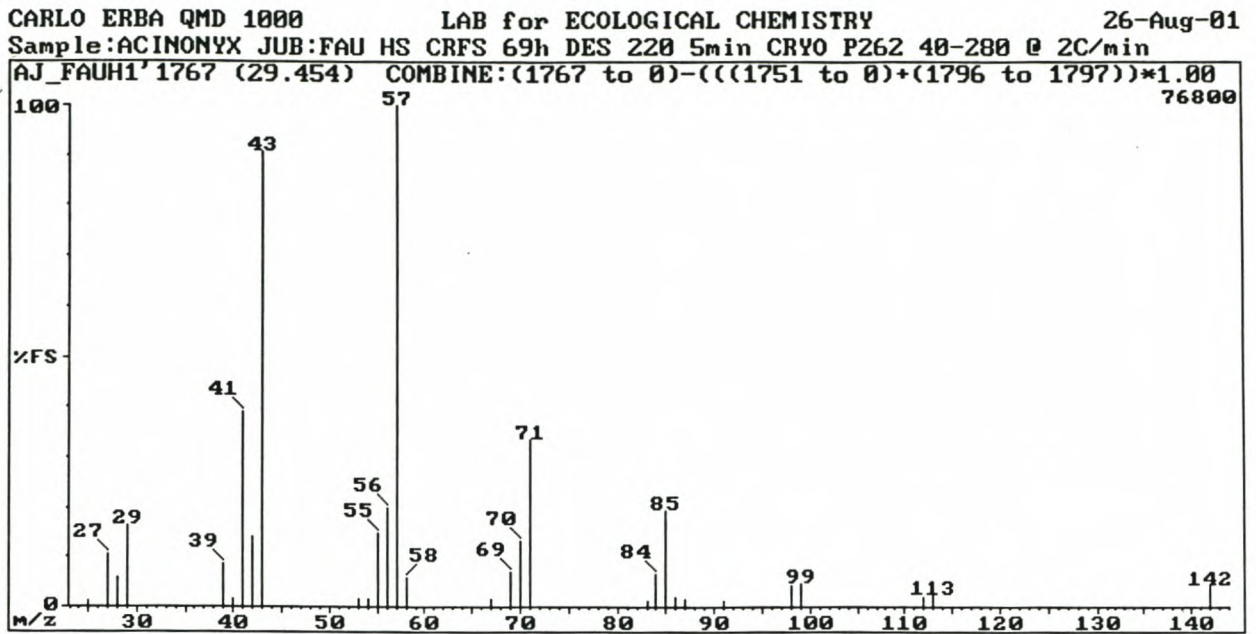


Fig. 4.2: EI mass spectrum of component 1767 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

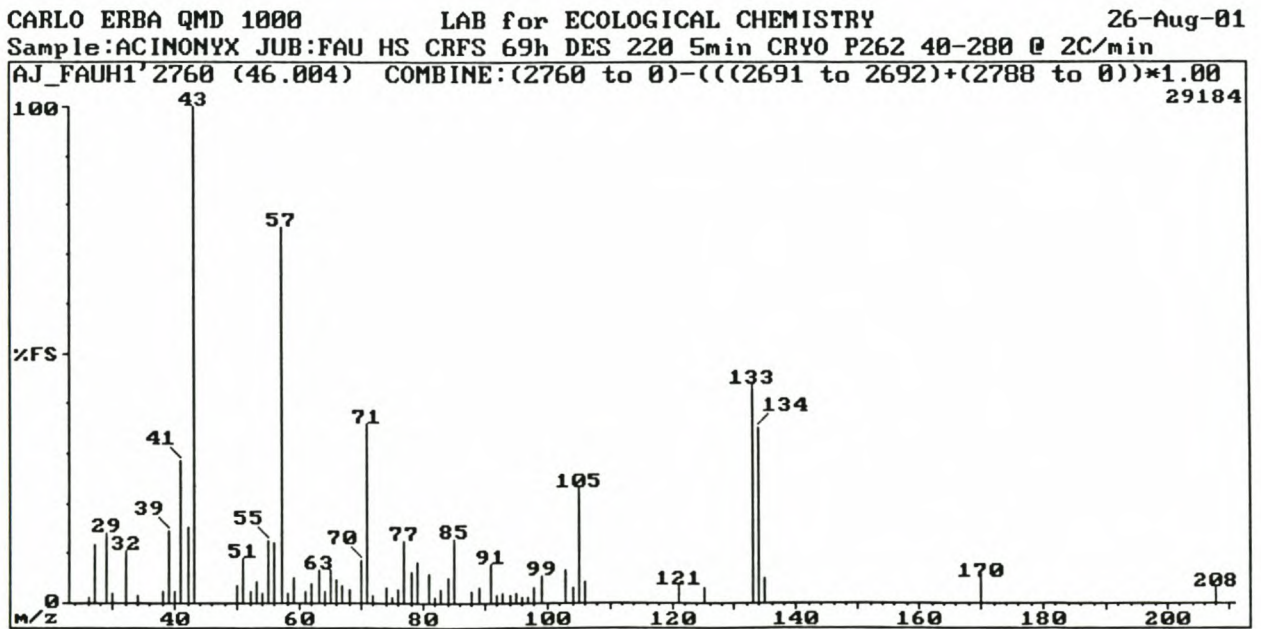


Fig. 4.3: EI mass spectrum of component 2760 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

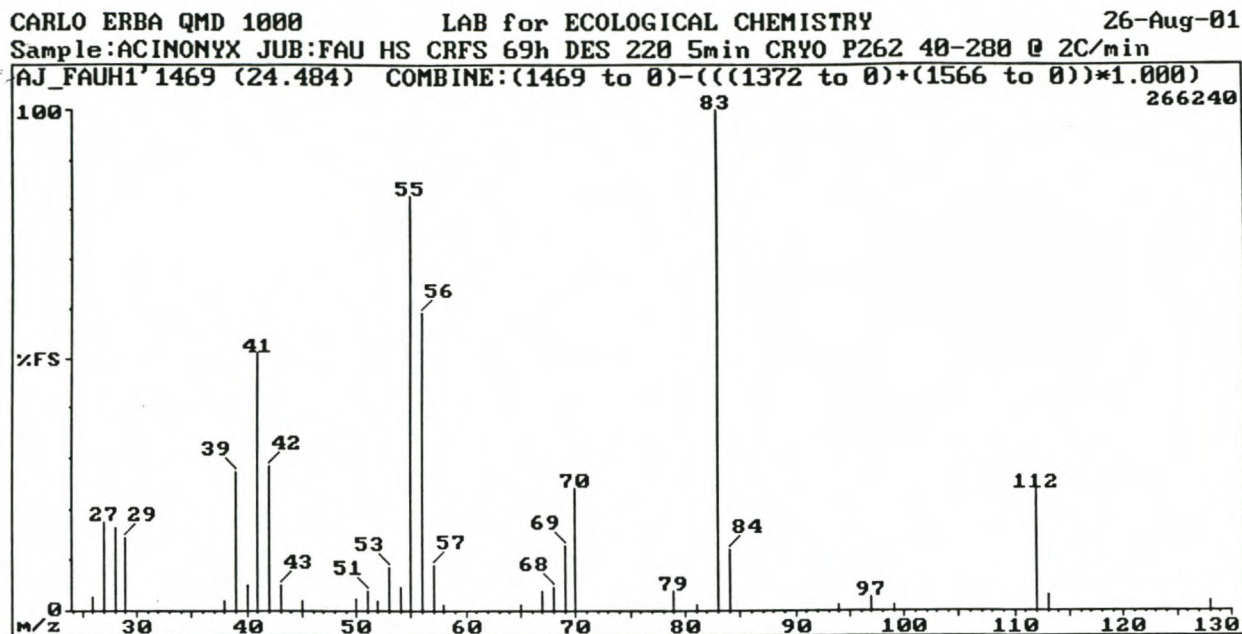


Fig. 4.4: EI mass spectrum of component 1469 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

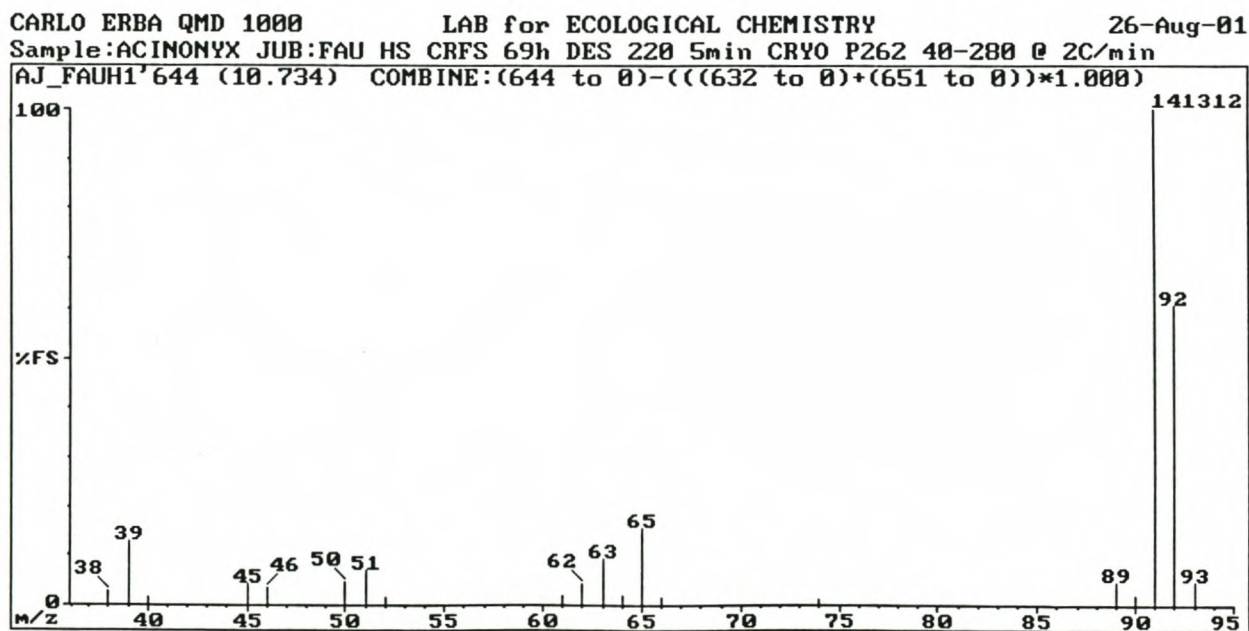


Fig. 4.5: EI mass spectrum of component 644 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

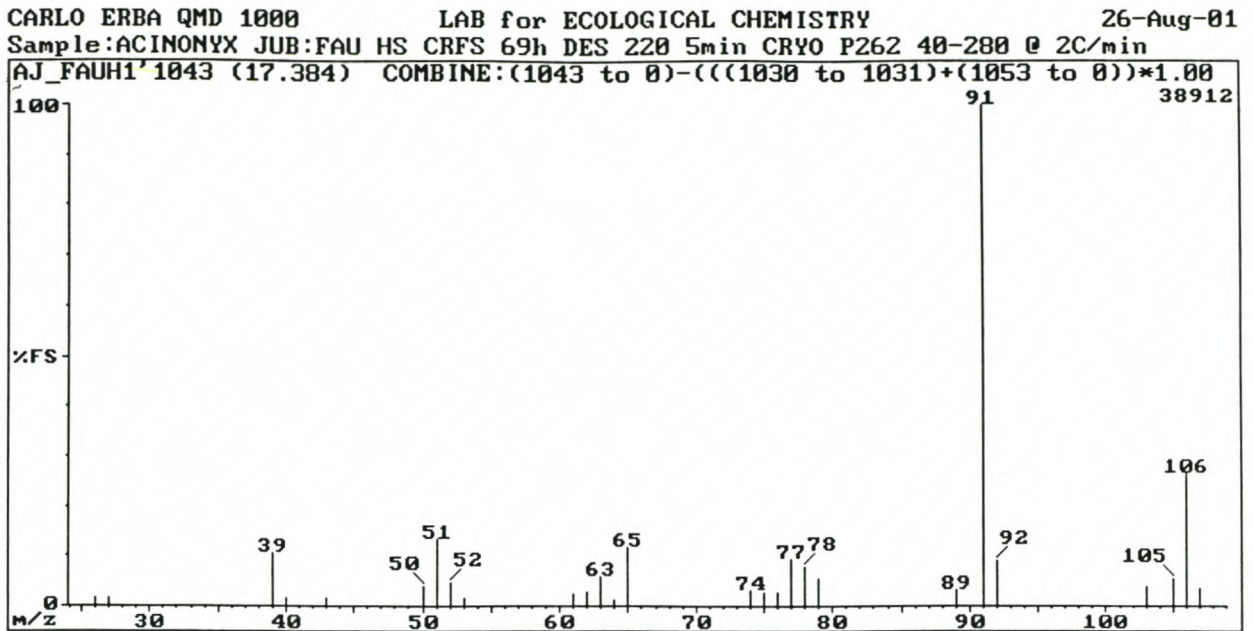


Fig. 4.6: EI mass spectrum of component 1043 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

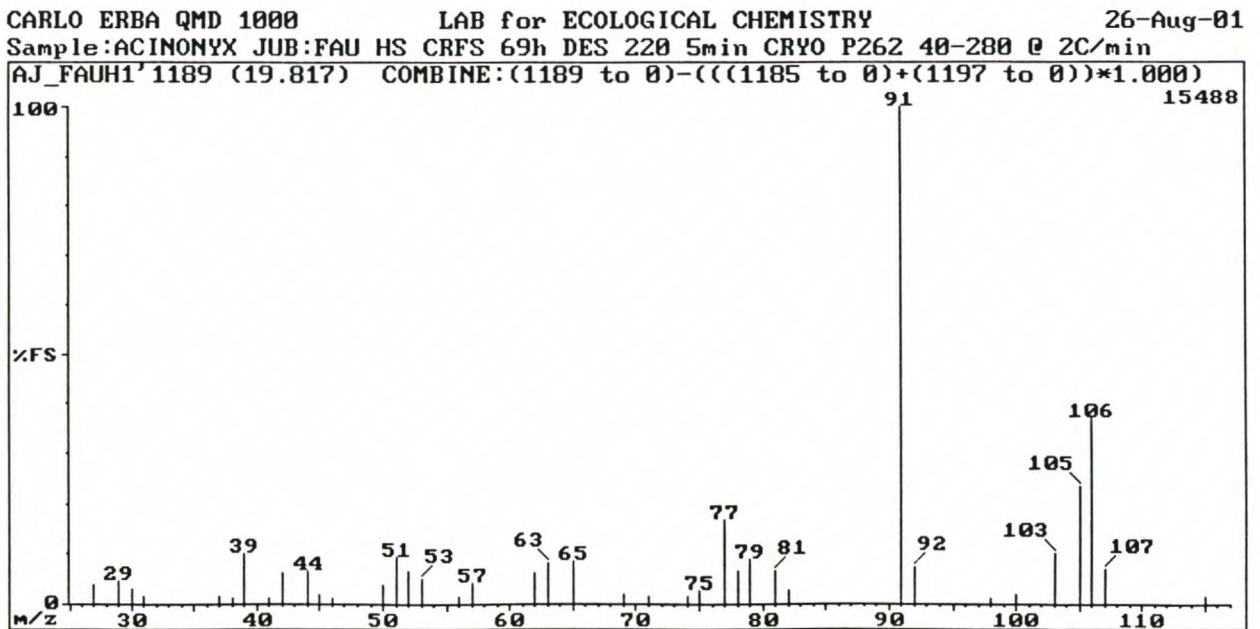


Fig. 4.7: EI mass spectrum of component 1189 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 26-Aug-01
 Sample: ACINONYX JUB:FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min
 AJ_FAUH1'1699 (28.320) COMBINE:(1699 to 0)-(((1684 to 0)+(1713 to 1714))*1.00

