IDENTIFICATION OF THE PUTATIVE URINARY INTRASPECIFIC RECOGNITION PHEROMONE OF THE CARACAL *Caracal caracal*

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 entirety or in part has been submitted at any other university for a degree.

.....…… 23rd December 2016

Aron Hailemichael Date

DEDICATION

To my late grandmother, Ghebriel Habtemichael, who was always highly supportive, with love and encouraging words to see me achieve success in higher education.

ABSTRACT

Sheep husbandry makes a considerable contribution to the economy of South Africa. However, it has been pressure during the last few decades due to the rapidly growing numbers of predators such as the caracal, *Caracal caracal*, and the red jackal, *Canis mesomelas*. Currently, sheep farming is hardly viable in the arid southern parts of the country. When experimenting with various methods of controlling caracal numbers, sheep farmers found that this problem animal could be effectively lured into traps using the urine of another male or female caracal. The main disadvantage here is that a sheep farmer could incur serious stock losses before he is able to obtain a starting sample of urine from another source (e.g., another farmer).

It was hypothesised that caracal urine contains a volatile organic substance, or substances (VOCs), that are involved in the semiochemical communication between members of this species. The objective of this investigation was to identify these putative attractants in the urine, for the subsequent formulation of a caracal lure composed of synthetic analogues of the natural VOCs. As sheep farmers reported that male and female urine were equally effective attractants, it was considered unlikely that the attracting agent could be a sex pheromone; rather, it could have an intraspecific signalling function.

Efforts were made to identify all the VOCs present in caracal urine samples donated by farmers from different sheep farming areas of the country. A total of 191 VOCs, including five steroid hormones, were identified. The identification of 86% of these VOCs was corroborated by gas chromatographic-mass spectrometric (GC-MS) comparison of the natural substances with authentic synthetic analogues.

Obligate proteinuria is known in certain animals, in which it is not an indicator of renal abnormality. In the house mouse, *Mus domesticus*, for example, so-called major urinary proteins (MUPs) bind dehydro-*exo*-brevicomin and 2-(*sec*-butyl)-4,5-dihydrothiazole elicit male aggression. The carboxylesterase-like urinary excreted pheromone-binding protein, also known as cauxin, has been identified in the urine of several felids, but was not found in caracal urine. However, the pheromone-binding proteins 'Transgelin fragment (M3WJ37)' and 'Uncharacterized protein (M3XEJ0)', both previously identified in *Felis catus* and *Felis silvestris catus*, were identified in caracal urine.

The macrocyclic C_{15} ketone, cyclopentadecanone, was identified as one of the VOCs present in the headspace gas of caracal urine. The headspace concentration of this ketone increased when the urine was heated at 95 ºC for 10 min. A similar, but more pronounced

increase in cyclopentadecanone concentration was observed when urinary protein present in caracal urine was denatured by subjecting it to similar treatment. This was interpreted as an indication that this ketone is a ligand of caracal urinary protein. The C_{13}, C_{14}, C_{16}, and C_{17} macrocyclic ketones were subsequently also identified as urinary protein ligands.

In bioassays, a mixture of synthetic analogues of a large number of the other ketones identified in caracal urine, including cyclopentadecanone, elicited typical feline behaviour in two male caracal in captivity. Similar behavioural patterns were observed when a mixture of the C_{13}, C_{14}, C_{15}, and C_{16} macrocyclic ketones were tested. It was concluded that these ketones, that are ligands of the caracal's urinary protein, could play an essential role in the intraspecific communication of the caracal.

OPSOMMING

Skaapboerdery maak ‘n aansienlike bydrae tot die Suid-Afrikaanse ekonomie, maar is die afgelope paar dekades onder toenemende druk as gevolg van die vinnige toename in rooikat- (*Caracal caracal*) en rooijakkals- (*Canis mesomelas*) bevolkings. Gevolglik is dié bedryf kwalik volhoubaar in die droë suidelike dele van die land. Skaapboere wat metodes ondersoek het om die skade wat rooikatte gereeld onder hulle skaapkuddes aanrig, het gevind dat hierdie probleemdier baie effektief gevang kan word deur die urien van ander wyfies of mannetjies as lokmiddel in vanghokke te gebruik. ‘n Nadeel van hierdie bestrydingsmetode is dat boere groot verliese kan ly voordat ‘n monster rooikat-urien bekom kan word om met so ‘n bestrydingsprogram te begin.

Gebaseer op die hipotese dat rooikat-urien moontlik ‘n verbinding, of verbindings bevat wat ‘n rol in die semiochemiese kommunikasie van hierdie diere speel, is ‘n navorsingsprojek van stapel gestuur met die doel om sodanige verbinding(s) te identifiseer, ten einde ‘n lokstof uit sintetiese analoë te formuleer. Aangesien skaapboere gevind het dat die urien van wyfies en mannetjies ewe effektiewe lokmiddels is, is voorlopig aanvaar dat dit hier om intraspesifieke kommunikasie gaan en dat steroïdhormone, wat waarskynlik in die urien aanwesig is, dus waarskynlik nie die hoofrol in die lokwerking speel nie.

Daar is gepoog om die chemiese samestelling van rooikat-urien so volledig as moontlik te bepaal deur al die vlugtige organiese verbindings (VOVs) in urien van verskillende geografiese gebiede waar met skape geboer word, te identifiseer. ‘n Totaal van 191 VOVs, wat die vyf steroïedhormone ingesluit het, is geïdentifiseer. Die identifikasie van 86% van hierdie verbindings is bevestig deur middel van gaschromatografies-massaspektrometriese vergelyking van die natuurlike verbindings met sintetiese analoë.

Obligate proteinuria kom by sommige soogdiere voor, maar is nie ‘n indikator van renale nierversaking nie. In die huismuis, *Mus domesticus*, byvoorbeeld, bind die sogenaamde major urinêre proteïene (MUP) dehidro-*exo*-brevikomien en 2-(*sek*-butiel)-4,5-dihidrothiasool wat aggressie in manlike muise ontlok. The karboksielesterase-soortige urinêre feromoonbindende proteïen wat as ‘cauxin’ bekend staan, is in die urine van verskeie katspesies geïdentifiseer, maar is nie in rooikat-urien gevind nie. Die twee feromoonbindende proteïene ‘Transgelin fragment (M3WJ37)’ en ‘Uncharacterized protein (M3XEJ0)’, wat voorheen in *Felis catus* en *Felis silvestris catus*, geïdentifiseer is, is nou ook as feromoon-bindende proteïene in rooikat-urien gevind.

Siklopentadekanoon is een van die VOVs wat in die bodamp van rooikat-urien gevind is. Met die verhitting van die urien by 95 °C vir 10 minute, het die bodampkonsentrasie van hierdie ketoon toegeneem. ‘n Soortgelyke, maar duideliker toename in bodampkonsentrasie is waargeneem wanneer die urinêre proteïen eers geïsolee is. Dit is as aanduiding aanvaar dat hierdie ketoon ‘n ligand van die rooikat se urinêre proteïen is. Die C_{13}, C_{14}, C_{16} en C_{17} makrosikliese ketone is later as verdere ligande van die proteïen geïdentifiseer.

‘n Mengsel van sintetiese analoë van verskeie ketone wat in rooikat-urien geïdentifiseer is en wat siklopentadekanoon ingesluit het, het die tipiese gedrag van kat-soortige diere ontlok in twee rooikatmannetjies wat in gevangenskap aangehou word. Soortgelyke gedragspatrone is weergeneem in toetse met ‘n mengsel van die C_{13}, C_{14}, C_{15} en C_{16} makrosikliese ketone. Daar is tot die gevolgtrekking gekom dat hierdie ketone, wat ligande van die rooikat se urinêre protein is, besmoontlik ‘n essensiële rol in die intraspesifieke chemiese kommunikasie van die rooikat speel.

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Aron Hailemichael Goitom
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ABBREVIATIONS

AGC	Automatic gain control
amu	Atomic mass unit
AOB	Accessory olfactory bulb
AOS	Accessory olfactory system
BCA assay	Bicinchoninic acid assay
BSA	Bovine serum albumin
°C	Degrees Celsius
CC	Caracal caracal
CID	Collision-induced dissociation
CV	Capillary voltage
Da	Dalton
DCM	Dichloromethane
DMDS	Dimethyl disulphide

EDTA	Ethylenediaminetetraacetate
EI	Electron impact
ESI	Electrospray ionization
ESI^+	Electrospray ionization in the positive mode
ESI-Q-TOF-MS	Electrospray ionization quadrupole time-of-flight mass spectrometer
FID	Flame ionisation detector
FC	*Felis caracal*
GC	Gas chromatograph/ Gas chromatographic
GC-EAD	Gas chromatographic-electroantenographic detection
GC-HRMS	Gas chromatography-high resolution mass spectrometry/ Gas chromatographic-high resolution mass spectrometric
GC-LRMS	Gas chromatography-low resolution mass spectrometry/ Gas chromatographic-low resolution mass spectrometric
GC-MS	Gas chromatography-mass spectrometry/ Gas chromatographic-mass spectrometric
GC-TOF-HRMS	Gas chromatography-time-of-flight high resolution mass spectrometry/ Gas chromatographic-time-of-flight high resolution mass spectrometric
HCD	High-energy collisional dissociation
HR	High resolution
HRMS	High-resolution mass spectrometry
i.d.	Inner diameter
IUCN	International union for Conservation of Nature
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LECUS	Laboratory for Ecological Chemistry, of the University of Stellenbosch
LR	Low resolution
LRMS	Low-resolution mass spectrometry
MOB	Main in olfactory bulb
MOE	Main olfactory epithelium
MOS	Main olfactory system
MALDI	Matrix-assisted laser desorption/ionization
MCK	Macrocyclic ketones
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry/mass spectrometric
MUP	Major urinary protein

MW	Molecular weight
MWCO	Molecular weight cut-off
NBS	National Bureau of Standards
NIST	National Institute of Standard Technology
Nano-ESI	Nano electrospray ionisation
OSN	Olfactory sensory neuron
PBP	Pheromone-binding protein
PEG	Polyethyleneglycol
PDMS	Polydimethylsiloxane
PSM	Peptide spectrum match
PTFE-faced	Polytetrafluoroethylene-faced
RI	Retention index
RPM	Revolutions per minute
RSLC	Rapid separation liquid chromatography
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEP	Sample enrichment probe
SPME	Solid-phase microextraction
TBME	*tert*-Butyl methyl ether
TEMED	N,N,N',N'-tetramethylethylenediamine
TIC	Total ion chromatogram
Tris-HCl buffer	*tris*(Hydroxymethyl)aminomethane
VNO	Vomeronasal organ
VOCs	Volatile organic constituents
UF	Ultra filtration
UPLC	Ultra performance liquid chromatography

CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1 Introduction

Humans interact with wildlife in many ways and for different reasons. The advent of modern farming practices has increased human-wildlife conflict, which could eventually have a profound effect on the planet's ecosystems. From the point of view of humans, the most serious problem is probably livestock depredation, particularly by carnivores. A study has shown that the loss of livestock is the most frequently cited (40%) reason for conflict involving carnivores (Sillero-Zubiri and Laurenson, 2001). Factors contributing to this situation may be a sharp increase in the domestic livestock population, resulting in increasing competition with other herbivores for grazing. This and the lack of anti-predator behaviour in herbivores results in a reduction in the wild herbivore population that would otherwise be prey for carnivores (Woodroffe *et al*., 2005: 17).