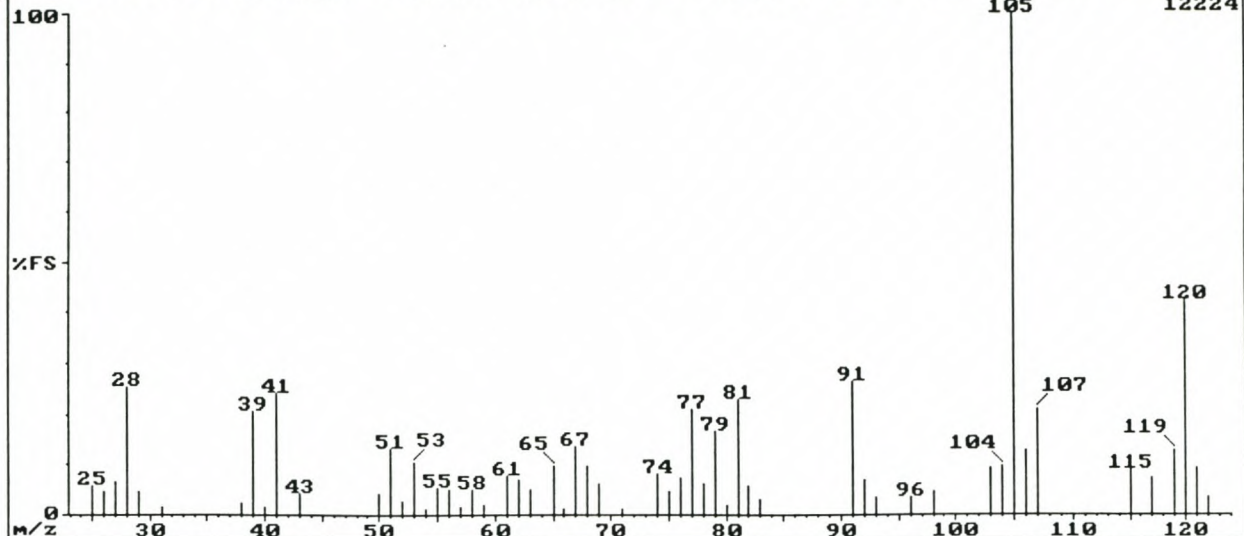
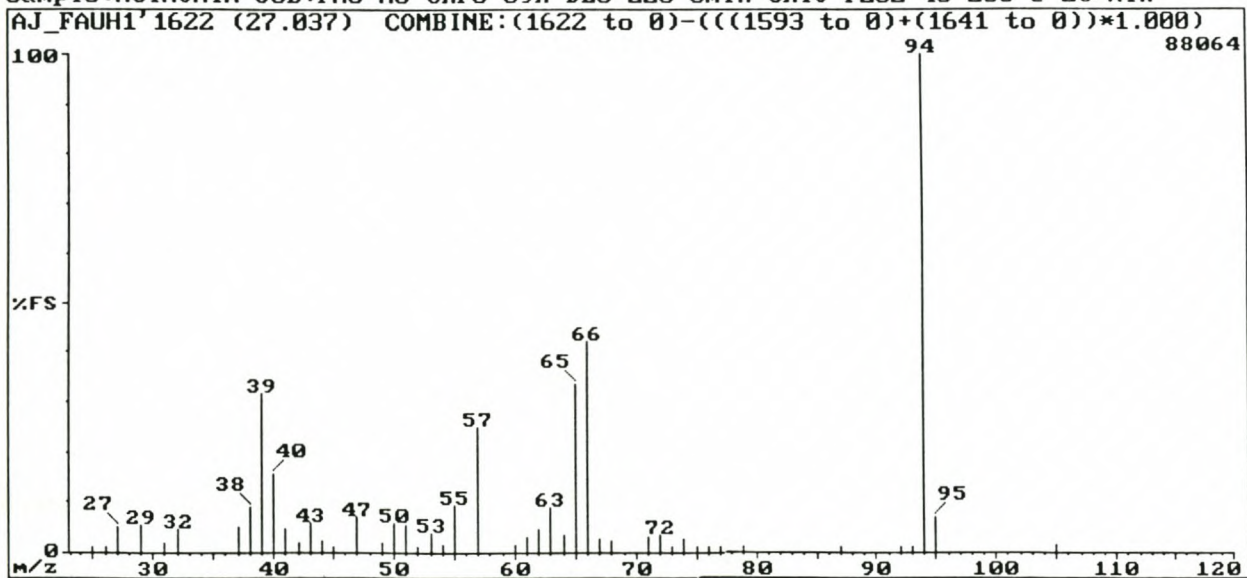


Fig. 4.8: EI mass spectrum of component 1699 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 26-Aug-01
 Sample: ACINONYX JUB:FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min



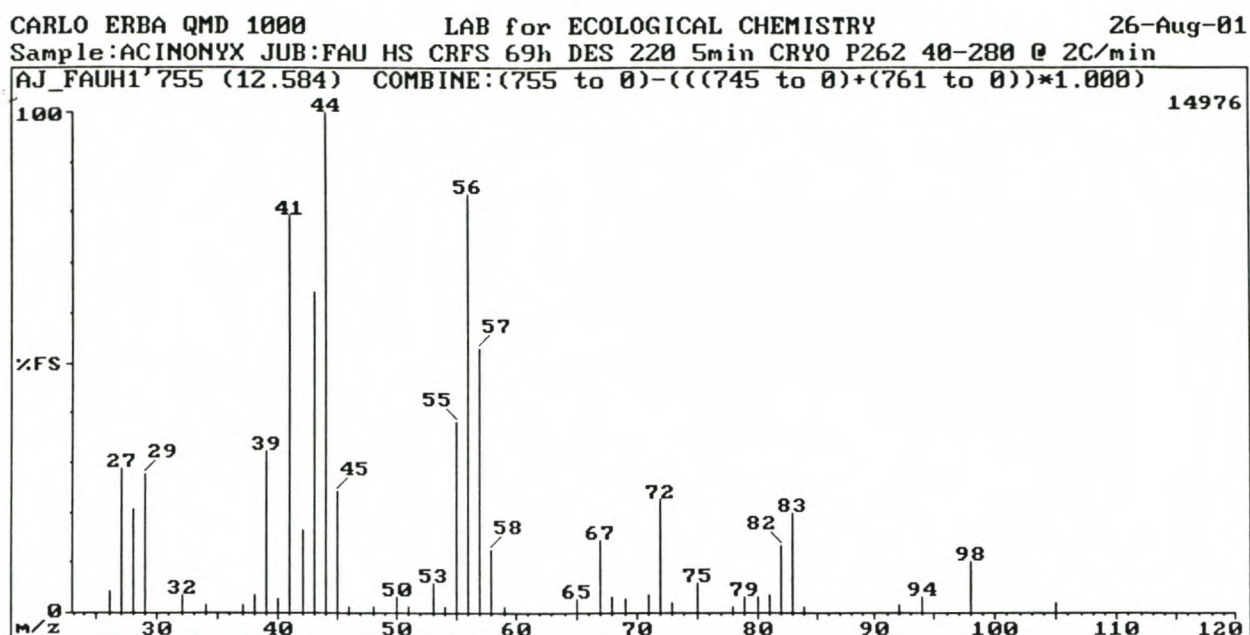


Fig. 4.10: El mass spectrum of component 755 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

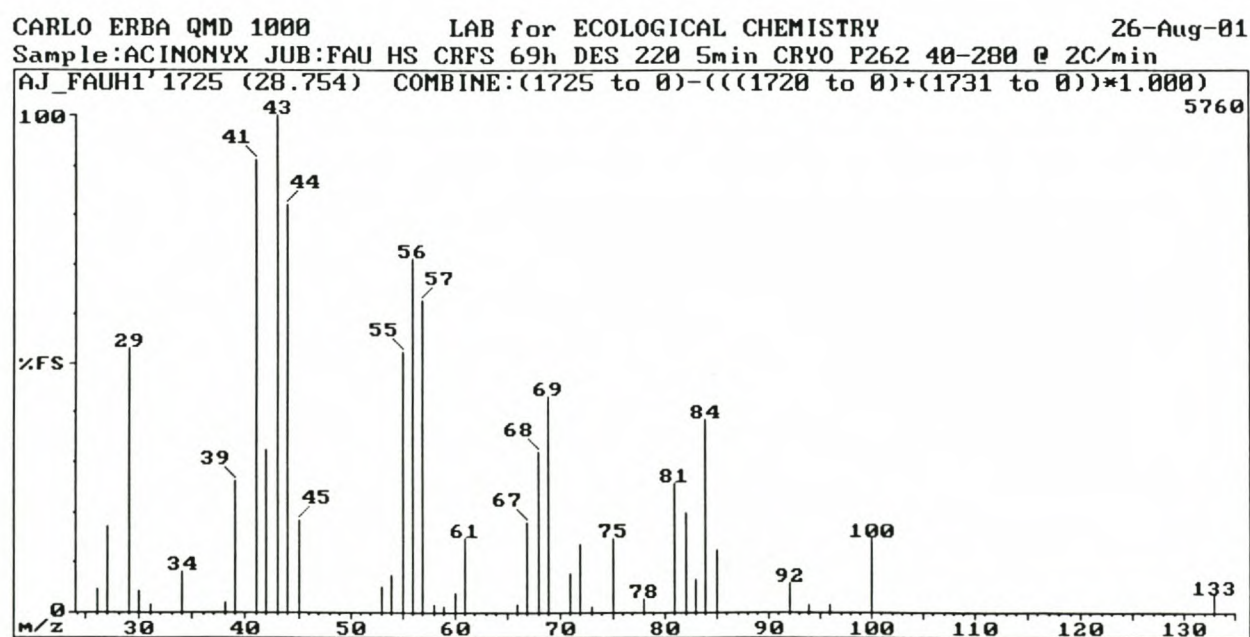


Fig. 4.11: El mass spectrum of component 1725 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 26-Aug-01
 Sample: ACINONYX JUB:FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min

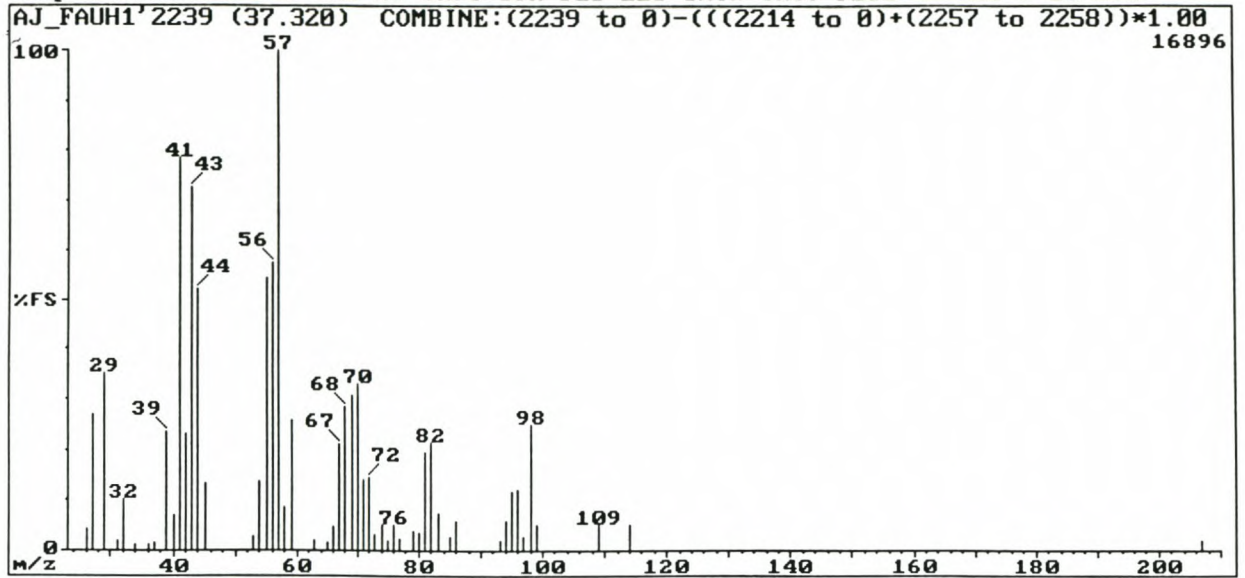


Fig. 4.12: EI mass spectrum of component 2239 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 26-Aug-01
 Sample: ACINONYX JUB:FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min

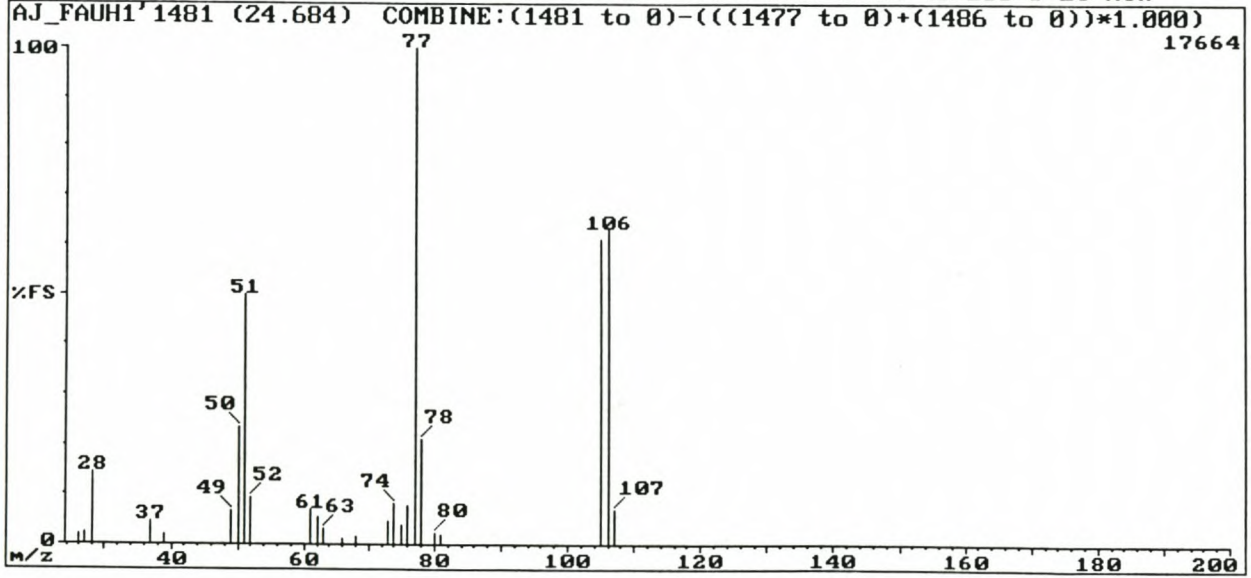


Fig. 4.13: EI mass spectrum of component 1481 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