Karlsson and Johansson (2010) have shown that the risk of predator attacks on sheep farms increases in intensity and that the frequency of attacks could increase 50-fold after the first few incidences. According to Avenant and du Plessis (2008), small stock farming is under threat in South Africa. Sheep farming has been under considerable pressure during the last few decades in certain parts of South Africa due to the rapidly growing numbers of predators, such as the caracal, *Caracal caracal*, and the red jackal, *Canis mesomelas*, which cause livestock losses amounting to several millions of rands annually. Already at this stage, sheep farming is not viable in certain parts of the Western Cape. This problem could have a negative effect on food security in this province and eventually also in other parts of South Africa. The basic problem is the perturbation of the ecological balance in sheep farming districts, resulting in an ample supply of defenceless prey (lambs) and practically no carnivorous competitors or natural enemies, except for the red jackal, which is an equally serious problem (Hey, 1964; Avenant *et al*., 2006). The absence of large predators such as hyena, cheetah and leopard in the sheep farming areas of South Africa could eventually result in a vast increase in the caracal population in these areas. Stahl *et al*. (2001) have suggested that reduction of the overall carnivore population density could be the most effective way to reduce stock losses.

1.2 The caracal

The caracal is classified as a problem animal in South Africa and Nambia. It is hunted, particularly in the Cape Province, because large caracal populations, pose a serious threat to sheep farming in parts of this province, especially in the Karoo where sheep farming is the only viable farming activity. Reports indicate that in South Africa, between 1931 and 1952, over 2000 caracal were killed annually in the Karoo, while in Namibia 2800 caracal were killed in 1981 alone (Nowell and Jackson, 1996). Despite the killing of large numbers of caracal, its population is increasing. The caracal problem is exacerbated by the fact that the caracal usually kills more prey than it can normally utilise; this phenomenon is known as surplus killing. It is quite common for the animal to kill more than ten lambs during one night (Skinner, 1979; Stuart and wilson, 1988), probably because it chases the lambs or plays with them almost like house cats play with mice.

The caracal problem is considered most problematic in the semi-arid regions of Southern Africa (Nowell and Jackson, 1996). Analyses of scat and stomach content of caracal from various unprotected areas in South Africa have shown that 17–55% of the caracal diet comprises domestic stock. In an investigation of the stomach contents of 394 caracal killed to reduce the caracal population in a part of South Africa, the stomachs of 37% of the animals were empty, while 28% of the other 248 animals' stomachs contained residues of sheep or goat (Pringle and Pringle, 1979; Stuart, 1982). Stuart (1984) and Brand (1989) have reported that small stock losses attributed to caracal in the Cape Province could be as high as 5.3 animals per 10 km^2 and that 82% of the farmers considered the caracal to be the principal predator of domestic livestock.

The caracal is a carnivore belonging to the order Carnivora and the widely distributed family felidae (Horak *et al*., 2000; Bininda-Emonds *et al*., 2001; Farhadinia *et al*., 2007). The name 'caracal' originated from the Turkish 'karakulak', for 'black ears'. In North India, the caracal is also known as 'syahgosh', a Persian term with the same meaning. In Afrikaans, the animal is called the 'rooikat', derived from its reddish colour (Rautenbach, 1976; Kohn *et al*., 2011). The caracal first became known to Western science when, in 1776, a German naturalist, Johann Christian Daniel von Schreber (1739–1810), illustrated and described a specimen from Table Mountain, overlooking Cape Town in the Western Cape (Willis and Morkel, 2007).

The utilisation of wildlife in its various forms is becoming a valuable form of land use, especially in areas where the inhabitants' livelihood depends on hunting or food gathering (Von Richter, 1970). Mattern and McLennan (2000) have very appropriately, described our relationship

with felids as follows: Due to limited conventional agriculture practices, "humans have had a long association with many members of the cat family. We hunt them for their fur and medicinal properties, display them in zoos, associate them with magic and witchcraft, worship them as gods, domesticate them for pest control, and keep them as pets. Although cats are an integral part of our history and our everyday lives, we know very little about most members of the felidae and their evolutionary relationships". Information is scant and generally biased toward the large, charismatic members of the genus *Panthera*, such as the lion and tiger, i.e., fascination with the larger cats, coupled with less interest being paid to the smaller, more secretive felids. It is, after all, much easier logistically to track and observe lions than to struggle through the jungles of Southeast Asia searching for the nocturnal leopard cat, *Prionailurus bengalensis*. Problems associated with data collection, combined with the observation that many felids appear to display a frustrating combination of primitive and specialised characters, have puzzled researchers interested in defining the relationships among cats ever since Linnaeus founded the genus *Felis* in 1758. Pocock (1917) described the results of early systematic attempts as 'chaotic confusion', a description that was echoed over half a century later in Collier and O'Brien's assessment (1985), stating that felid family relationships are still widely disputed.

There are various approaches with regard to the classification of the caracal, which is sometimes called a desert lynx or caracal lynx, based on morphological similarities. As a result, the caracal was initially classified together with the lynx, despite the two species being quite different. Based on phylogenetic studies, Werdelin (1981) found no relationship between these species and placed the caracal in the genus *Felis*. The systematic name, *Felis caracal*, has often been used for the caracal. However, recent phylogenetic studies (e.g., Mattern and McLenann, 2000) indicate that the original name *Caracal caracal* is preferable.

The majority of the felids are nocturnal and terrestrial, with a wide habitat range. Some felids are nevertheless diurnal. A few are terrestrial with the extra ability of swimming, for example, the so-called Geoffroy's cat, *Leopardus geoffroyi*. Some caracal are terrestrial, but also display arboreal activity (Mattern and McLenann, 2000). The scientific names, common names, habitat and activities, as well as the ecological characteristics of felid species are summarised in Table 1.1.

Table 1.1: Scientific names, common names, habitat and activities of felid species[a]

Scientific name	Common name	Habitat	Activity
Acinonyx jubatus	Cheetah	Bush/grassland	Diurnal
Caracal caracal	Caracal	Forest/savannah	Nocturnal
Felis bieti	Chinese desert cat		
Felis catus	Domestic cat	Widespread	Nocturnal
Felis chaus	Jungle cat	Forest (open)	Diurnal
Felis libyca	African wild cat	Widespread	Nocturnal
Felis margarita	Sand cat	Deserts	Nocturnal
Felis nigripes	Black-footed cat	Forest (open)	Nocturnal
Felis silvestris	European wild cat	Widespread	Nocturnal
Herpailurus yagouaroundi	Jaguaroundi	Lowland forest	Both
Ictailurus planiceps	Flat-headed cat	River banks	Both
Leopardus geoffroyi	Geoffroy's cat	Open bush	Nocturnal
Leopardus pardalis	Ocelot	Forest (dense to open)	Nocturnal
Leopardus tigrinus	Little tiger cat, tigrina	Forest	?
Leopardus wiedii	Margay	Forest	?
Leptailurus serval	Serval	Savannah, around streams	Nocturnal
Lynchailurus colocolo	Pampas cat	Forest/grasslands	Nocturnal
Lynx canadensis	Canadian lynx	Widespread (dense to open tundra)	Nocturnal
Lynx lnyx	Eurasian lynx	Widespread (dense to open tundra)	Nocturnal
Lnyx pardnus	Spanish lynx		
Lynx rufus	Bobcat	Widespread (forest/semi desert)	
Mayailurus iriomotensis	Iriomote cat		
Neofelis nebulosa	Clouded leopard	Forest	Diurnal
Oncifelis guigna	Kodkod	Forest	Nocturnal
Oreailluru jacobita	Andean mountain cat		
Otocolobus manul	Pallas' cat	High steppes and deserts	Nocturnal
Panthera leo	Lion	Savannah	Nocturnal
Panthera onca	Jaguar	Widespread	Both
Panthera pardus	Leopard	Widespread	Nocturnal
Panthera tigris	Tiger	Widespread	Nocturnal
Panthera uncia	Snow leopard	High mountains	Diurnal
Pardofelis badia	Borean bay cat	Forest	
Pardofelis marmorata	Marbled cat	Forests	Nocturnal
Prionailurus bengalensis	Leopard cat	Forest (high-low)	Nocturnal
Prionailurus rubiginosus	Rusty spotted cat	Widespread	Nocturnal
Prionailurus viverrinus	Fishing cat	Mangrove swamps	?
Profelis aurata	African golden cat	Forest and mountains	Diurnal
Profelis temmincki	Asian golden cat	Forest	?
Puma concolor	Cougar, mountain	Widespread	Both

[a]Classification follows Ewer (1973: 279–292), Collier and O'Brien (1985) and Mattern and McLennan (2000).

The caracal is a slender, yet muscular, medium-sized cat, long-legged and with hind legs that are somewhat longer than its forelegs for running faster than other cats. It has a short tail and an outstanding capability of leaping up to 3 m high, and it climbs trees well. It is an uncharacteristic felid species due to its tufted ears (Sunquist and Sunquist, 1988: 38–39; Narasimmarajan *et al*., 2011). Other remarkable features are its large paws with strong, heavily built claws that are retractable. The caracal differs from the majority of the other members of the felid family in that it has a uniform fawn coat, shading ventrally to white and to black at the tips of the ears (Eizirik *et al*., 2003), as depicted in Fig. 1.1. According to Kohn *et al*. (2011), the body sizes of male and female caracal differ in that the males are heavier, longer and larger in most body dimensions than the females. Depending on whether male caracal are observed in captivity or in the wild, their weight generally ranges from 6 to 19 kg.

Fig. 1.1: Photograph of a caracal, *Caracal caracal*.

The caracal can occupy and tolerate a wide range of habitats due to its good ability to adapt to various environmental conditions. It is found in dry habitats, such as the drier woodlands, savannah, semi-deserts and steppes, as well as in vegetated areas associated with water (Dayan *et al*., 1990). Its distribution extends over large parts of Africa, where it is absent only in the humid central African forest areas and the central Sahara. Its habitat also extends beyond the Arabian Peninsula to South-eastern Turkey and North-western India. However, it is particularly abundant in parts of the the Cape Province of South Africa (Dayan *et al.*, 1990; Skinner and Smithers, 1990;

Ray *et al*., 2005; İlemin and Gürkan, 2010). Caracal generally prefers more open spaces than most other cats, in particular desert mountains and hilly terrain that offers them some cover by trees, bushes or rocks (Sunquist and Sunquist, 2002: 39).

A study carried out by Marker and Dickman (2005) on commercial farmlands in Namibia revealed that the caracal has large home ranges that enable the recolonisation of vacant areas when local extinction occurs. In South Africa, for example, the ranging areas of the caracal amount to about 65 square miles for males and 18.2 square miles for females (Norton and Lawson, 1985; Farhadinia *et al*., 2007; İlemin and Gürkan, 2010). The caracal male and female home range areas in relation to prey density areas in the West Coast National Park (Cape Province) is depicted in Fig. 1.2.

Caracal are generally noctrunal, secretive, solitary predators and phenomenal athletes, capable of jumping very high, as mentioned earlier, sprinting, stealth and stalking (Skinner and Chimimba, 2005; Kohn *et al*., 2011; Singh *et al*., 2014). Generally, caracal have a very large prey spectrum, ranging from mice to birds and antelope. Caracal hunt mainly during the night, but in the regions where they live undisturbed they can also be observed hunting during the day. When scared by people, they usually search for cover, where they lie flat on the ground, and are then hardly visible due to their coat colour. They hunt by stalking with a final short spurt of generally less than 5 m. Large prey-like antelope and duiker are stifled by a targeted bite to the throat, whereas small prey such as rabbits and mice are usually killed with a neck bite (Drake-Brockman, 1910: 21; Liebenberg, 2000: 81; Farhadinia *et al*., 2007).