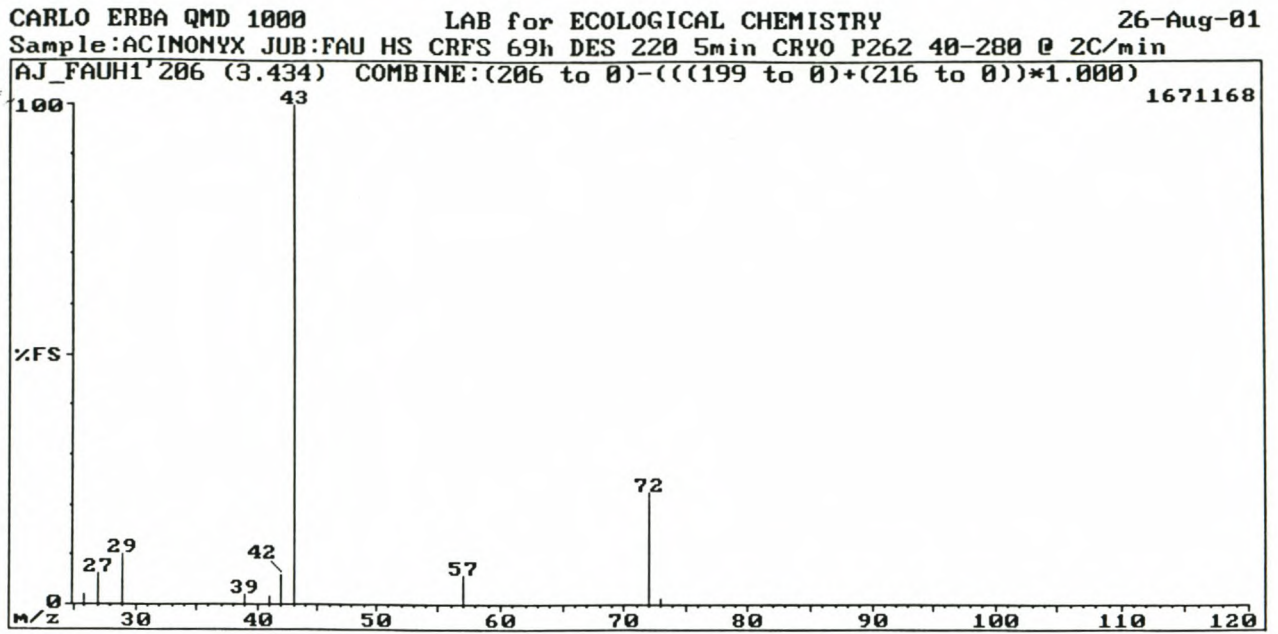


Fig. 4.14: El mass spectrum of component 206 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

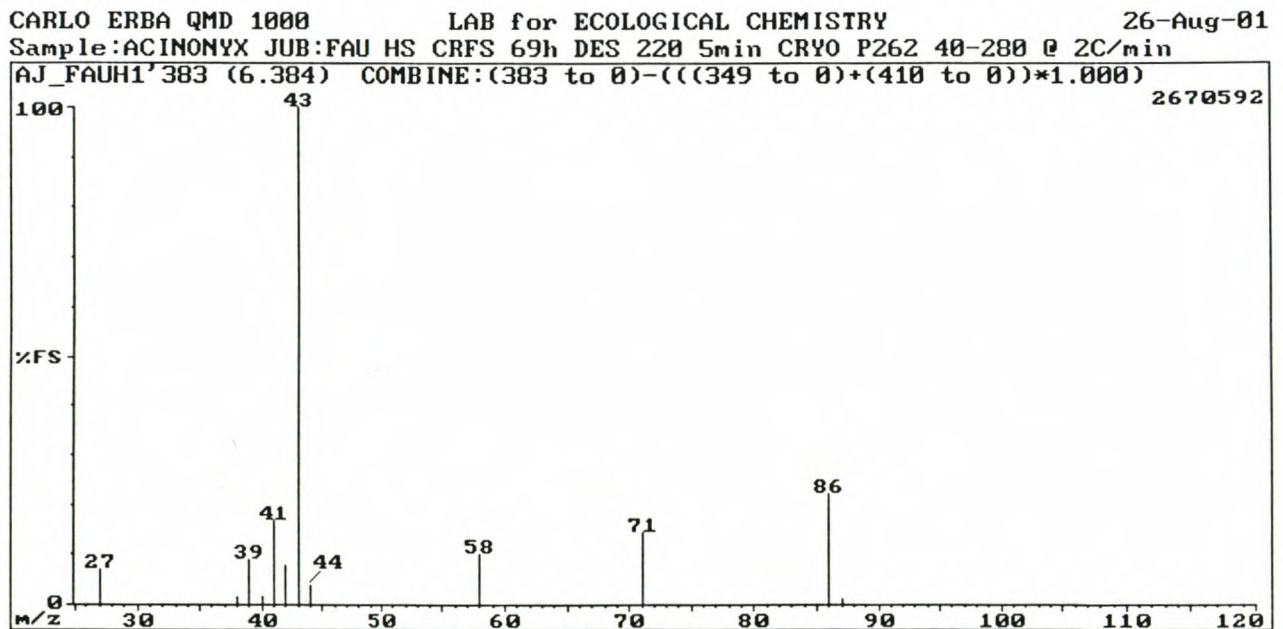


Fig. 4.15: El mass spectrum of component 383 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 26-Aug-01
Sample:ACINONYX JUB:FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min
AJ_FAUH1'715 (11.917) COMBINE:(715 to 0)-(((707 to 0)+(727 to 0))*1.000) 79872

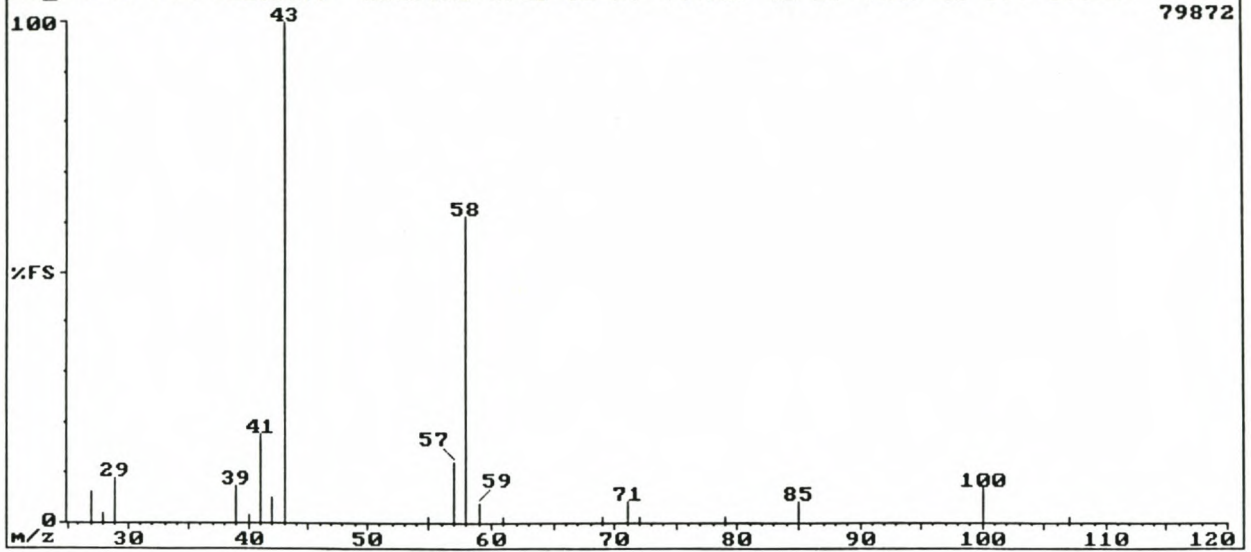


Fig. 4.16: El mass spectrum of component 715 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 26-Aug-01
Sample:ACINONYX JUB:FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min
AJ_FAUH1'1161 (19.350) COMBINE:(1161 to 0)-(((1142 to 0)+(1181 to 0))*1.000) 266240

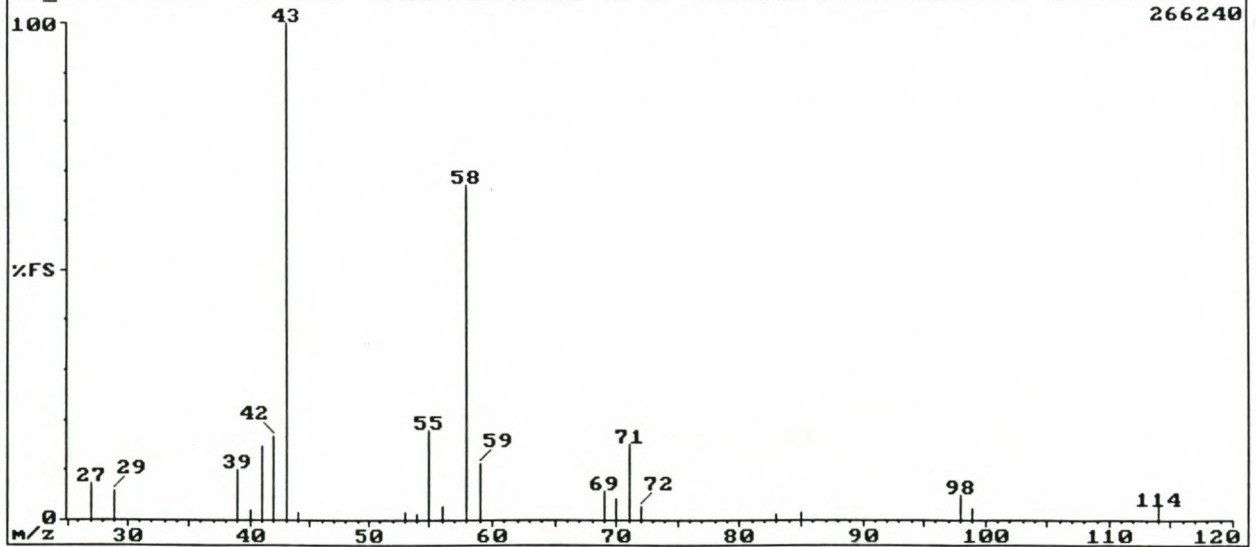


Fig. 4.17: El mass spectrum of component 1161 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

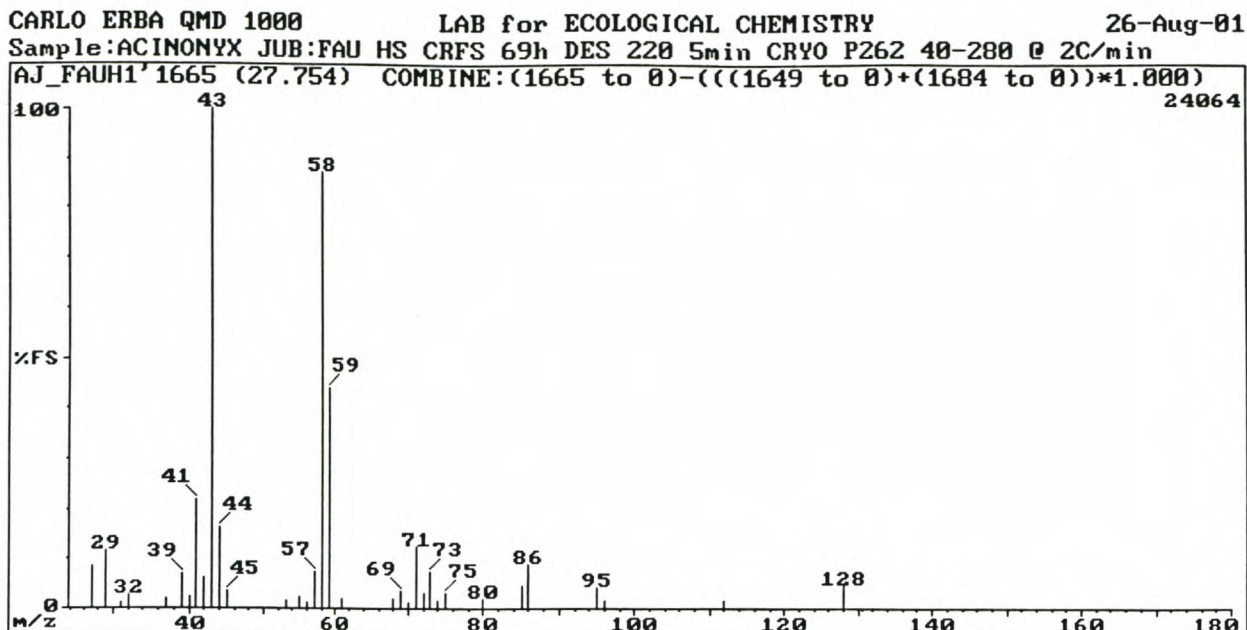


Fig. 4.18: EI mass spectrum of component 1665 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