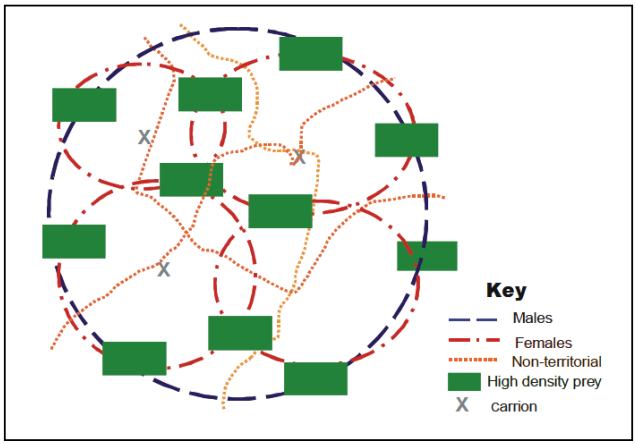


Fig. 1.2: Caracal male and female home range areas in relation to prey density areas in the West Coast National Park, 1990/91 (Avenant *et al.*, 2006).

The International Union for Conservation of Nature (IUCN) has categorised caracal as a species of least concern (Nowell, 2002). The caracal is of low concern because it remains widespread and relatively abundant throughout Southern and Eastern Africa, with only minor range losses in Northern and Western Africa due to high human pressure and extreme habitat change. The caracal is the largest of Africa's smaller cats and adapts well to livestock areas. Generally, the animal has little legal protection due to the damage it causes to livestock and its abundance. However, caracal is endangered in Asia due to habitat destruction through agriculture and desertification (Nowell, 2002: 4; Ray *et al*., 2005).

According to Farhadinia *et al*. (2007), females give birth in spring with litter sizes ranging from 2 to 3 cubs. However, a study of Bernard and Stuart (1987) on the reproduction of the species in captivity has shown that females can give birth throughout the year, with a pronounced peak in summer. The mean litter size of the captive and wild caracals was 2.2 and 2, respectively. The mean duration of gestation was 79 days. According to other reports, wild cubs can be born at any time throughout the year, with a peak between October and February (Skinner *et al*., 2002). In Southern Africa, cubs are generally born in summer. The key reproductive features of caracal are summarised in Table 1.2.

Table 1.2. Key reproductive features of caracal[a]

Activity	Duration
Breeding interval	Once a year
Number of offspring	1 to 3
Gestation period	78 to 81 days
Birth mass	98 to 250 grams
Average weight of an adult	8 kg (female) and 11 kg (male)
Head-body length	80 cm (female) and 90 cm (male)
Weaning age	4 to 6 months
Onset of puberty	7 to 10 months
Reproductive maturity	12 to 14 months

[a]Bernard and Stuart (1987), Skinner *et al*. (2002), Sunquist and Sunquist (2002: 37–40) and Stuart and Stuart (2006: 284).

Depending on the availability and variety of food, and protection from predators, the lifespan of the caracal is generally different in the wild and in captivity. According to Livingston

(2009), the caracal is believed to have a lifespan of approximately ten years in the wild, but it may live longer in captivity.

Bernard and Stuart (1987) found that a male caracal in captivity can identify a female in oestrus from urine spray sites, after which the male approaches the female. The male does not approach a non-receptive female and it backs off when approached by such a female. Oestrus lasts about two weeks and is not restricted to a specific season. The young are usually born in a quiet place such as a cave or somewhere with other protective cover. Cubs are weaned at about 15 weeks. When raised in captivity, they start trying to catch prey for themselves from about three months. Presumably, at the age of nine to ten months they leave the home range of the female and they are then sexually mature.

As highlighted above, carnivores such as the caracal and red jackal cause enormous losses in livestock husbandry. Controlling and protecting the livestock from predators is imperative. Generally, the problem animals are controlled by lethal and non-lethal methods. Herding, hunting, bell collars, poisoning, traps and electric fencing as caracal-controlling methods are non-selective and not cost-effective, with only limited success rates (Bowland *et al*., 1993).

The use of semiochemicals might eventually be a more effective method for controlling these problem animals (Herbst and Mills, 2010; Zöttl *et al*., 2012).

1.3 Semiochemicals

In the 1870s, the French naturalist Jean-Henri Casimir Fabré hypothesised that the peculiar behaviour of male peacock moth (*Saturnia pyri*) could be ascribed to some still unknown type of communication between the male and female moths. Almost a century later, in 1959, the German chemist Adolf Butenandt first succeeded in isolating a sex attractant, bombykol, *trans,cis*-hexadeca-10,12-dien-1-ol, that is responsible for similar behaviour in the silkworm moth, *Bombyx mori*. Since then, knowledge of chemical communication and understanding the animal world has been growing exponentially.

Karlson and Lüscher (1959) introduced the term ‘pheromone’, derived from the Greek *pherein*, to carry or transfer, and *hormōn*, to excite or stimulate. Pheromones belong to a subclass of semiochemicals (see below) that are used to exchange chemical information between members of the same species. A pheromone is released into the environment by an individual and received by a second individual of the same species, in which it then releases a specific reaction, for

instance, a definite behavioural pattern; this is in contrast to the action of hormones as integral signals within an individual organism.

As the understanding of chemical communication progressed, new terminology came into use. Regnier (1971) proposed the term 'semiochemical', which is derived from the Greek *semeion*, for a sign or signal. Semiochemicals are compounds or mixtures of compounds involved in animal communication. Semiochemicals are usually subdivided into, for example, pheromones and allelomones.

Pheromones are for communication within a species (intraspecific signals) whereas chemicals that take part in interspecific interactions (predator–prey, for example) are called allelomones. Semiochemicals are different from hormones and nucleic acids that are used for chemical communication within an organism (Regnier, 1971). Pheromones are released in secretions or excretions of living organisms. They are a most important feature in the ecology of nocturnal animals. In terrestrial animals, for example, elephant and mice secrete pheromones in the urine (Mucignat-Caretta *et al*., 1998; Wyatt, 2003: 9). Mucignat-Caretta and Caretta (1999) suggested that pheromones influence the behaviour and endocrine status of conspecific mammals, which affects the social organisation of some species such as mice.

After the introduction of the term pheromone by Karlson and Lüscher (1959), Regnier (1971) proposed a further subdivision of semiochemicals into 'releaser' and 'primer' pheromones. The difference between the two types of pheromones is based on the behavioural changes in the receiver. Releaser pheromones have an immediate effect on the behaviour of the receiver. There are many types of releaser pheromones and they are often subdivided according to their function. The most commonly known examples are sex attractants, to which the opposite sex immediately responds, and alarm, trail, aggregation and territorial marking pheromones (Wilson, 1963; Brown and Godin, 1997). Primer pheromones have long-term physiological effects on the receiver. Primer pheromones are used in, for example, puberty acceleration, caste determination, and the inhibition of ovulation in mammals and social insects such as mice, termites, ants, bees and wasps (Caretta *et al*., 1995; Novotny *et al*., 1999 and Reinhard, 2004).

'Allelomones' are a subclass of semiochemicals that are used for interspecific communication, for example, in semiochemical interactions between predator and prey. Allelomones are further subdivided into allomones and kairomones, based on the costs or benefits for a sender or a receiver (Sbarbati and Osculati, 2006). Nordlund and Lewis (1976) originally

defined allomones as chemical substances produced by an organism and interacting with another organism, causing behavioural or physiological response favouring the emitter, i.e., they are semiochemicals that benefit the sender at the cost of the receiving species. Semiochemicals are secreted for defence, deceit or propaganda. Ticks, for example, use a cuticular secretion as dog-deterring allomones to prevent a dog from removing a tick from its body, which allows the tick to complete its blood meal (Burger *et al*., 2006), while bolas spiders secrete moth pheromones to lure male moths of those species into range for capture by the spiders (Wyatt, 2003: 1–2).

Semiochemicals that favour the receiving species are known as 'kairomones', which could be compounds or mixtures of compounds providing information to the advantage of the receiver. Kairomones are chemical cues that help a predator to locate or recognise its prey and also compounds that attract insects to certain food plants. The predatory beetle (Coleoptera: Scolytidae) finds its prey by detecting the pheromone, ipsdienol, that the bark beetle, pine engraver, *Ips pini*, uses for intraspecific communication. When considered from the perspective of the predatory beetle, the pheromone can be classified as a kairomone (Lewis *et al*., 1976; Birch, 1978; Albone and Shirley, 1984: 4; Stanley *et al*., 1994).

Wyatt (2003: 2) has suggested the term 'synomones' for semiochemicals that benefit both signaller and receiver in mutualisms, such as those between sea anemones and anemonefish. A classification of semiochemicals is given in Fig. 1.3.

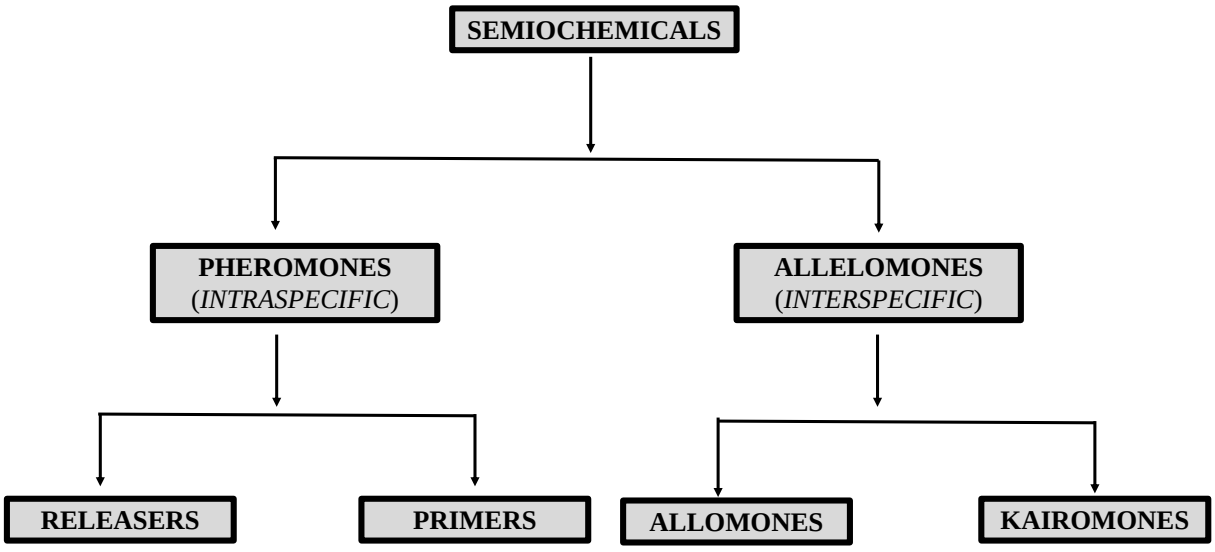


Fig. 1.3: A classification of semiochemicals.

1.4 Mammalian semiochemistry

Since the first identification of an insect sex attractant by Butenandt *et al*. (1959), hundreds of sex attractants and other semiochemicals have been identified in insects and other animals. Some of these compounds have relatively simple unbranched structures with one or more double bonds, while others have complex structures that in some cases took many years to unravel. It was to be expected that biologists and chemists would eventually start wondering whether it would be possible to identify the semiochemicals that were presumably responsible for well-known behavioural patterns in mammals, and whether synthetic analogues of these chemicals could possibly be used to influence the behaviour of mammals, for example, to control problem animals.

Brownlee *et al*. (1969) identified *cis*-4-hydroxydodec-6-enoic lactone, a component of the tarsal secretion of the black-tailed deer, *Odocoileus hemionus colombianus*, as the first putative pheromone of a mammal. The same compound was subsequently identified in the complex interdigital secretion of the bontebok, *Damaliscus pygargus pygargus* (previously *Damaliscus dorcas dorcas*) (Burger *et al*., 1977). Several research groups initiated projects in this new field of research. However, for various reasons, the first wave of optimism evaporated rather quickly.