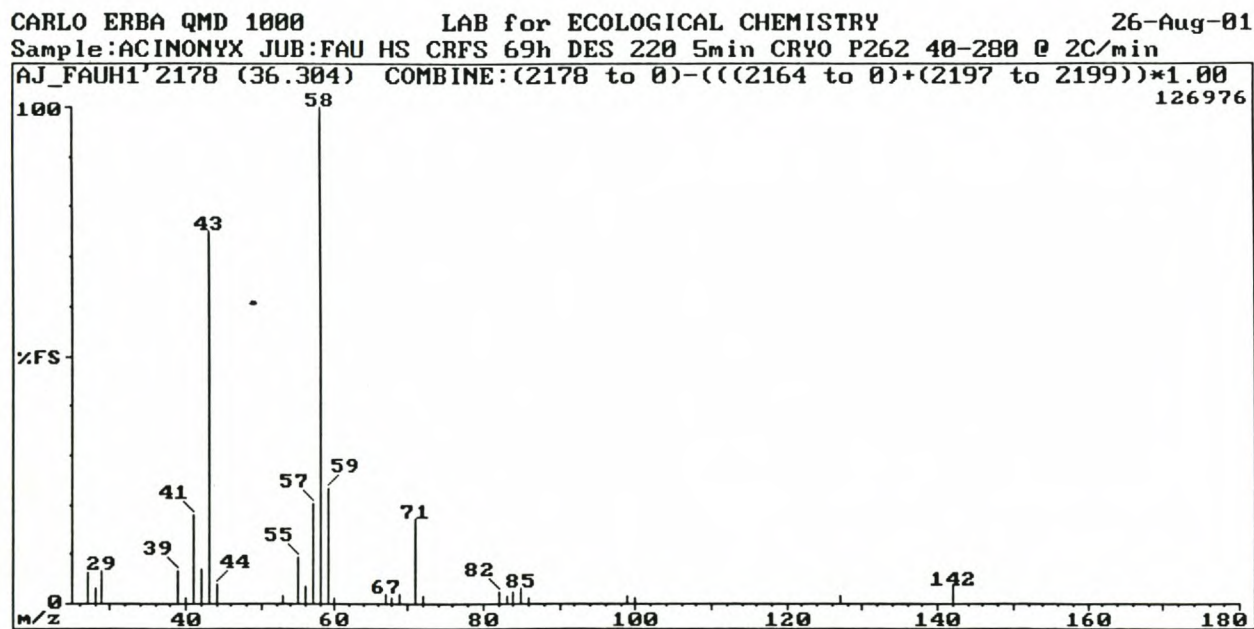


Fig. 4.19: EI mass spectrum of component 2178 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

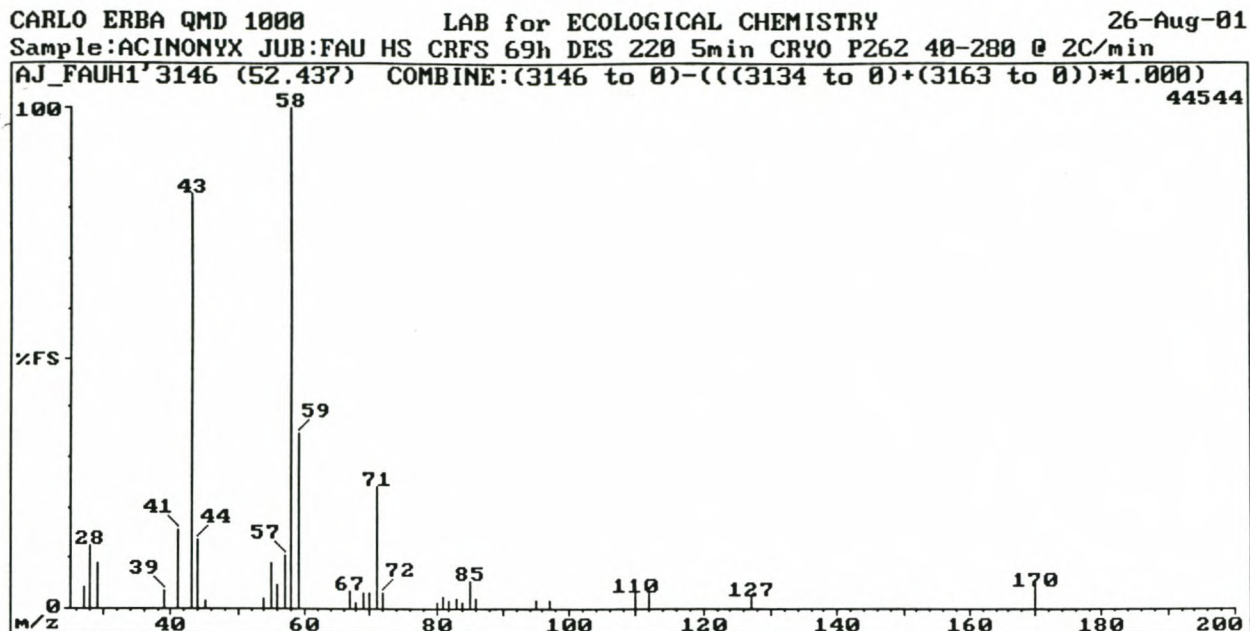


Fig. 4.20: EI mass spectrum of component 3146 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

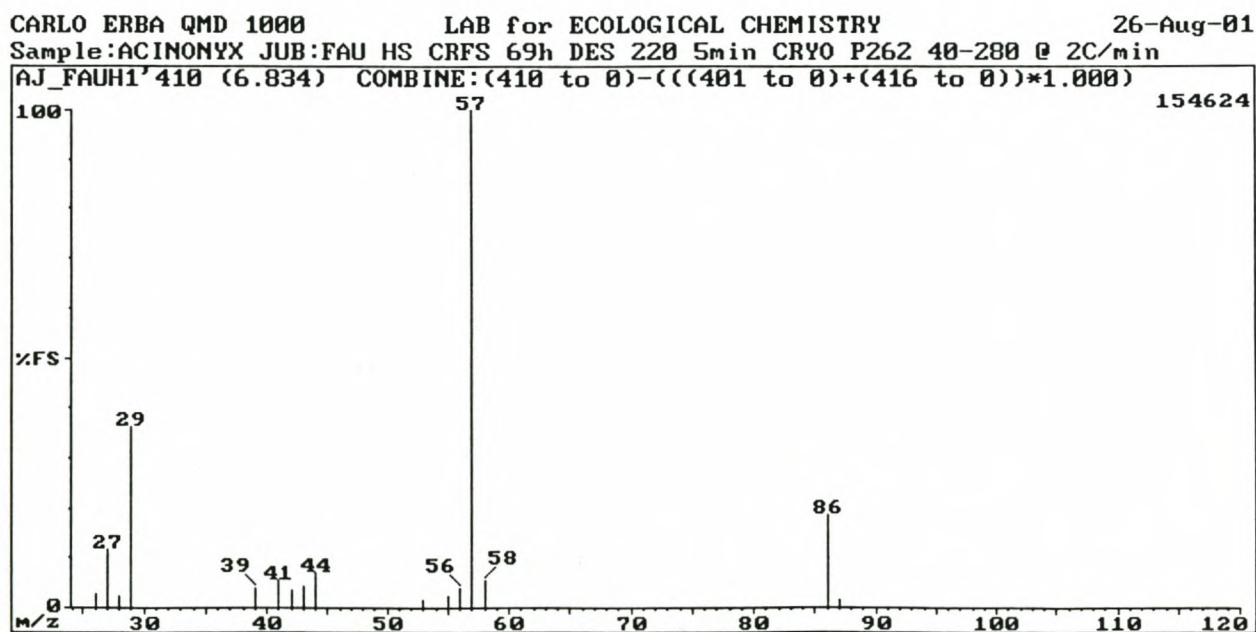


Fig. 4.21: EI mass spectrum of component 410 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

CARLO ERBA QMD 1000

LAB for ECOLOGICAL CHEMISTRY

26-Aug-01

Sample:ACINONYX JUB:FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min

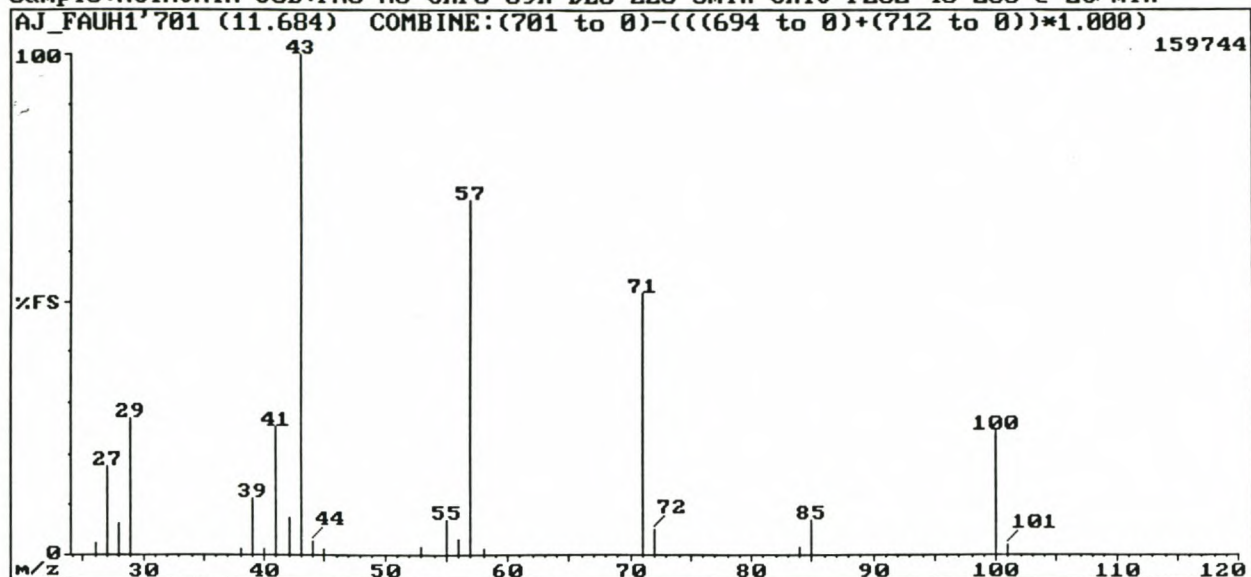


Fig. 4.22: EI mass spectrum of component 701 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

CARLO ERBA QMD 1000

LAB for ECOLOGICAL CHEMISTRY

26-Aug-01

Sample:ACINONYX JUB:FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min

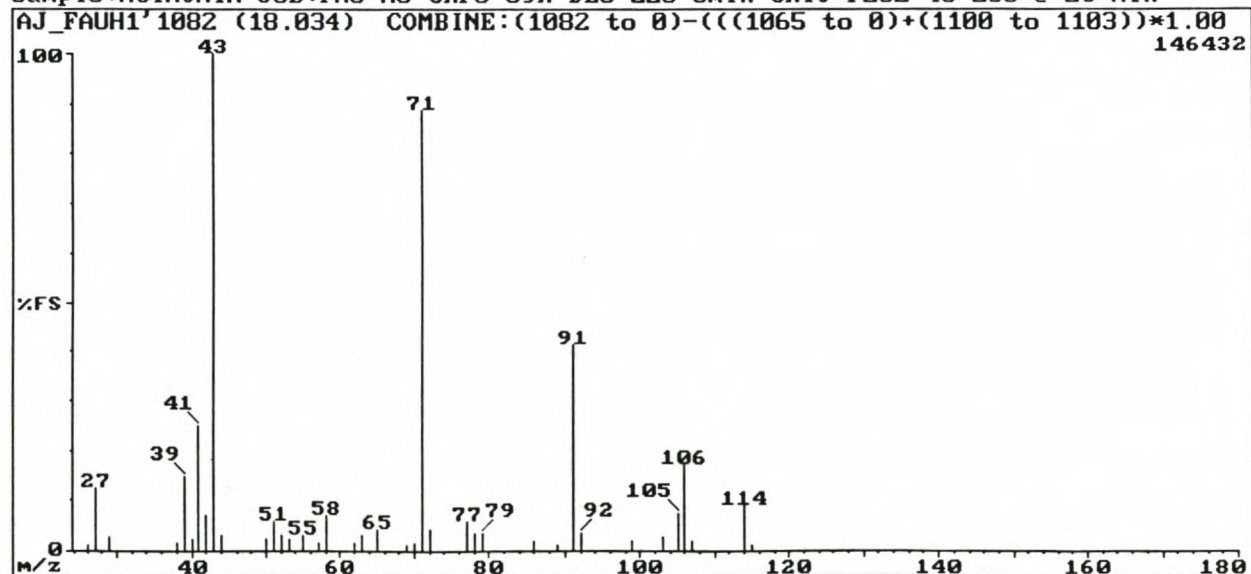


Fig. 4.23: EI mass spectrum of component 1082 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

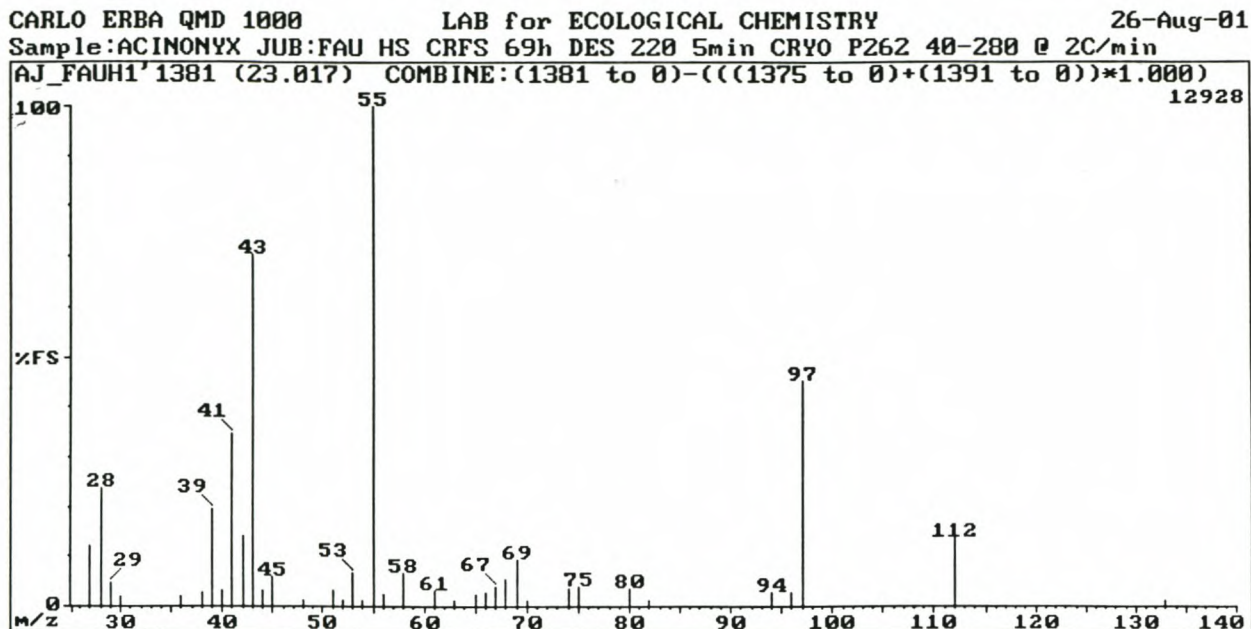


Fig. 4.24: EI mass spectrum of component 1381 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