The exocrine secretions of mammals are mostly complex mixtures, the constituents of which are often not commercially available. Field tests with synthetic analogues are problematic or are simply not feasible in the case of large wild animals. Furthermore, with the exception of mice, rats and other experimental animals that are readily available in research institutions, domestic animals and other small pets, it is very difficult to isolate and identify mammalian semiochemicals by applying a response-guided strategy (Albone and Shirley, 1984: 5–8). The most serious obstacle in this type of research is probably the lack of technology that is comparable with gas chromatographic-electroantennographic detection (GC-EAD). This analytical technique has revolutionised insect semiochemistry and made possible ground breaking research on the identification of insect semiochemicals aimed at the utilisation of their synthetic analogues in pest control strategies (Burger, 1991: 1; Cork and Hall, 1998). However, the interesting results emanating from research on the influence of components of the urine of rats and mice on the behaviour and physiology of these animals (e.g., Hurst *et al*., 1998; Novotny *et al*., 1999; Mucignat-Caretta *et al*., 2004) initiated what could be described as a renaissance in the field of mammalian semiochemistry. Chemical communication in mammals has been reviewed by Beruter *et al*. (1974), Brinck *et al*. (1978), and more recently by Burger (2005).

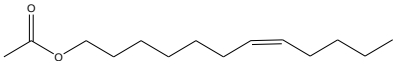
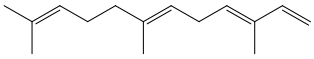
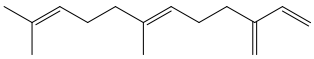
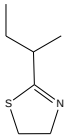
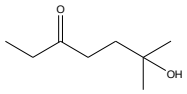
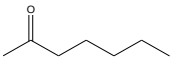
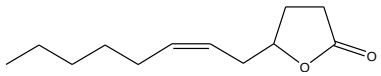
Eisenberg and Kleiman (1972) pointed out the importance of chemical communication in mammals. Olfactory communication, i.e., communication by smell and taste, plays a crucial role in communication between sender and receiver. Using specialised receptors, the receiver can identify, integrate and respond behaviourally to the chemical signal. The relationship between the sender and the receiver is in some way the result of natural selection, in which the signal production by the sender results in an increased probability that it could benefit from the transmitted message. Scent marking, which involves odour deposition by an animal in its environment, is one of the most conspicuous behaviours of many mammals, and it is a common method of olfactory communication among mammalian species (Bossert and Wilson, 1963; Peters and Mech, 1975).

For their survival, mammals rely on a wide variety of semiochemicals that play important roles in their lives, including marking and maintaining territory, maintaining social structure, reproduction, alarm signalling, defence and food procurement. The use of chemical signals for exchanging information among animals is widely used. Chemical signalling is the most effective mode of communication in both vertebrates and invertebrates (Hefetz, 1987).

In mammals, semiochemicals play an essential role in providing information regarding the sender's physical and social environment. These compounds may function at the individual and/or species level for different purposes, including marking and maintaining territory, mate choice, male–male competition, detection of predators and prey, advertisement of social and reproductive status and for kin recognition (Eisenberg and kleiman, 1972; Burger *et al*., 1981).

Most mammals possess a highly developed olfactory sense and use chemical signals present in urine, faeces, genital secretions, saliva, and cutaneous scent glands for the purpose of social communication (Ralls, 1971). Chemical signals may transfer information about the species, sex, age, health, social status, emotional state, reproductive state, and group and individual identity of an animal (Brown and Macdonald, 1985). Scent marking may be involved in male–male aggression, in repelling conspecific males, territorial marking and courtship activities, and for familiarisation with a new environment (Beruter *et al*., 1974; Kimball *et al*., 2009). A few examples of mammalian pheromones and their functions are given in Table 1.3.

Table 1.3: Some examples of mammalian semiochemicals and their functions

 *cis*-Dodec-7-en-1-yl acetate[a] Sex attractant pheromone present in the urine of female elephants, Asian elephant (*Elephas maximus*)	 *trans*,*trans*-α-Farnesene[b]  *cis*-β-Farnesene[b] Oestrous induction and inter-male aggression; produced in preputial gland; production is testosterone dependent in male mouse (*Mus domesticus*)	 2-*sec*-Butyl-4,5-dihydrothiazole[c] Oestrous induction and inter-male aggression; female attraction in the male mouse (*Mus domesticus*)
 6-Hydroxy-6-methyl-3-heptanone[d] Primer pheromones in male mouse urine (*Mus domesticus*) responsible for puberty acceleration	 2-Heptanone[e] Primer pheromones in female mouse (*Mus domesticus*) urine responsible for puberty delay	 *cis*-Dodec-6-en-4-olideo[f] Component of the tarsal hair scent of the male black-tailed deer, the interdigital secretion of the bontebok and the marking fluid of the male Bengal tiger (*Panthera tigris*)

[a,c]Rasmussen, *et al*. (1985); [c]Kelly (1996); [b,d,e]Novotny *et al*. (1999); [b,f]Burger (2005).

Animals may use different channels of communication, including visual (Shorey, 1976: 1–15), acoustic (Owings, 1998: 129), tactile (Frings and Frings, 1964: 11) and olfactory or chemical signalling (Feldhamer and Feldhamer, 1999: 346). The choice of communication channel depends on the distance between the sender and receiver (Forrest *et al*., 1993). There are two types of animal signals, namely discrete and graded. A discrete signal could be sent in a simple manner to express the state of an animal, whereas a graded signal could provide information on the intensity of a particular state. The territorial song of a gibbon is a discrete signal with a typical form and grade of aggressive vocalisation. However, it often consists of a series of graded signals, in which variations may reflect the emotional state of the sender (Poole, 1985: 26–27).

The choice of a channel of communication depends on the animal's environment and the type of information being sent (Feldhamer and Feldhamer, 1999: 346). A chemical or olfactory signal has advantages over the other types of communication signals in that it can transmit information over long distances, even during the night, at a slow transmission rate, with a slow fade-out time, at low cost and potentially with very high specificity. It may also be safer for the

sender to use chemical communication in cases where it is difficult to locate the sender in the presence of predators. The major characteristics of different channels of communication are summarised in Table 1.4.

Although the mechanism by which semiochemicals are produced might be varied and complex, the identification of their sources, and understanding the processes of production and detection of these chemicals are imperative in mammalian semiochemical research (Vander Meer, 2012).

Various types of glands located in different parts of the bodies of mammals are sources of semiochemicals (Albone and Shirley, 1984: 40–41, 74). The sources include the skin and its associated glands, the oral and nasal cavities with their glands, the lungs, the urinary and genital tracts, and the anal region. Skin or specialised glands (epidermal glands) such as dorsal glands (Burger *et al*., 1978; Burger *et al*., 1981), different exocrine secretions (Burger *et al*., 2008),apocrine glands located in the axillae and pubic region in humans (Grammer *et al*., 2005), urine (Albone and Shirley, 1984: 165) and anal sac gland secretions (Ewer, 1973) are all examples of some of the potential sources of semiochemicals. The sources also include secretions associated with the eye and perhaps even those of the external ear (Albone and Shirley, 1984: 41). Urine, faeces and the secretions of skin are sources of semiochemicals that are of special behavioural importance in that they may be deposited in the animal's environment, where they could form a semi-permanent record (Burger, 2005: 231-278). In the male mouse, the preputial gland is the source of volatile organic compounds with pheromonal activity, such as α-and β-farnesenes that are responsible for oestrous induction or inter-male aggression (Dulac and Torello, 2003). Although urine marking is common in mammals, little is known regarding the origin of urinary compounds. Some species have glands in the distal portion of the urogenital tract as a source of chemical compounds. Harvey *et al*. (1989) and Novotny *et al*. (1990) studied the contribution of secretions of the preputial glands to the effect of bladder urine on the semiochemical interaction between male mice.

Table 1.4: Characteristics of different sensory channels of communication

Signal properties	Type of signal			
	Olfactory	Auditory	Visual	Tactile
Range	Long	Long	Medium	Short
Transmission rate	Slow	Fast	Fast	Fast
Travel around objects	Yes	Yes	No	No
Night use	Yes	Yes	Little	Yes
Fade-out time	Slow	Fast	Fast	Fast
Locate sender	Difficult	Varies	Easy	Easy
Cost to send signal	Low	High	Medium	Low
Specificity	Potentially very high	High	More limited	Limited

[a]Wyatt (2003: 12).

1.5 Detection of mammalian semiochemicals

Mammals detect semiochemicals either by the main olfactory system (MOS) or by the accessory olfactory system (AOS). The MOS is involved in the detection of the volatile compounds in the air, whereas the AOS is useful in the detection of non-volatile cues sampled by direct physical contact. These systems have the remarkable ability of detecting and identifying thousands of compounds that provide valuable information about the animal's environment (Dulac and Torello, 2003). The organizational principles underlying the recognition of olfactory stimuli were reviewed by Munger *et al*, 2009.

The two main olfactory sensory organs for chemical communication in the nasal cavity of most terrestrial vertebrates are the main olfactory epithelium (MOE) and the vomeronasal organ (VNO), as shown schematically in Figure 1.4. These two sensory organs, MOE and VNO, receive information from the environment and transmit signals to higher cortical centres in the brain for the detection of all chemosensory cues that pass through the nose (Kelliher, 2007).

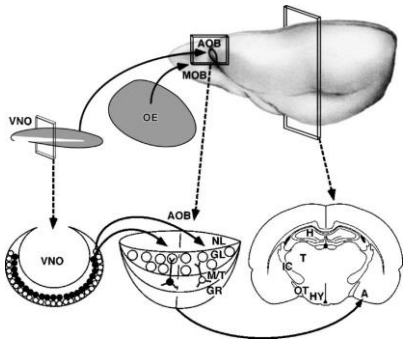


Fig. 1.4: General organisation of the vomeronasal system (VNS) in mammals. The upper figures illustrate the relationships between the vomeronasal organ (VNO), the olfactory epithelium (OE), and the main olfactory and accessory olfactory bulbs, MOB and AOB, respectively. The lower figures represent the coronal (VNO and cerebral hemispheres) and parasagittal (AOB) sections, showing apical and basal populations of vomeronasal receptor cells projecting to the anterior and posterior portions of the AOB, respectively. The AOB in turn projects to the amygdaloid complex (A). *Abbreviations*: NL, nerve; GL, glomerular; M/T, mitral/tufted; GR, granule cell layers of the AOB; H, hippocampus; IC, internal capsule; T, thalamus; HY, hypothalamus; OT, optic tract (from Halpern and Martinez-Marcos, 2003).

1.5.1 The main olfactory system

Olfactory sensory neurons form the MOE, which is the main sensory organ within the posterior nasal cavity that is involved in chemical communication by detecting odorous small volatile compounds (Kelliher, 2007). The axons from sensory neurons of the MOE transmit information to the main olfactory bulb (MOB), the first relay station in the brain. The olfactory bulb is found inside the skull beyond the olfactory epithelium and forms part of the limbic system of the brain. MOB sends most of its fibres to distinct brain nuclei that form the primary olfactory cortex, which in turn projects to higher sensory neurons (Buck, 2004; Munger *et al*., 2009), as shown in Fig. 1.5 (Dulac and Torello, 2003).

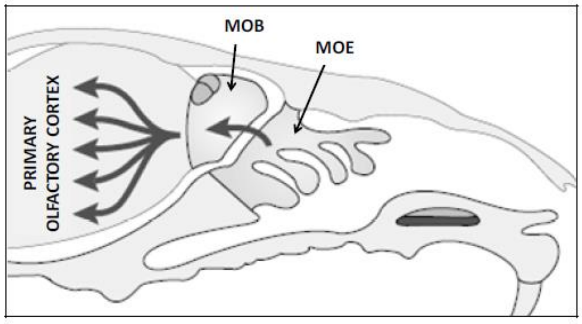


Fig. 1.5: Functional and anatomical segregation of the main olfactory system (from Dulac and Torello, 2003).