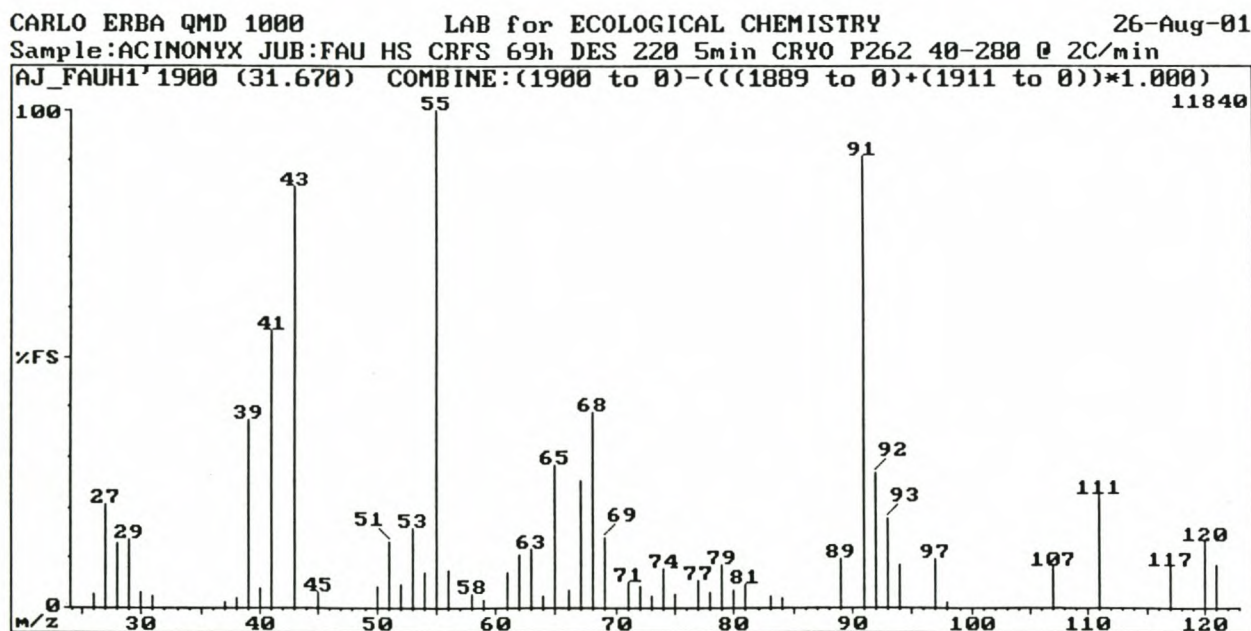


Fig. 4.25: EI mass spectrum of component 1900 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

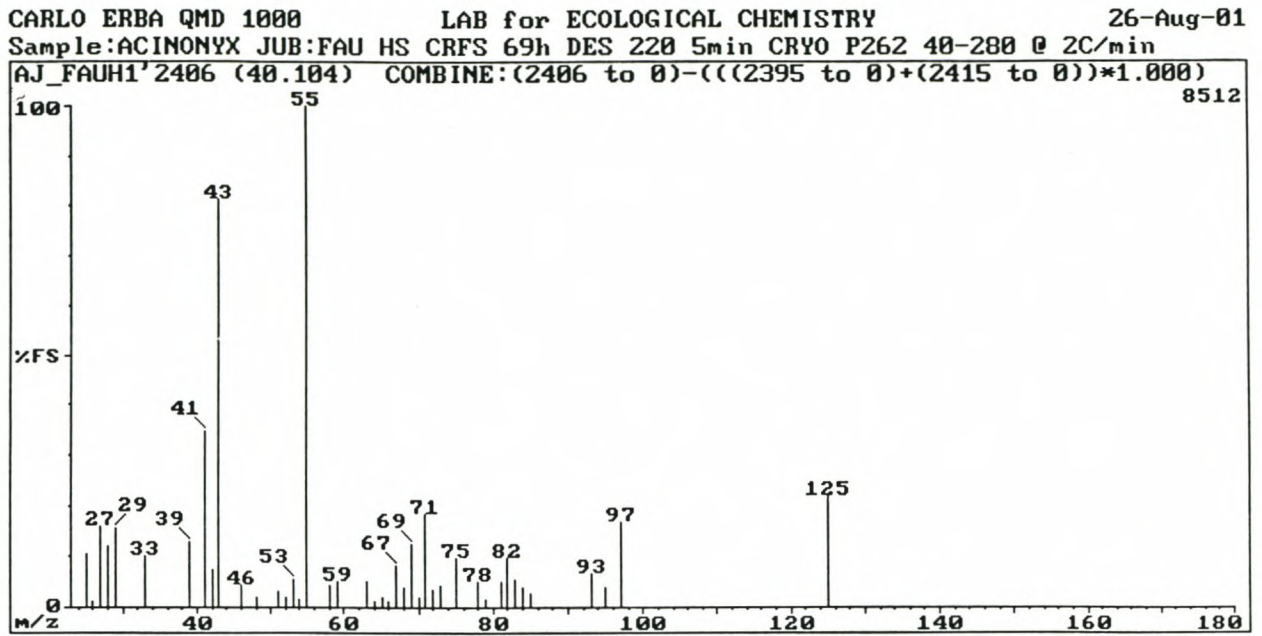


Fig. 4.26: EI mass spectrum of component 2406 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

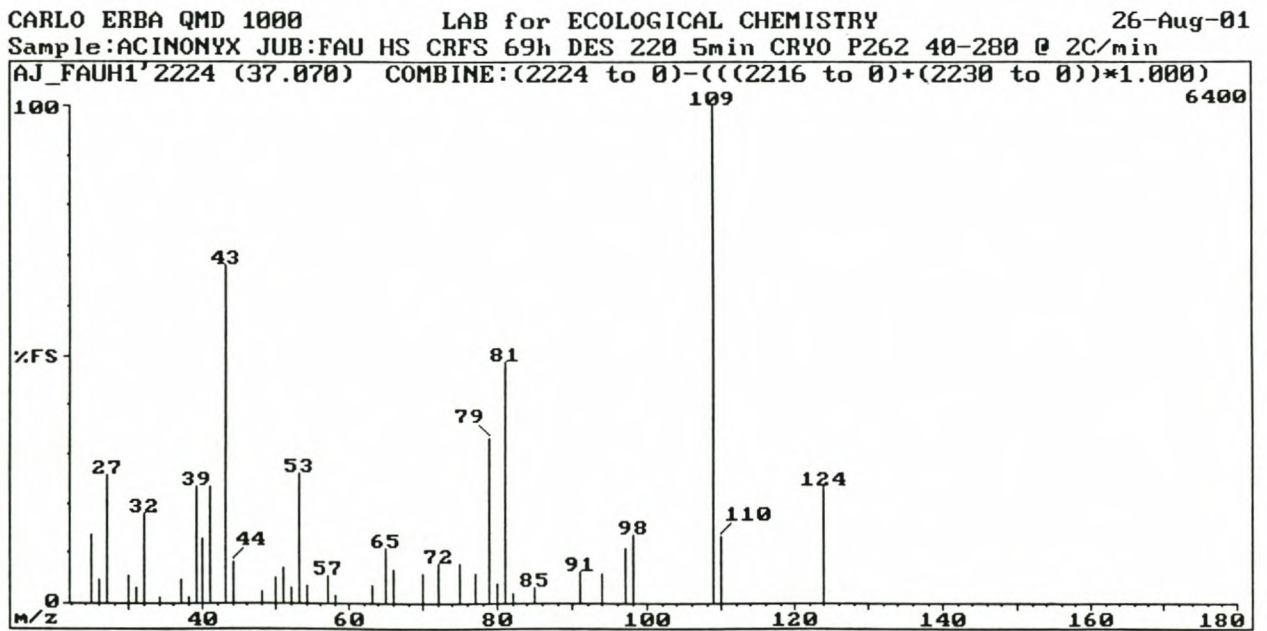


Fig. 4.27: EI mass spectrum of component 2224 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

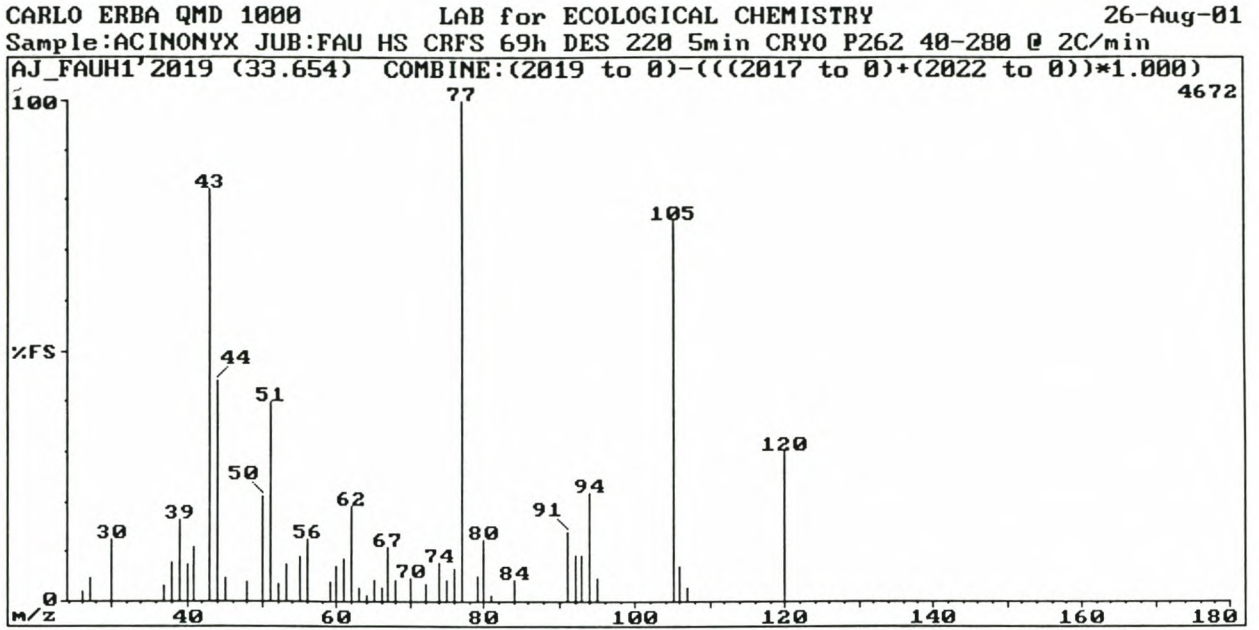


Fig. 4.28: EI mass spectrum of component 2019 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

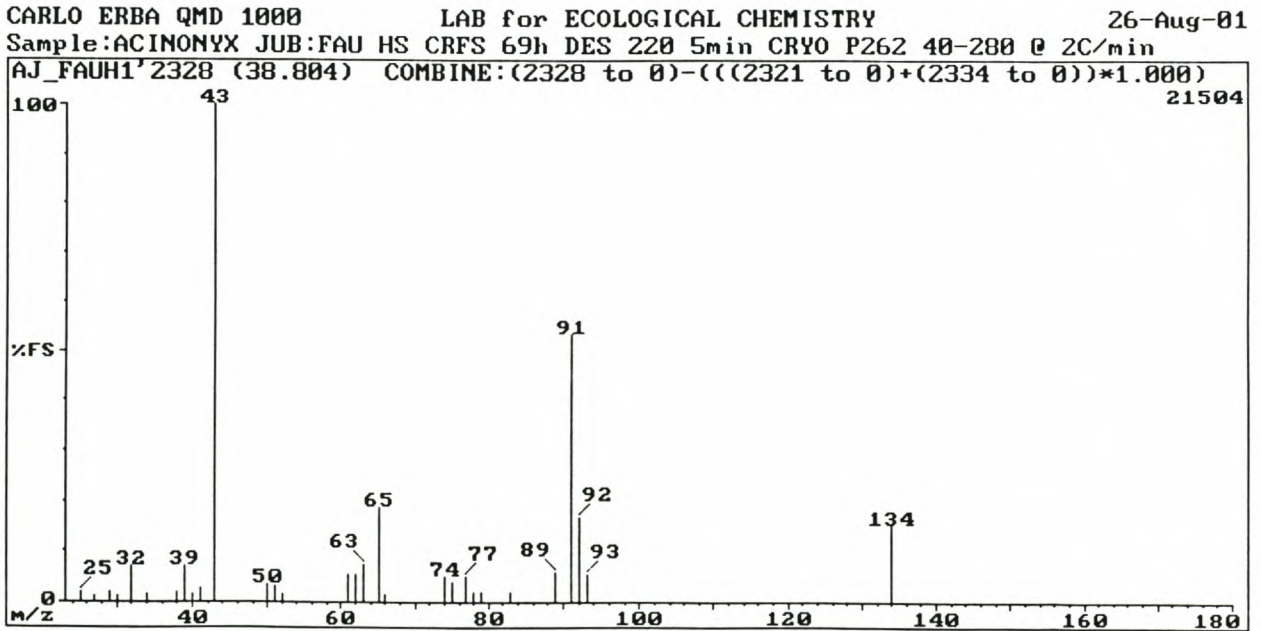


Fig. 4.29: EI mass spectrum of component 2328 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 26-Aug-01
 Sample:ACINONYX JUB:FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min
 AJ_FAUH1'1150 (19.167) COMBINE:(1150 to 0)-(((1119 to 0)+(1186 to 1188))*1.00
 229376

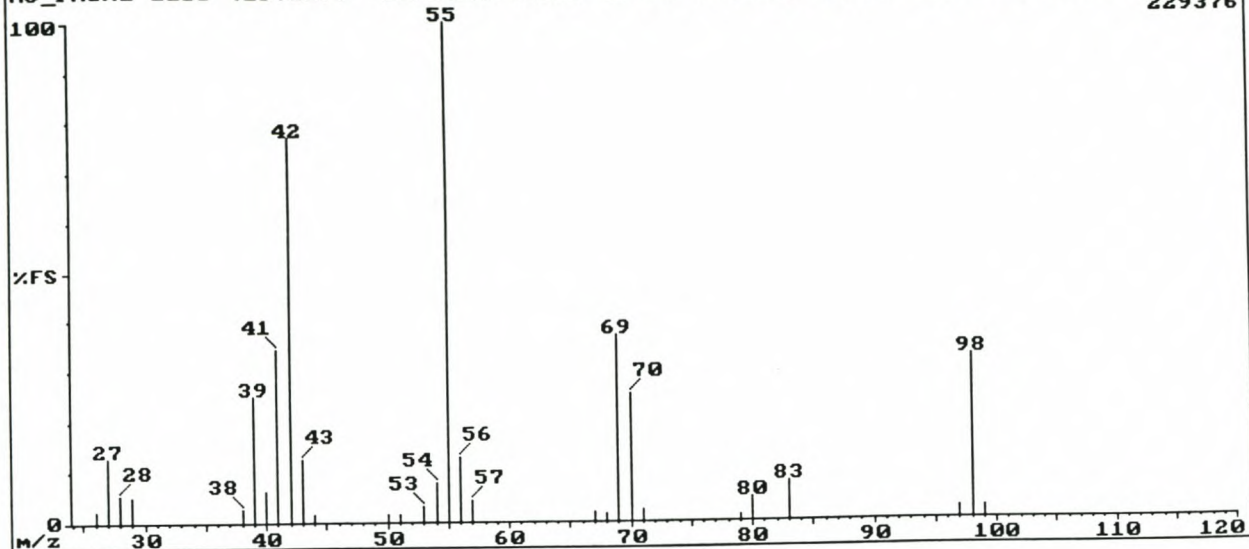


Fig. 4.30: EI mass spectrum of component 1150 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

CARLO ERBA QMD 1000 LAB for ECOLOGICAL CHEMISTRY 26-Aug-01
 Sample:ACINONYX JUB:FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min
 AJ_FAUH1'938 (15.634) COMBINE:(938 to 0)-(((923 to 0)+(949 to 0))*1.000)
 230400

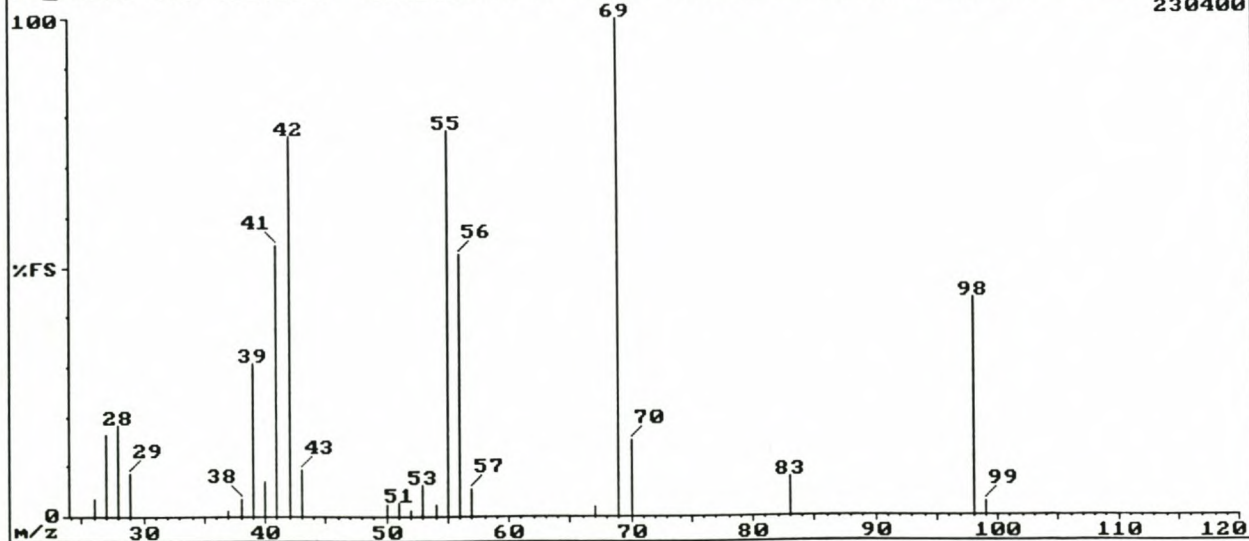


Fig. 4.31: EI mass spectrum of component 938 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

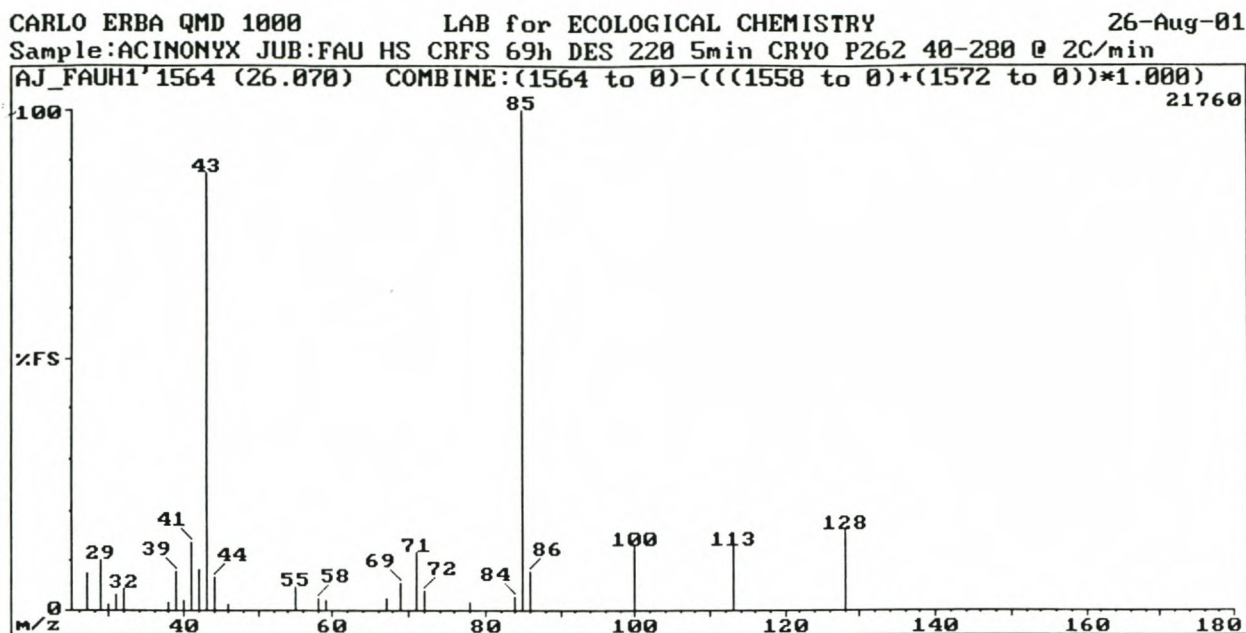


Fig. 4.32: EI mass spectrum of component 1564 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

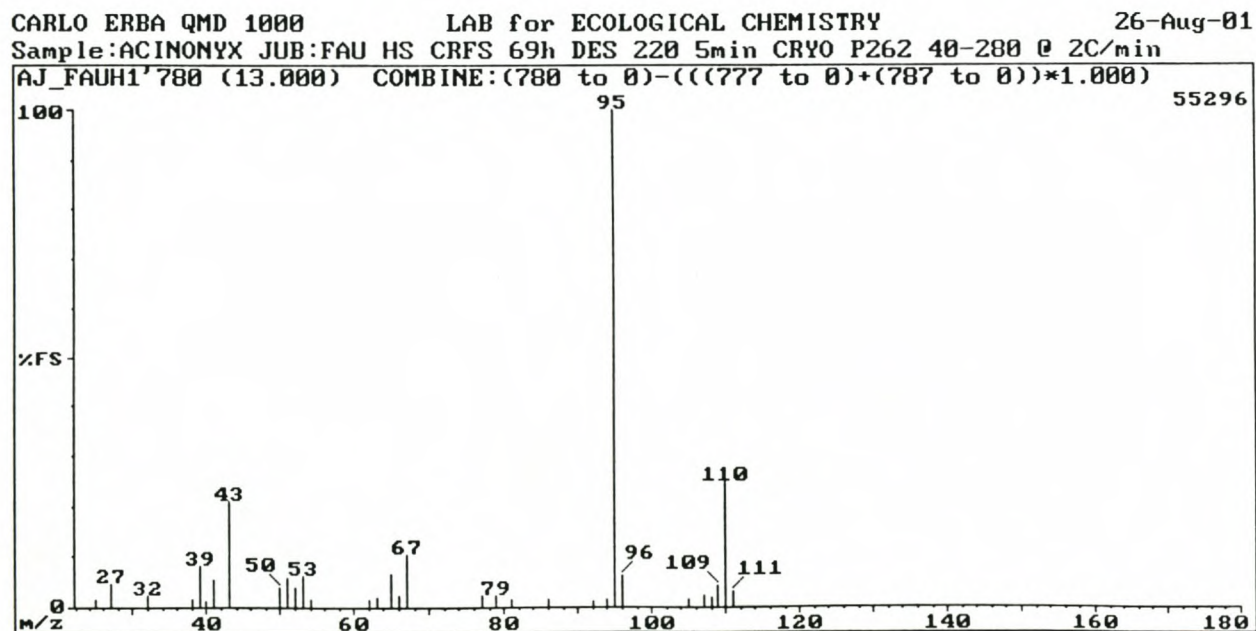


Fig. 4.33: EI mass spectrum of component 780 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

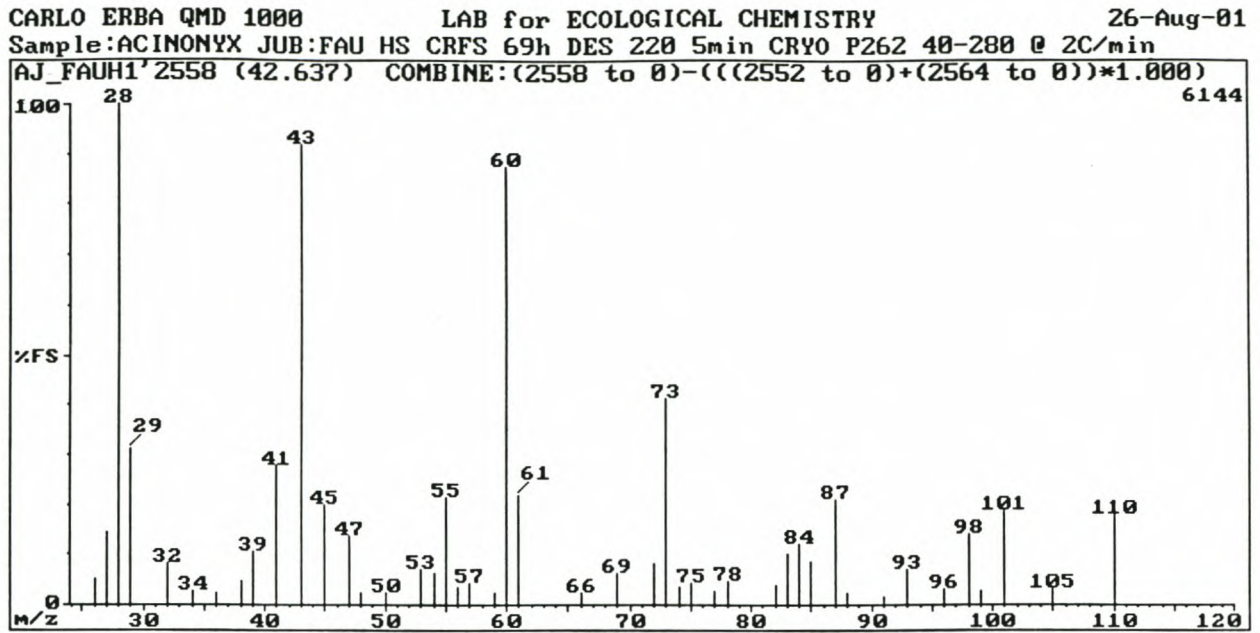


Fig. 4.34: EI mass spectrum of component 2558 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

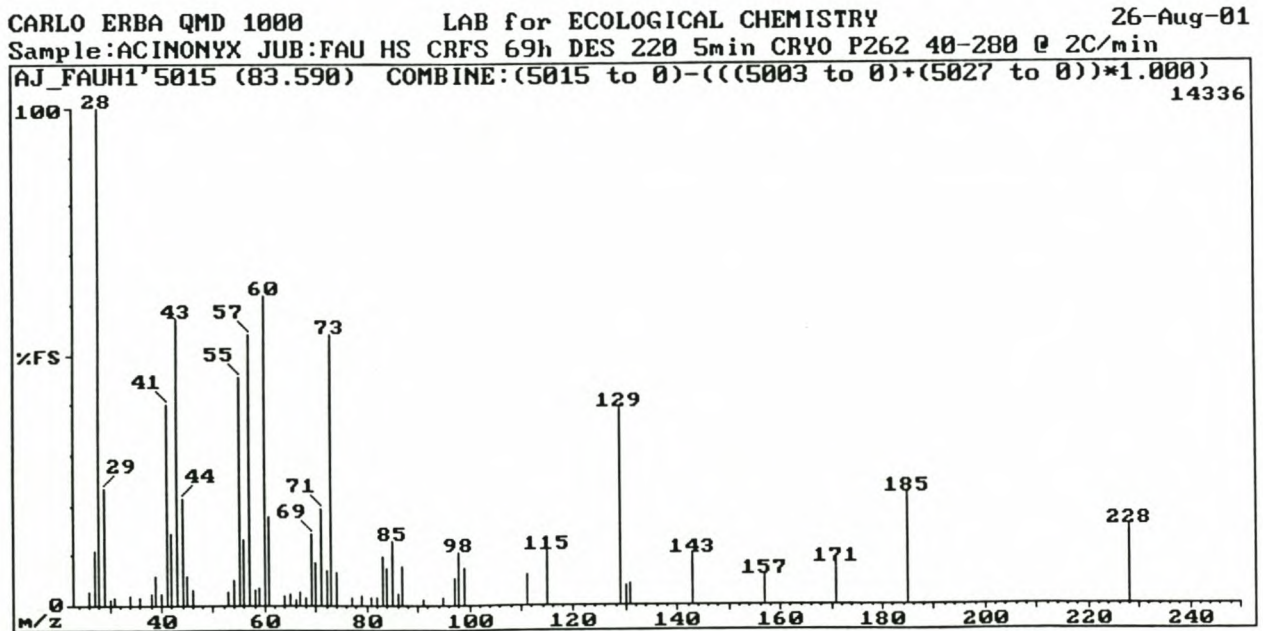


Fig. 4.35: EI mass spectrum of component 5015 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

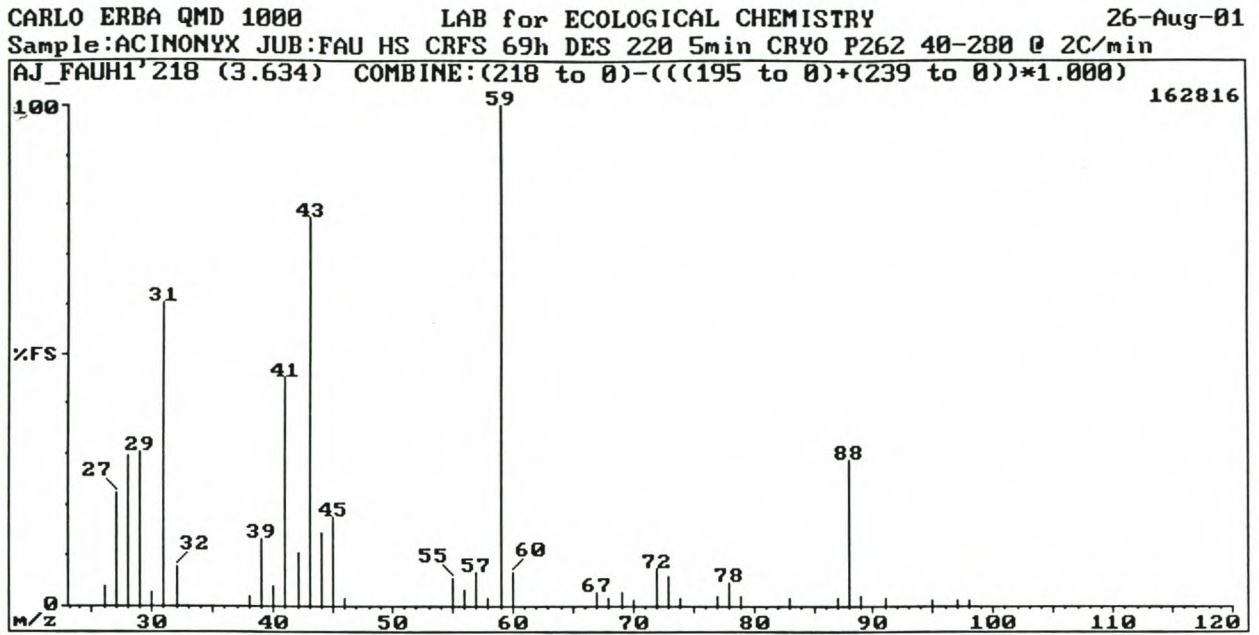


Fig. 4.36: EI mass spectrum of component 218 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