1.5.2 The accessory olfactory system

Many mammals also possess another chemical sensory organ, the VNO, also known as Jacobson's organ (Grammer *et al*., 2005). The VNO organ is a bilateral tubular structure in the anterior part of the nasal cavity of most territorial species, with the exception of the higher primates and bats. The structure of the VNO differs widely from species to species. The VNO nerve cells project axons to the accessory olfactory bulb (AOB), which in turn transmits sensory information to the vomeronasal amygdala, and then to specific nuclei of the hypothalamus that are involved in regulating genetically pre-programmed physiological and reproductive responses. The information from pheromone signals is detected by sensory neurons in the VNO, as depicted in Fig. 1.6 (Dulac and Axel, 1995; Dulac and Torello, 2003).

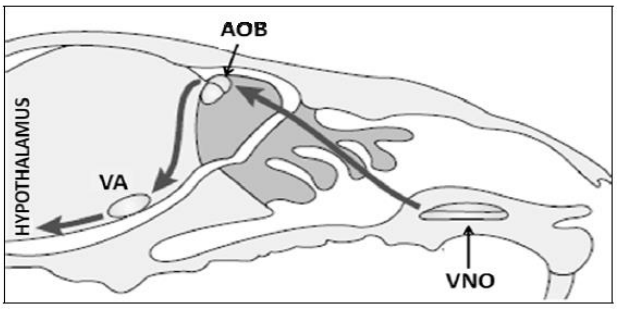


Fig. 1.6: Functional and anatomical segregation of accessory olfactory system (from Dulac and Torello, 2003).

1.6 Semiochemicals in territorial marking

Bossert and Wilson (1963) studied the modes of chemical transmission in animals, including the emission of the chemical signals and the rate of diffusion processes when these signals are released into the environment. Many mammalian species communicate by the deposition of odorous material, i.e., by so-called scent marking (Peters and Mech 1975). The ability to mark territory and defend home ranges against intruders is important in all social structures, particularly among higher mammals that possess the ability to interpret semiochemical information and use this information to adjust their behaviour in subsequent interactions. In the Steenbok, *Raphicerus campestris*, for example, both sexes create well-defined territories (Burger *et al*., 1999). Olfactory cues are used in territorial marking for recognising a particular home range belonging to a certain species or individuals, and in reducing conflict with intruders (Hurst, 1990). Many mammals,

including rodents, ungulates, carnivores and prosimians, mark portions of their home ranges with scent from excrement and exocrine glands (Hölldobler and Wilson, 1977).

Territorial marking, either by scent marking or countermarking, involves the overt defence or advertisement of occupation of a territory by deposition of a visual or olfactory trace or signal in an animal's home range (Thiessen and Rice, 1976; Rich and Hurst, 1999). Jaffe and Puche (1983) and Jaffe and Sanchez (1984) have described the criteria that are involved in the definition of a territorial pheromone. Normally, territorial marking pheromones are a mixture of chemicals that are secreted by the organism to define a territory. The chemical cue has to have intraspecific differences (e.g., concentration differences) detectable by the animal. The animal has to recognise its own mark and differentiate it from marks of conspecifics. The presence of the territorial signal has to give some advantage to the animal producing the signal and the marked area should be respected by conspecifics for the reduction of aggressive interactions between such organisms. The chemicals cause behavioural changes in conspecific intruders.

Scent marks contain important information with regard to the physical and social environment of the sender. Therefore, the role of semiochemicals in the communication between mammals is crucial in providing information regarding the status of the sender. Depending on the purpose of their release, the semiochemicals are beneficial to mammals at the individual and/or species level for marking and maintaining territory, locating predators and prey, reproductive status, kin recognition and social advertisement (Eisenburg and kleiman, 1972; Burger *et al*., 1981).

Wide varieties of organic compounds are used for chemical communication. The medium (water or air) through which the signal has to be transmitted and the manner in which the signal is transmitted, whether by diffusion or contact, determine which chemical composition would best serve the purpose of the signal. Semiochemicals that are released into the air have to be volatile, which is a function of their molecular size and polarity. Volatile molecules have an upper molecular weight of approximately 300 Da, i.e., they do not contain more than approximately twenty carbon atoms. Most airborne odour molecules contain between five and twenty carbon atoms. Airborne semiochemicals containing more than twenty carbon atoms are energetically expensive to produce and have low diffusion rates, while molecules containing less than five carbon atoms are uncommon because they are too volatile and possess too few options for species-specific structural variation. Semiochemicals vary greatly in terms of functional group position,

double bond position, and the occurrence of branches and rings in the carbon chain. Although these variations have minor effects on volatility, they greatly affect the shape of the molecule and the molecular detection by receptor cells (Bradbury and Vehrencamp, 1998: 281–283).

Alberts (1992) studied mammalian chemical signals and found that territorial marking pheromones have higher molecular masses and are less volatile and thus more persistent than sex attractants and kin recognition pheromones. The rate of evaporation is higher at higher temperatures, which reduces the persistence or longevity of a chemical signal. It is probably due to this temperature effect that the molecular masses of territorial marking pheromones in tropical forest species are higher than in species from temperate forests. In chemical communication systems in terrestrial vertebrates, evolutionary selection is expected to favour the use of signalling strategies that convey messages efficiently and minimise energetic costs to the signalling animals. Semiochemicals involved in territorial marking should satisfy additional physical requirements. When used primarily for defining territory, they have to provide information about the marked home area and individual identity, including the emotional status or health condition of the individual, and the compounds should thus have sufficient molecular diversity to encode complex messages. It is expected that semiochemicals used as marking signals should have lower rates of diffusion and a higher persistence than semiochemicals used for sex attraction or kin recognition, which should have higher rates of diffusion that result in rapid rise times so that they can be detected easily and quickly (Alberts, 1992). The comparison of sex attractants, kin recognition and territorial marking semiochemicals in Fig. 1.7 shows that the semiochemicals used for the territorial marking possess higher molecular masses than those used for sexual attraction and for individual recognition within, as well as across, mammalian orders.

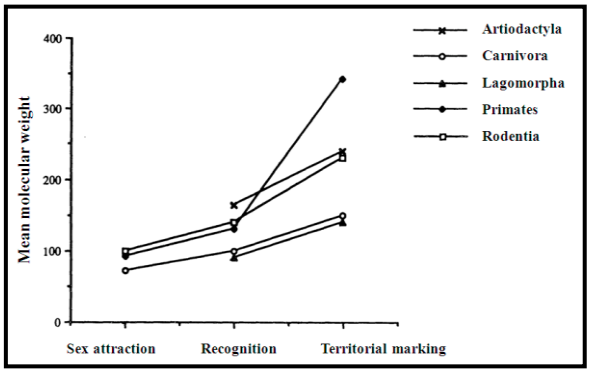


Fig. 1.7: Comparison of the mean molecular weight of sex attraction, recognition and territorial marking pheromones in five mammalian orders (from Alberts, 1992).

Many mammalian chemical signals are probably more persistent due to the presence of carrier compounds that slow down the diffusion of volatile constituents. Sebum, the lipid-rich, oily substance produced by sebaceous glands (Albone and Shirley, 1984: 45–46, 60–64), acts as a controlled-release substance that delays the evaporation rate of volatile constituents (Regnier and Goodwin, 1977). Squalene, one of the long-chain compounds present in the secretions of many species, may also act as a controlled-release substance. It is present in high concentrations in the lipid fraction of the marking fluid of the male Bengal tiger (*Panthera tigris*) and it has been shown that the lipid fraction of the marking fluid of this species displays a high affinity for the volatile constituents it contains (Burger *et al*., 2008).

In addition, the spatial range over which the signal is active is also important. The range can be increased by increasing the amount of material emitted, which, however, is energetically unfavourable for the animal. By increasing the surface area available for signal evaporation, the amount of the secreted material need not be increased (Alberts, 1992), but the lifetime of the signal may be reduced.

1.7 Semiochemical communication in felids

Felids, among the carnivores, release chemical signals from glands such as their anal, facial, interdigital and supracaudal glands, as well as in their urine and faeces (Macdonald, 1985: 619-722; Apfelbach *et al*., 2005).

Miyazaki *et al*. (2003) discovered that, as in the mouse (*Mus musculus*) and the rat (*Rattus norvegicus*), the domestic cat (*Felis catus*), also displays obligatory proteinuria involving the species-specific excretion of major urinary proteins (MUPs) that are members of the lipocalin superfamily and function as carriers of volatile urinary pheromones (Cavaggioni and Mucignat-Caretta, 2000; Beynon and Hurst, 2003). As the urinary excretion of MUPS had not been reported in the cat, Miyazaki *et al*. (2003) investigated the cause of proteinuria in the cat and identified a novel 70 kDa protein, which they named cauxin. Cauxin itself, as well as the decomposition products produced by cauxin, are excreted in a species-, sex-, and age-dependent manner (Miyazaki *et al*. (2006a). Domestic cats spray urine with a species-specific odour for territorial marking. Cauxin, which is a carboxylesterase, regulates the production of felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid), a putative pheromone precursor. Cauxin and felinine are excreted age-dependently after three months of age. Cauxin and felinine levels are sex-

dependently correlated and were higher in males than in females. In the headspace gas of cat urine, 3-mercapto-3-methyl-1-butanol, 3-mercapto-3-methylbutyl formate, 3-mercapto-3-methylthio-1-butanol and 3-methyl-3-(2-methyldisulfanyl)-1-butanol were identified as candidates for feline derivatives (Miyazaki *et al*., 2006b). These compounds contribute to the catty odour of cat urine.

The caracal might use scent marking for communication purposes by marking bushes or logs with scent from glands located between their toes or raking the scent into the ground with their hind feet (Cavallini *et al*., 2009: 73). Unlike in human beings, urine plays an important role in many mammals for communication between members of the same species. As territorial animals, caracal mainly use urine scent marking to define their territory. Generally, they deposit fluid from the bladder by normal urination and spray marking. The urine is sprayed on the target object while the animal stands with its tail lifted vertically (Mellen, 1993; Sunquist and Sunquist, 2002: 42).

Albone and Shirley (1984: 165–209) has discussed in detail the importance of urine in mammalian semiochemical communication. Many mammalian metabolic waste products are eliminated in the urine of the animals. The waste consists of volatile and non-volatile components belonging to different compound classes. Some odorous steroidal hormones and their urinary metabolites that are involved in semiochemical communication can also be excreted in the urine as waste; some of these components may be releaser or primer pheromones. Urine is used for different purposes, for example as sex attractants, for the reduction of aggression between mature males, territorial marking, puberty acceleration, oestrus induction and pregnancy block (e.g., Hurst *et al*., 1998; Novotny *et al*., 1999; Mucignat-Caretta *et al*., 2004). In general, caracal can communicate with one another using various channels, including visual, acoustic, tactile and chemical signals. They can, for example, spit, hiss, growl and snarl as different forms of vocal communication (Sunquist and Sunquist, 1988: 423).

1.8 Caracal olfactory communication

The caracal is a predator that enjoys a wide distribution and it poses a serious threat to the small livestock farming industry (Farhadinia *et al*., 2007). Despite its abundance, very little is known about its ecology in general and its semiochemistry in particular. Although some information on population and ecological characteristics has been obtained by camera trapping, the caracal is the least studied member of the felid family (Singh *et al*., 2014). The IUCN 2012 report placed the

animal in the Data Deficiency Category. Most of the previous work on the caracal focused mainly on its taxonomy, physical description, and ecological aspects such as its habitat and geographical distribution, its behaviour and feeding habits, reproduction and, specifically, human-predator conflict. Although information is available on the ecology of the caracal, no data are available with regard to olfactory communication in this animal. Only in a few reports it is suggested that the caracal probably employs semiochemicals present in the urine olfactory communication (Sunquist and Sunquist, 2002: 42).