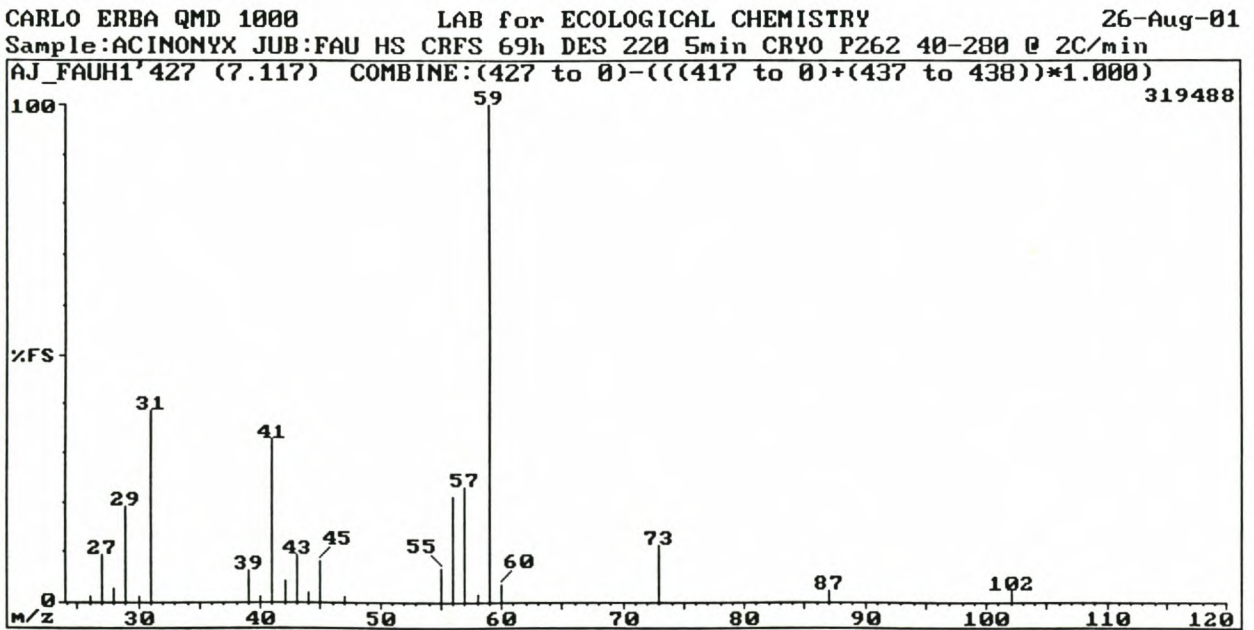


Fig. 4.37: EI mass spectrum of component 427 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

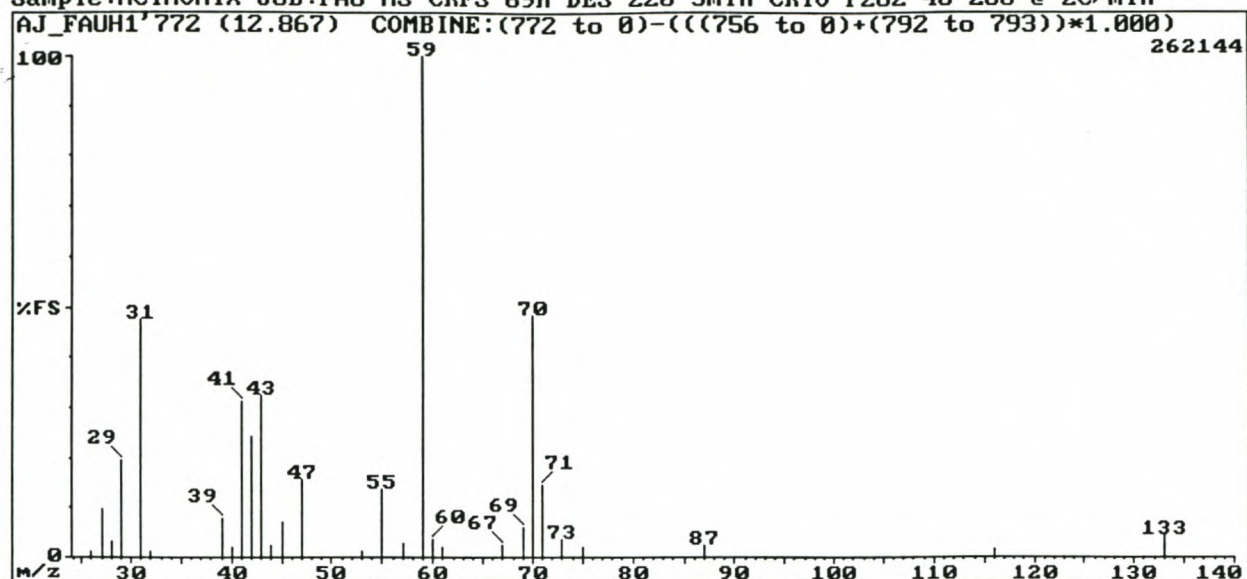


Fig. 4.38: EI mass spectrum of component 772 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

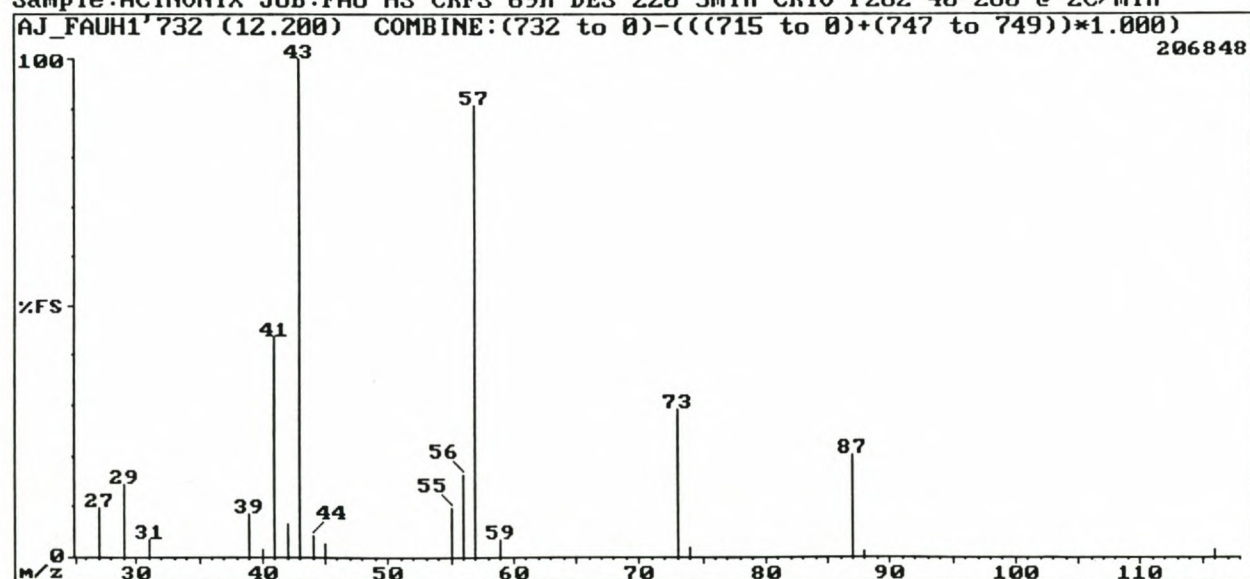


Fig. 4.39: EI mass spectrum of component 732 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

Sample: ACINONYX JUB: FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min

AJ_FAUH1'406 (6.767) COMBINE: (406 to 0) - (((401 to 0) + (412 to 0)) * 1.000)

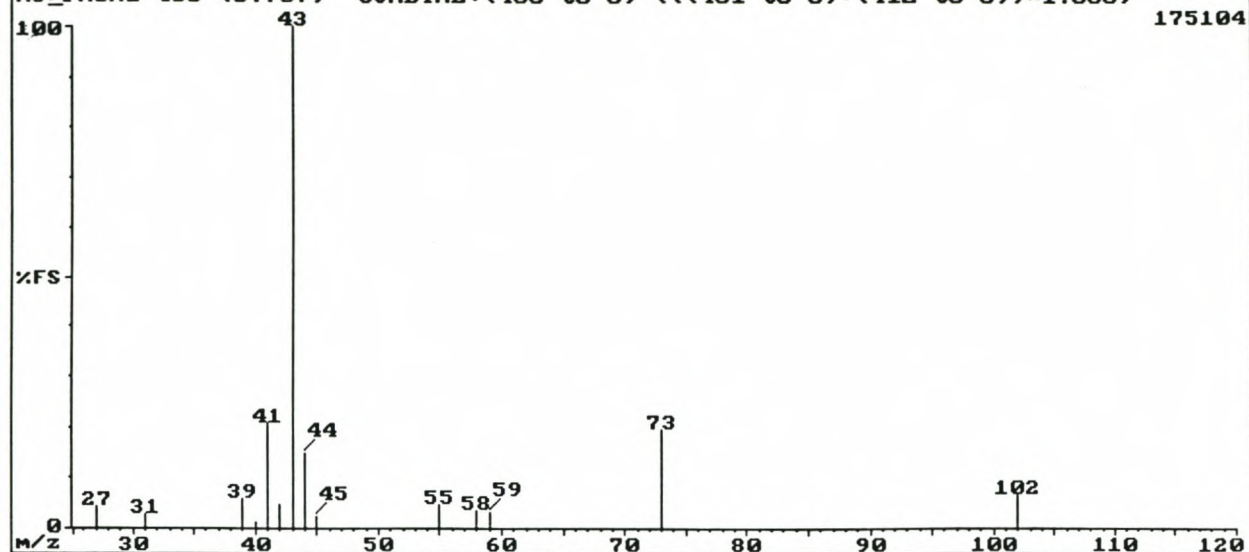


Fig. 4.40: EI mass spectrum of component 406 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

Sample: ACINONYX JUB: FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min

AJ_FAUH1'529 (8.817) COMBINE: (529 to 0) - (((523 to 0) + (536 to 0)) * 1.000)

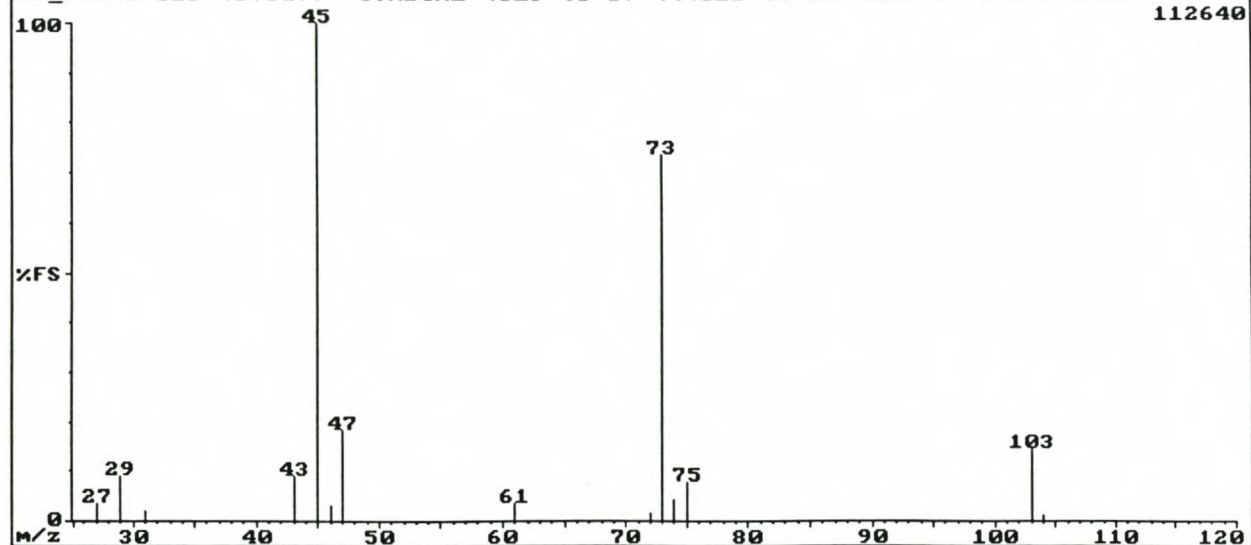


Fig. 4.41: EI mass spectrum of component 529 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

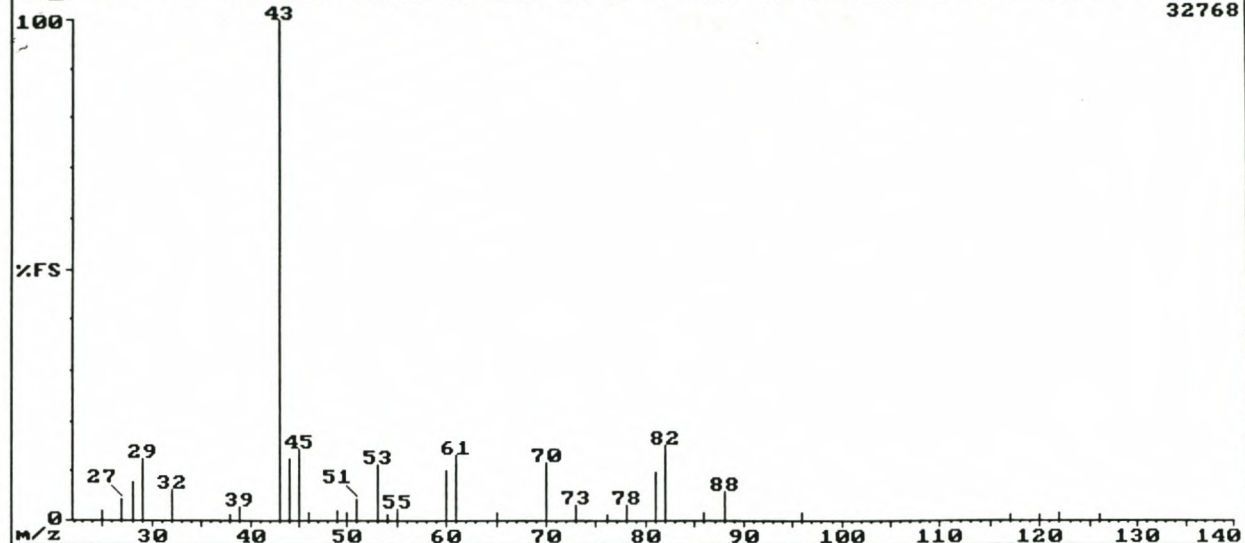


Fig. 4.42: El mass spectrum of component 240 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

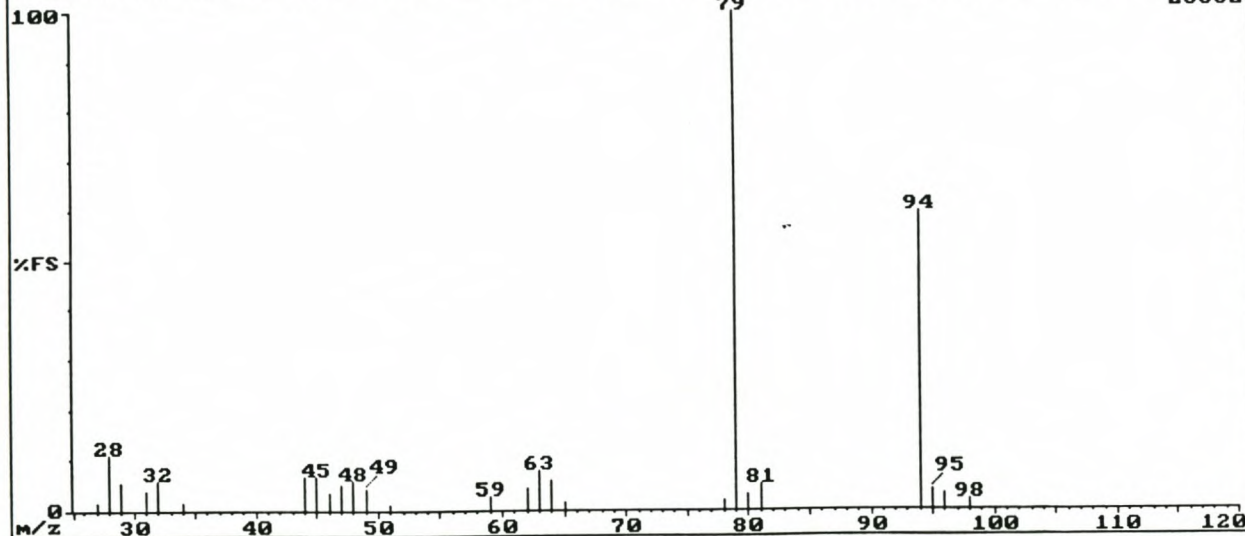


Fig. 4.43: El mass spectrum of component 1240 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

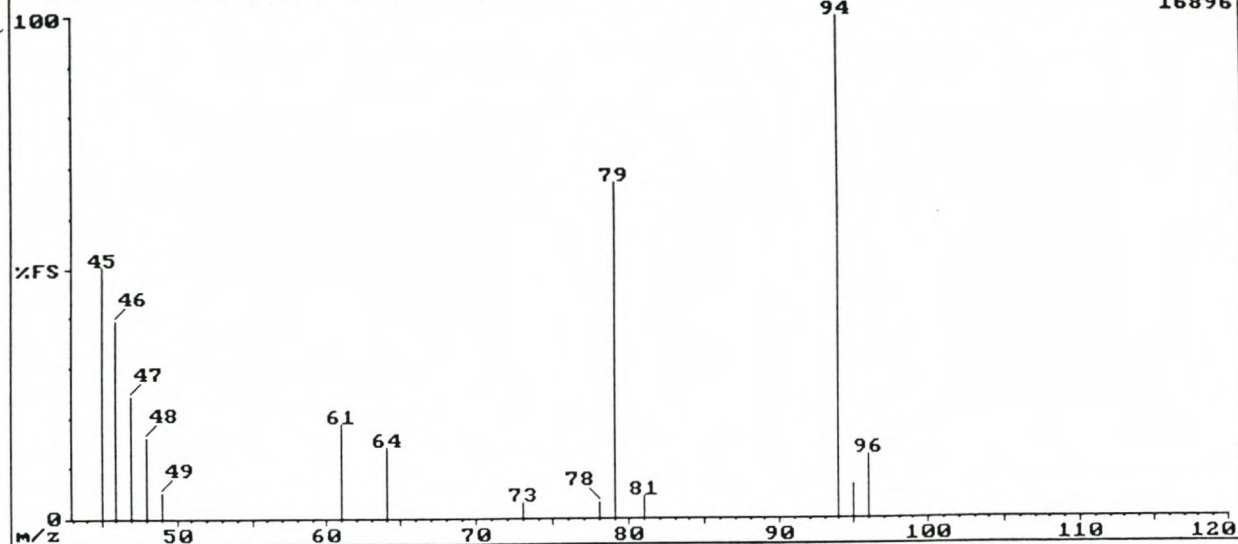


Fig. 4.44: EI mass spectrum of component 554 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

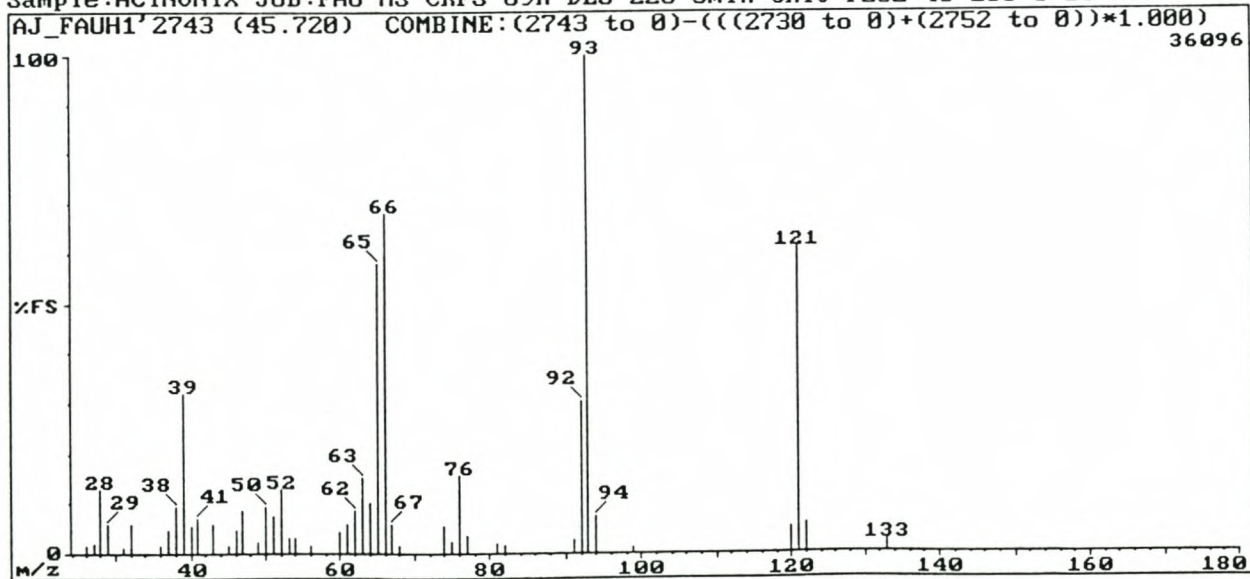


Fig. 4.45: EI mass spectrum of component 2743 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

Sample: ACINONYX JUB: FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min

AJ_FAUH1'4005 (66.754) COMBINE: (4005 to 0) - (((3993 to 0) + (4017 to 0)) * 1.000)

27392

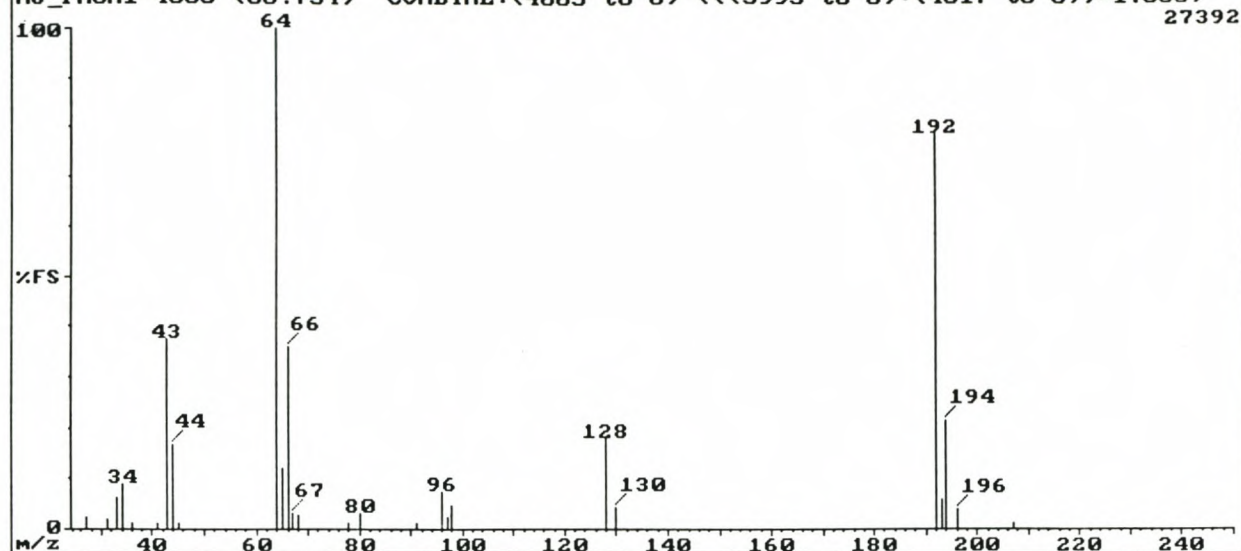


Fig. 4.46: EI mass spectrum of component 4005 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

Sample: ACINONYX JUB: FAU HS CRFS 69h DES 220 5min CRYO P262 40-280 @ 2C/min

AJ_FAUH1'5950 (99.174) COMBINE: (5950 to 0) - (((5894 to 5901) + (6008 to 0)) * 1.00)

109568

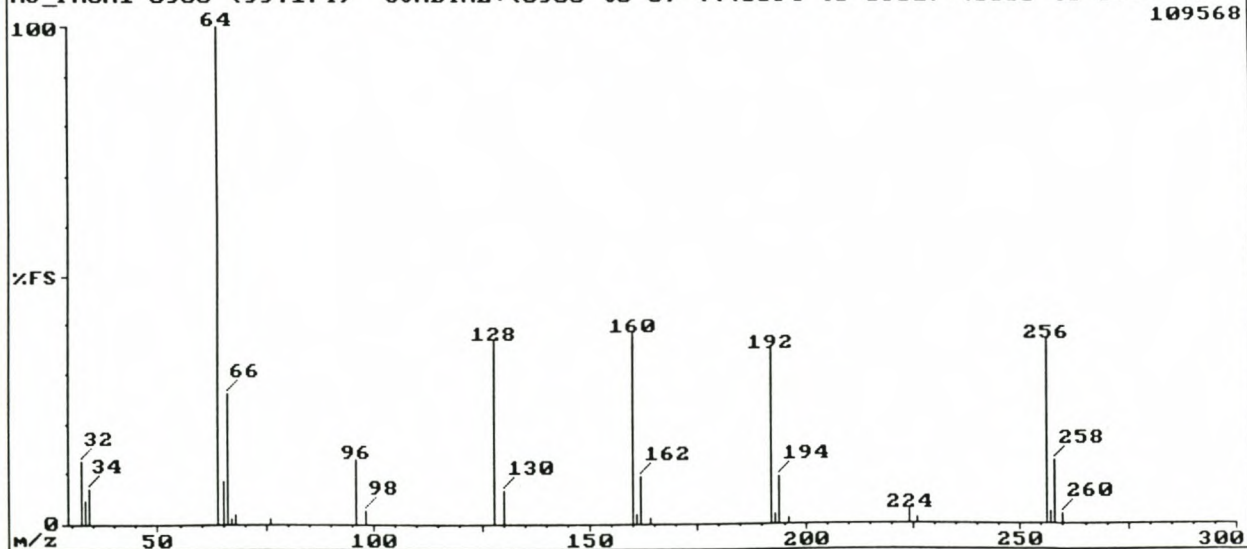


Fig. 4.47: EI mass spectrum of component 5950 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)

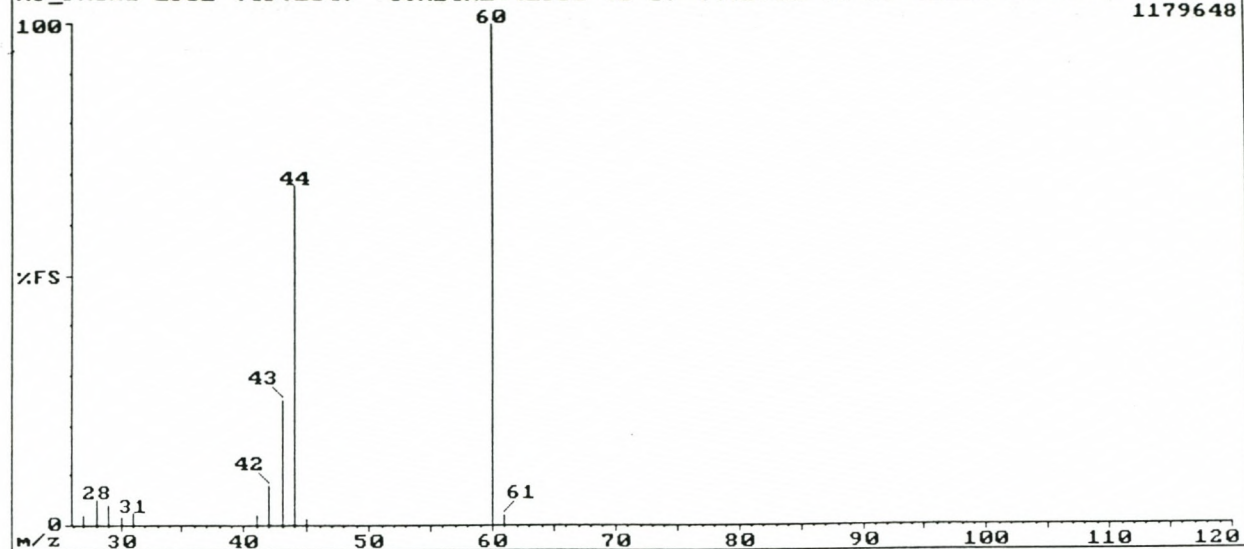


Fig. 4.48: EI mass spectrum of component 2952 of the urine of female cheetah (numbering as in Fig. 3.6, Table 4.1)