In addition to the research of Miyazaki *et al*. on scent marking in domestic cats (2006a; 2006b), previous investigations into the composition of felid urine focused mainly on the chemical characterisation of constituents of the urine of large cats such as the lion, *Panthera leo* (McLean *et al*., 2007), Bengal tiger, *Panthera tigris* (Burger *et al*., 2008) and cheetah, *Acinonyx jubatus* (Burger *et al*., 2006).

The caracal is a territorial animal and defines its territories by depositing urine in its environment, although it is not yet clear whether this is an example of defensive territorial marking, or supplying information with a date stamp to conspecifics. Comprehensive identification and characterisation of the constituents of the caracal's urine could thus provide more specific information on the semiochemical communication of this interesting animal.

1.9 Motivation for and objectives

1.9.1 Motivation

As mentioned in the introduction to this chapter, Stahl *et al*. (2001) have suggested that reduction of the overall carnivore population density could be an effective way of reducing livestock losses. Various methods are currently used to control the caracal problem in sheep farming districts, including lethal and non-lethal methods. Only limited success has been achieved with methods such as herding, hunting, fencing, poisoning, trapping and using bell collars, largely due to their non-selective and uneconomical nature. Although non-lethal methods, for example the use of electric strained-wire fences, could be employed to reduce livestock losses, the available methods are not particularly cost effective. Poisoning the problem animals is a non-selective and unacceptable method of control because several other bird species and scavenging mammals are mostly also poisoned in the process, which could result in further disturbing the ecological balance. The use of traps for control, such as cage traps and springs or so-called 'gin traps', are ineffective

as the cats are primarily predators and not scavengers, resulting in less attraction to baited traps. There is also a strong public lobby against using poison and spring traps to control problem animals (Hey, 1964). Some sheep farmers have started experimenting with other methods to protect their sheep herds. Using semiochemicals to control problem animals has been suggested as a promising alternative to the above methods (Herbst and Mills, 2010; Zöttl *et al*., 2012).

Research was carried out by the Laboratory for Ecological Research of the University of Stellenbosch (LECUS) in the 1980s to investigate this possibility of controlling the jackal, *Canis mesomelas*, which also poses a serious problem in sheep farming areas, by semiochemical means. The urine of the female jackal contains a very potent sex attractant. However, fractions of the female's urine attracted males only during the short mating season of the animal when the females are in oestrus. Because canines' sense of smell is so keen that they can detect odours that are at least three orders of magnitude below the smallest quantities detectable by state-of-the-art analytical instrumentation, the possibility exists that it might, anyway, have been impossible to identify the territorial marking pheromone of the jackal with the technology then available.

1.9.2 Objectives

An investigation into the possibility of controlling the caracal by utilising its urine or, preferably, constituents of its urine to attract this species appeared to be a more viable approach than continuing research on the jackal. In this regard, it was encouraging that a few farmers have achieved almost total control of caracal on their farms by trapping male as well as female caracal in cages baited with the urine of the animal. It is claimed that male as well as female urine can be used to bait the traps. As farmers first have to catch a caracal to obtain a sample of urine, a considerable number of lambs could be lost by the time urine could be collected from a trapped caracal to start a caracal-catching programme during the current lambing season. It would therefore be preferable to identify the active attractant(s) present in the animal's urine and to formulate a synthetic attractant, which emulates the animal's urine to such an extent that it would attract the target animals.

This aim of this research is the comprehensive chemical characterisation of the constituents of caracal urine and the eventual formulation of synthetic mixtures that could be tested for their efficacy in the envisaged semiochemical control of the caracal. If successful, this project could

lead to further investigations into other aspects of the semiochemical communication of this interesting animal.

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CHAPTER 2

EXPERIMENTAL

The experimental work reported in this thesis can be roughly subdivided into: (a) identification of VOCs extracted from the headspace gas of urine samples, (b) identification of urinary proteins and their putative ligands and (c) bioassays with synthetic analogues of the VOCs, including the ligands, to establish which of these compounds would elicit behavioural responses in caracal. The instrumentation used for the identification of the VOCs is availacle in LECUS and is used exclusively by workers in this laboratory. The instrumentation used for the purification and separation of proteins is available in the Department of Biochemistry and was used under supervision of scientists with experience in the field of proteomics, while the mass spectrometric identification of the proteins was carried out in the Medical Faculty of the University by a specialist in this field.

2.1 General

All Pyrex (borosilicate) glassware was washed with distilled water, rinsed with acetone and dried at 120 °C in a ventilated oven prior to use. The Pyrex glass bottles, flasks and beakers intended for use in the sampling and handling of caracal urine were further heated to 500 °C in an annealing oven, immediately prior to use, to remove any possible remaining traces of organic material, and then cooled. All reagents and solvents were of analytical grade or equivalent and unless stated otherwise, were purchased from Sigma-Aldrich, Schnelldorf, Germany.

Syringes used for gas chromatographic sample injection were cleaned by flushing with dichloromethane (DCM) (residue analysis or Pestanal grade). The plungers and needles were rinsed with the same solvent. Forceps and other equipment used for sample handling were also rinsed with DCM and dried prior to use. DCM and *tert*-butyl methyl ether (TBME) (Merck, Darmstadt, Germany) were used for the extraction of volatile organic compounds (VOCs) from some of the urine samples.

2.2 Sample collection

Sheep farmers from various parts of the Western Cape Province, the Northern Cape Province and the Karoo donated samples of caracal urine for this research. Practically all the urine used in this project was donated by farmers who had caught caracal in traps baited with caracal urine. Clean and sterilised glass bottles with polytetrafluoroethylene-faced septa [PTFE (Teflon)-faced septa] were supplied to farmers for sample collection. Having caught a caracal, the farmer would inform the LECUS directly or via the nature conservation organisation, CapeNature.

The following procedure was used to collect the urine of a trapped caracal. The farm owner or an official of CapeNature shot the animal with a small-calibre hand weapon. The animal's bladder was removed and the urine it contained was allowed to drain into a clean and sterile sample bottle. The sample was cooled on ice and transported to LECUS where it was stored at –23 °C until analysed or otherwise appropriately processed. The urine was stored in glass bottles with screw caps and septa. Plastic bottles and rubber septa without a PTFE protective layer were not used. Contamination of the urine with antioxidants and plasticisers present in rubber or plastic and extraction of volatile organic contaminants from the urine by the rubber seals in the caps of some commercially available bottles was thus avoided (Burger, 2005: 233). Some farmers preferred removing the bladder, tying a knot in the urethra to prevent the urine from draining out of the bladder, and freezing the bladder in a glass bottle until it could be transported to the laboratory.

Several samples of male and female urine were kindly donated by Ms Marine Drouilly, manager of a large-scale wildlife population survey that is being carried out by the Centre for Social Science Research (CSSR). (This survey covers an area of about 80 000 hectares between Laingsburg and Prince Albert, to the north of the Langeberg mountain range.) Information on the sources of some of the urine samples that were used in this study is given in Table 2.1.

Table 2.1: Urine samples of caracal collected for analysis

Location	Gender, Volume (ml)	Location	Gender, Volume (ml)
Porterville	Female, 9[a]	Prince Albert	Female, 5[a]
Porterville	Male, 95[a]	Zoutpan	Male, 1[c]
Aurora	Male, 10[a]	Wellington	Male, 31[a]
Fraserburg	Male, 45[a]	Wellington	Male, 84[a]
Porterville	Female, 9[c]	Porterville	Female, 28[a]
Redelinghuys	Male, 10[a]	Prince Albert	Female, 20[a]
Redelinghuys	Female, 10[a]	Prince Albert	Male, 400[a]
Laingsburg	Female, 60[a]	Prince Albert	Male, 75[a]
Laingsburg	Female, 5[a]	Prince Albert	Female, 5[a]
Laingsburg	Male, 50[a]	Prince Albert	Male, 6[a]
Laingsburg	Male, 10[a]	Heidelberg	Male, 1[a]
Laingsburg	Male, 5[a]		

[a]Collected in glass bottle; [b]combined samples; [c]bladder transported in a plastic bottle.

2.3 Sampling

Of the methods available for the enrichment of VOCs from the headspace gas of analytical samples, solid-phase microextraction (SPME) and sample enrichment probes (SEPs) were compared with respect to sensitivity, reproducibility and convenience. Stir bar sorptive enrichment (SBSE) (Baltussen *et al*., 1999) was not included in the comparison because essential ancillary equipment, *viz*., a desorption-cryotrapping unit, was not available for use in this project.

SPME sampling was carried out according to the general SPME sampling and analytical procedures as described by Arthur and Pawlizsyn (1990). However, instead of covering sample bottles with aluminium foil, sampling bottles with a gas-tight closure (see Fig. 2.1) were used to prevent VOCs from escaping from the bottles if, for example, protracted sampling at room temperature was required.

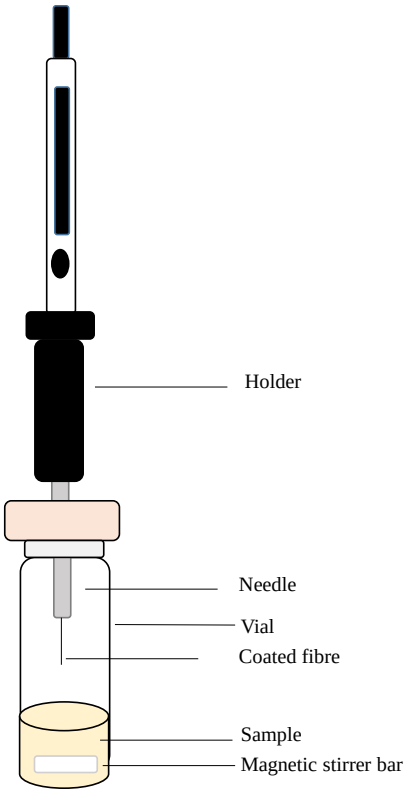

Fig. 2.1: Schematic diagram of SPME device with fibre exposed to the headspace of the sample.

There is a range of SPME fibre coatings available for the enrichment of VOCs from various types of analytical samples. For extraction, the efficiency of polydimethylsiloxane (PDMS) and a mixture of divinyl benzene (DVB) with PDMS (PDMS/DVB) were compared. The PDMS fibre with a film thickness of 100 μm was selected as the most appropriate for analysis of the extraction of non-polar urine VOCs. The fibre was conditioned at 250 °C for 30 min. The PDMS/DVB fibre with a film thickness of 65 μm was tested for the extraction of the more polar components (Prosen and Kralj, 1999; Kataoka *et al*., 2000). The fibres were conditioned at 250 °C for 30 min.

In the enrichment experiments, the headspace volatiles of a sample of urine (15 ml) in a bottle (50 ml) were sampled by SPME for up to 24 h at 23 °C on a conventional laboratory magnetic stirrer (Heidolph, MR 3001K, Germany). Due to the thin sorptive PDMS fibres used in this technique, equilibrium was achieved quite rapidly and relatively short enrichment times were required. The enriched VOCs were desorbed at 230 °C in the injector of a Finnigan MD 800 GC-MS instrument (Finnigan, Palo Alto, USA). The fibre was removed from the injector 10 min after commencement of the gas chromatographic-mass spectrometric (GC-MS) analysis.

Due to the small volume of sorptive material that is used in this technique, the advantage of the short enrichment time that is required by SPME has to be weighed up against the low sensitivity of the technique (Lloyd *et al*., 1998; Lord and Pawliszyn, 2000; Wilkowska and Biziuk, 2011). It was mainly for this reason that SPME was not used in this project.

2.3.1 Sampling by sample enrichment probe (SEP)

A second generation SEP50 (Burger *et al*., 2011) was used to extract (enrich) the volatile organic material from the headspace of urine samples for gas chromatography (GC) and GC-MS analyses. A fresh sample of clear (no turbidity) caracal urine (15 ml) was placed in a 50-ml glass bottle with a cap that had a stainless steel insert with the same thread as that of the injector cap of the GC (Burger *et al.*, 2006). The urine sample was stirred at room temperature (23 °C) on a magnetic stirrer. The sample was thermally isolated from the stirrer; a sheet of polystyrene foam (2 cm) was used to prevent heat transfer from the stirrer motor to the contents of the sample bottle. Sampling at a low temperature (< 50 °C) produced better results than sampling at a higher temperature. At temperatures above room temperature, water droplets condense on the PDMS sleeve of the SEP and the slow diffusion of VOCs through the condensed water impedes the enrichment of VOCs in the PDMS. A SEP50 (130 × 0.70 mm) stalk carrying a 50-mm sleeve of PDMS tubing (0.64 mm i.d. × 1.19 mm o.d. and weighing 47 mg) near its lower tip was thus exposed to the headspace gas of the urine for 24 h to sample the VOCs present in the urine. The volatile compounds were then desorbed at 230 °C in the injector of the GC-MS instrument and the SEP was left in the injector until completion of the analysis (Burger *et al.*, 2006). A key advantage of using a bottle cap with the same thread as the injector of the GC is that removing the cap-SEP assembly from the sample bottle and installing it in the injector does not take more than a few seconds; hence, the loss of sample, even of very volatile analytes, is negligible when using this procedure. The SEP50 with its accessories installed in a sample bottle containing caracal urine is shown schematically in Fig. 2.2.

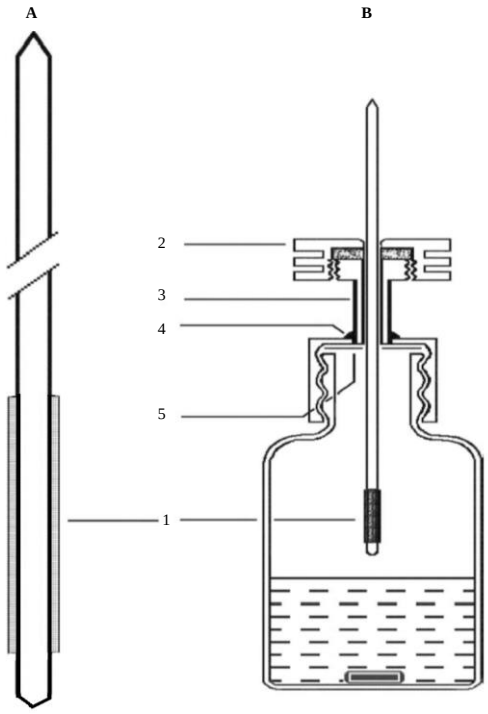

Fig. 2.2: (A) Schematic diagram of sample enrichment probe (SEP) made from inert material with (1) a sleeve of PDMS rubber. (B) sample bottle with a SEP installed for enrichment of headspace volatiles from an aqueous solution; (2) standard septum cap of the GC with its central hole enlarged to 2.4 mm; (3) stainless steel insert in the phenolic cap of a standard reagent bottle; (4) epoxy glue applied to the outside surface of the cap around the insert; (5) Teflon gasket.

A SEP and sampling accessories installed in a bottle containing the caracal urine and a SEP installied in the injrctor of the GC-MS is depicted in Fig. 2.3.

Fig. 2.3: SEP (with PDMS-sleeved stalk) installed in a sample bottle containing caracal urine and in a GC injector at 230 °C.

2.3.2 Conventional sample extraction

Although headspace analysis produced excellent results in VOC analyses of the urine, solvent extraction was used as sample preparation technique in the analyses of high-boiling steroids expected to be present in the urine. DCM (1 ml) was added to a sample of urine (5 ml) in a glass centrifuge tube. The tube was then closed with its screw cap and a Teflon-faced silicone rubber septum, and vigorously shaken for 2 min. Phase separation was facilitated by centrifuging the mixture for 45 min at 2000 rpm. The bottom DCM layer was removed from the centrifuge tube with a syringe and needle[1] and transferred to a 5-ml Reacti-Vial. The Reacti-Vial with its contents and then transferred to a clean vial. In difficult cases, the water droplets can be frozen, after which the DCM extract can be decanted from the frozen droplets adhering to the glass surface. If too dilute, the extract was concentrated for analysis in an atmosphere of pure N_2 in the following manner (Reiter *et al*., 2003). The vial containing the extract was placed in a beaker that was small

[1] Due to its surface tension and capillary forces, some of the supernatant aqueous layer enters the needle as soon as the needle comes into contact with the supernatant water layer. A thin film or a trail of the aqueous layer tends to adhere to the outside surface of the needle as it moves through the bottom DCM layer. In rare instances, this trail conducts some of the upper aqueous layer down into the DCM layer and into the needle and syringe. This can mostly be avoided by drawing a small volume of fresh DCM into the needle before it is brought into contact with the aqueous layer on its way into the DCM extract. When the needle tip has reached the bottom of the centrifuge tube, or, if applicable, the bottom of a vial, the DCM is expelled from the needle while keeping the needle tip in the DCM layer. This procedure also helps to disrupt the aqueous film on the needle. The DCM layer (extract) can then be drawn up into the syringe and removed from the supernatant aqueous layer. Even better results are obtained by using a blunt needle, i.e., a needle without a bevelled tip. If an emulsion is formed between the aqueous and DCM layers, it can be broken up by cooling the centrifuge vial with its contents to –25 °C and centrifuging the contents for about 30 min. Due to the changing densities of the DCM and the melting ice, complete separation of the aqueous layer and DCM extract can be achieved.

enough to prevent the vial from falling over. The top of the beaker was covered with aluminium foil and a thin glass capillary (or fused silica tube) was inserted through the aluminium foil into the beaker in such a way that it did not enter the vial. A slow stream of N_2 was blown into the beaker, without blowing it directly into the vial. Thus the N_2 simply served to provide an N_2 atmosphere and to remove the solvent vapours from the beaker. From time to time, the contents of the vial were swirled carefully to dissolve and return the less volatile extracted compounds accumulating on the wall of the vial into the residual extract. In the case of DCM, the evaporation of 1 ml of the solvent took about 6 h to yield a final volume of *ca*. 10 µl of the concentrated extract. Concentrated extracts were subjected to GC-MS analysis for the determination of steroids in the urine or for the analysis of VOCs by conventional GC-MS analysis using conventional liquid sample injection.

2.3.3 Quantitation of VOCs

With the exception of cyclopentadecanone, the macrocyclic ketones identified as ligands of the urinary proteins of the caracal are present in very low concentrations in the animals' urine. For comparison of the relative ratios in which these compounds are present in the urine of individual animals, the VOCs were extracted from urine samples, the extracts were concentrated and then analysed by GC-MS. The quantitative data obtained also served as guidelines for the formulation of mixtures of the synthetic VOCs that were later used in bioassays.

Two samples of urine (3 ml each), freshly removed from an animal's bladder, were transferred to 5-ml Reacti-Vials. One of the vials with its contents was heated at 95 °C for 15 min while the other vial was held at room temperature. After cooling the first vial with its contents to room temperature in cold water, DCM (1 ml) was added to the contents of both the vials. The vials were shaken for 2 min and then centrifuged at 2300 rpm for 30 min. If, after centrifugation, a sharp separation of the phases was not achieved, the vials were cooled to –25 °C and centrifugation was resumed for a further 20 min. This treatment invariably resulted in a sharp separation of the phases. The DCM layer was removed from the vials using a 1-ml syringe with a snub-nosed needle and transferred to a clean 4-ml vial. The DCM extract was concentrated in a N_2 atmosphere (Reiter *et al*., 2003), transferred to a small Reacti-Vial, and concentrated to a final volume of 20 µl. Aliquots of 1 µl were analysed by GC-MS. Comparison of shapes of the peaks of the macrocyclic ketones in the TICs and in single-ion chromatograms constructed for the peaks

at *m/z* 58, 96, and 98 was used to help detect the presence of overlapping background peaks. Except for two analyses, no sign of the presence of background peaks overlapping with those of macrocyclic ketones was detected in any of these analyses, in which these ions were integrated and the results compared with those of the corresponding synthetic ketones to get an estimated integration value for the natural compound.

2.4 Analytical instrumentation

The identification of compounds that could possibly be involved in intraspecific communication in the caracal essentially involved the isolation and/or separation of the VOCs present in the urine, followed by identification of the compounds. The following combinations of different separation techniques and methods of chemical identification, so-called hyphenated analytical techniques, were employed for the identification of the urinary VOCs of the caracal: gas chromatography with flame ionisation detection (GC-FID) (in which the FID is only of limited value for identification); gas chromatography in conjunction with low-resolution mass spectrometry (GC-LRMS); gas chromatography in conjunction with high-resolution mass spectrometry (GC-HRMS) or in conjunction with a time-of-flight mass spectrometer (GC-TOFMS); ultra-performance liquid chromatography with tandem mass spectrometry detection (UPLC-MS/MS); ultra-performance liquid chromatography with electrospray ionisation mass spectrometric detection (UPLC-ESMS) and nanoelectrospray ionisation (Nano-ESI-Orbitrap analysis).

2.4.1 GC-FID

GC-FID analyses were carried out on a Carlo Erba HRGC 5300 instrument (Carlo Erba, Milan, Italy) using one of the following 30-m columns: Zebron ZB-5MS (5% phenyldimethylpoly-siloxane), Zebron ZB-1701 or Zebron ZB-Wax (Phenomenex, Torrance, USA). H_2 was used as carrier gas at a linear flow velocity of 50 cm/s. The injector and FID of the instrument were operated at 220 °C and 280 °C, respectively. GC data were acquired on a DELTA Chromatography Data System, Version 5.0 (Digital Solutions, Brisbane, Australia). All GC analyses (including the GC stage of GC-MS analyses) were carried out following a standard procedure. The oven was left open with the oven fan running (this requires arresting the oven door microswitch in the 'on' position) until a temperature as close as possible to room temperature was reached. With the oven door still open, the analytical sample was injected (or the SEP installed in

the injector), the door immediately closed and the oven immediately ballistically heated to 40 °C, at which temperature the temperature program and data acquisition were started simultaneously[2]. Only two temperature programs were used: 2 °C/min for analyses requiring high separating efficiencies, and 4 °C/min for analyses of starting materials and synthetic products.

Retention time comparison of the urinary VOCs with synthetic reference standards was carried out using this GC-FID instrument. Mixtures of reference standards were prepared in relative concentrations emulating the concentrations present in caracal urine. Conventional liquid injection was employed as sample introduction technique. Retention indices were determined to substantiate the identification of the VOCs.

GC-FID was also used for retention time comparison of the urinary VOCs with their synthetic analogues using the SEP technique described above. After the VOCs had been enriched, 1 or 2 µl of an appropriately diluted mixture of the synthetic references were carefully streaked on the PDMS sleeve of the SEP, after which the SEP was introduced into the injector of the instrument.

2.4.2 GC-LRMS

GC-LRMS analyses were carried out on a Fisons GC 8000 series GC coupled to a Fisons MD800 quadrupole mass spectrometer (Fisons Instruments, Milan, Italy) employing the columns specified above. He was used as carrier gas at a linear velocity of 28.6 cm/s, measured at a column oven temperature of 40 °C. The columns were temperature programmed at a rate of 2 °C/min from 40 to 280 °C (to 260 °C in the case of the wax column) and held isothermally at the final temperature for 20 min. The injector of the GC 8000 was operated at 230 °C and the GC-MS interface was held at 250 °C. The temperature of the ion source of the MS temperature was set at 180 °C and the pressure in the source housing was ca. 2×10^{-5} mm Torr at a column temperature of 40 °C, decreasing to ca. 1×10^{-5} mm Torr towards the end of the temperature program. Electron impact (EI) mass spectral data were acquired at 70 eV scanning from *m/z* 25 to 500. A scan rate of 0.9 s/scan and an interscan time of 0.1 s were used. After completion of the analyses, the SEP was left in the injector until the oven had cooled down and could be used for the next analysis. This GC-LRMS instrument was also used for the determination of the purity of commercially available

[2]For every 15 °C the oven is heated ballistically, the peak width of the early-eluting peaks is halved. The effect of small variations in *manual* injection rhythm on the retention time of analytes is minimised using this procedure. Retention time differences of < 2 s were routinely obtained.

reference standards, reference standards available in-house and the starting materials for the synthesis of three macrocyclic ketones, as well as for retention time comparison (co-injection) of the natural VOCs and synthetic reference compounds as described in the previous paragraph.

2.4.3 GC-TOFMS

Additional diagnostic high-resolution mass spectrometric information on the urinary VOCs was obtained by GC-TOFMS. A GC 8000 series gas chromatograph (Fisons Instruments, Milan, Italy) coupled to a GCT Premier Micromass MS Technologies high-resolution time-of-flight mass spectrometer (Waters, Milford, USA), and furnished with one of the columns specified above, was available for these analyses. He was used as carrier gas at 1.0 ml/min. The inlet and interface temperatures were 230 and 250 °C, respectively. Headspace sampling with a SEP50 for 24 h at 22 °C was employed as sample enrichment technique. Samples were introduced in the split mode (10:1) at a column temperature below 30 °C, after which the temperature was raised ballistically to 40 °C, as explained above. The column was temperature programmed at 2 °C/min from 40 to 280 °C (held for 20 min). EI data were acquired at 70 eV scanning from *m/z* 35 to 650 at a scan rate of 0.18 s/scan with an interscan delay of 0.02 s. The ion source was operated at 180 °C. Perfluoro-tri-*n*-butylamine was used as mass reference.

2.4.4 UPLC-MS/MS

UPLC-MS/MS identification of the urinary steroids was carried out on a UPLC ACQUITY UPLC instrument (Waters, Milford, USA) using a Phenomenex UPLC Kinetex PFP column (100 mm × 2.1 mm, 2.6 μm) coupled to an API Xevo triple quadrupole mass spectrometer (Waters, Milford, USA). The urinary steroids extracted from caracal urine were analysed by multiple reaction monitoring (MRM) using the electrospray probe in the positive ionisation mode (ESI^+) according to the protocol of Storbeck *et al*. (2013). The retention times were compared with those of authentic standards that were purchased from various suppliers (Sigma-Aldrich, St. Louis, USA; Wilton, USA; Andover, USA). The following instrumental parameters were used: capillary voltage 3.5 kV, cone voltage 15–35 V, collision energy 4–32 eV, source temperature 120 °C, desolvation temperature 400 °C, desolvation gas 900 l/h and cone gas 50 l/h. The mobile phases were 1% formic acid (solvent A) and acetonitrile/methanol/isopropanol (49:49:2) (solvent B). The steroids were eluted at a flow rate of 0.4 ml/min, using a linear gradient from 15% solvent B to

38.5% solvent B in 3.5 min, followed by a linear gradient to 100% B in 0.1 min. The total run time was 5 min and the injection volume was 5 µl.

2.4.5 UPLC-ESMS

An UPLC instrument (ACQUITY UPLC) fitted with a C18 column (Waters HSS C18, 2.1 mm × 150 mm, 1.7 µm) and coupled to a Synapt G2 quadrupole TOF mass spectrometer (Waters, Milford, USA) was used for exploratory analyses of any polar constituents, such as proteins, that were expected to be present in caracal urine. Positive electrospray ionisation with a capillary voltage of 3 kV and cone voltage of 15 V was employed. Leucine-enkephalin was used as lock mass reference for accurate mass determination. MassLynx 4.1 software program was used for data acquiring and processing.

2.4.6 Dionex nano-rapid separation liquid chromatography

Liquid chromatography was performed on a Thermo Scientific Ultimate 3000 RSLC (Thermo Scientific, Waltham, USA) equipped with a C18 trap column (2 cm × 100 µm) and an in-house manufactured C18 column (35 cm × 75 µm) (Luna C18, 5µm) (Phenomenex, Torrance, USA) analytical column. More detailed information on analysed carried out on this instrument is given in Chapter 4.

2.4.7 Orbitrap mass spectrometric analysis

Orbitrap mass spectrometric characterisation of the protein(s) that were presumed to have cyclopentadecanone as a ligand is discussed in Chapter 4.

2.5 Synthesis of macrocyclic ketones

Cyclohexadecanone **3.102** was synthesised according to Prelog *et al*. (1947). A 3-neck round-bottomed flask was fitted with an electrically driven Hershberg stirrer, a distillation head, and Liebig condenser. A slow flow of purified N_2 was introduced into the flask through the third neck. Xylene (xylene isomer mixture) (350 ml) was poured into the flask, then 50 ml of this solvent was distilled off to remove any traces of moisture present in the solvent. The distillate was collected in two fractions (see below). The flask with its contents was allowed to cool to 110 °C. The distillation head was replaced by a reflux condenser. Sodium (1.5 g, 0.065 mol) was introduced

into the flask, where it melted and was converted into relatively small globules by the rapidly rotating stirrer. Diethyl hexadecanedioate (5.0 g, 0.015 mol) was diluted to 20 ml with xylene taken from the second (dry) fraction mentioned above. This solution was introduced into the rapidly stirred sodium suspension from a 20-ml syringe mounted in an infusion pump (Model SE 200B, VIAL Médical, St Etienne de St Geoirs, France) at a rate of 5 ml/h via a needle that was long enough to deliver the ester into the stirred reaction mixture below its surface. It was only after about 2 ml of the ester solution had been added that the sodium particles suddenly broke up and sodium sand was formed. Presumably, this phenomenon could be ascribed to the mechanism of the reaction proposed by Prelog *et al*. (1947), according to which the reaction proceeds via the attachment of the electrophilic ester groups to the electron-dense surfaces of the molten sodium particles. We presumed that as soon as the formation of a relatively apolar layer on the surface of the sodium particles reached a certain stage, the molten sodium particles could no longer coagulate, and sodium sand was formed. After completion of the addition of the hexadecanedioate solution, the reaction mixture was stirred for a further 1 h at 110 °C. The sodium excess was 'dissolved' by the addition of methanol (10 ml), after which the reaction mixture was acidified with dilute H_2SO_4 (1.5 M). The supernatant xylene solution was washed with water, dilute $NaHCO_3$ solution, and again with water, then dried over anhydrous Na_2SO_4. The drying agent was filtered off and the filtrate was concentrated under reduced pressure to yield 2-hydroxycyclohexadecanone (2.7 g).

The crude 2-hydroxycyclohexadecanone was dissolved in glacial acetic acid (7 g). This solution was added to zinc wool (7 g) in a 150-ml Erlenmeyer flask fitted with a reflux condenser. The flask with its contents was heated in an oil bath at 100 °C and concentrated HCl (7 ml) was added to the contents of the flask. After 30 min, the same volume of concentrated HCl was added to the reaction mixture; this addition was repeated twice at 30 min intervals. After the reaction mixture had cooled, *t*-butyl methyl ether was added to the contents of the flask. The unreacted zinc was filtered off and thoroughly washed with this solvent. The combined filtrate and washings were washed with water, dilute $NaHCO_3$, again with water, and then dried over anhydrous Na_2SO_4. The drying agent was filtered off. Removal of the solvent from the filtrate under reduced pressure yielded cyclohexadecanone (2.4 g, 69% overall). ^{13}C NMR (600 MHz, $CDCl_3$): δ (ppm) = 212.66 (1-CO), 42.13, 27.74, 27.29, 27.07, 26.84, 26.63, 26.58, 23.54. Cyclotridecanone and cyclotetradecanone were synthesised in a similar manner and had similar ^{13}C NMR spectra.

The macrocyclic ketones were recrystallised from methanol. Their mass spectra were identical to those of the natural compounds. The GC-MS analyses revealed the presence of many trace impurities, such as cyclopentadecanol, cyclopentadecene and various long-chain ester derivatives. The target products could be further purified by converting them into the corresponding semicarbazones, which can be recrystallized from methanol, after which the ketones can be recovered from the semicarbazones by steam distillation in the presence of phthalic anhydride. Due to time constraints, however, these purification steps were not carried out and these compounds were used as such in the biotests carried out at Giraffe House. Cyclopentadecanone is commercially available and was procured from Sigma-Aldrich.

2.6 Bioassays

The evaluation of the behavioural responses of the caracal towards the available synthetic urinary VOCs was carried out at Giraffe House Wildlife Awareness Centre near Stellenbosch. The Giraffe House management allowed us to carry out tests during the early morning hours from 07:00 to 09:00 and during the afternoon from 16:00 until the centre closed about an hour later, when the animals are fed and no visitors are around. Unfortunately, at present, the centre has only two male caracal that share the same enclosure. Their enclosure is neighboured by similar enclosures holding a large number of small carnivores and other animals, some of which are typical caracal prey species. Hence it was doubtful from the start whether any credible results, or even any results at all, could be expected.

The VOCs, singly or as mixtures of compounds belonging to the compound classes as grouped in Table 3.2, were dissolved in 10 ml pentane, or in DCM (if a more polar solvent had to be used). Squares (15 cm × 15 cm) of cotton fleece cloth were treated with these solutions. On average, the resulting lures contained *ca*. 1 mg of each of the compounds. The wet cloth squares were left outside overnight to allow complete evaporation of the solvent and the lures were then stored in clean glass jars until they were used in the experiments at Giraffe House. Clearly, the evaporation of the solvent from the cloth squares resulted in a significant change in the quantitative composition of the VOCs left on the cloth squares.

The caracal enclosure (14 m × 12 m) faces an open area (*ca*. 100 m × 50 m) from where visitors can watch the caracal and other animals in their enclosures. A family of black-backed jackal (*Canis mesomelas*), and a family of Cape foxes (*Vulpes chama*) are the next-door

neighbours of the caracal. There are also other animals on the other side of an alley at the back of their pen. The caracal enclosure is normally overgrown with tall grasses and small bushes, but the grasses had been cut to facilitate the observation of the caracals' behaviour. Inside the fence separating the pen from the open space, the ground has been compacted by the caracal patrolling that side of their enclosure for hours on end in a way that could be described as 'oscillating' between the two front corner posts.

The first tests were carried out by pinning a square of cotton fleece cloth (5 cm × 5 cm), treated with either male or female urine, or a solution of VOCs, to one of the two kennels that the caracal use as shelter near the middle of their pen. These lures, including fleece cloth squares treated with the urine of male or female caracal, elicited absolutely no response, regardless of where the lures were placed. Similar lures were suspended from a thin black string at the entrances to the kennels in such a way that the animals could not enter a kennel without coming into contact with a lure. It is possible that the caracal did not respond to these lures because, suspended in the air, they could be considered out of context.

Squirting female urine (20 ml) onto the compacted path did elicit a few minutes of hectic patrolling. The next series of tests were carried out by placing two squares treated with different VOC mixtures on the compacted path near the two corner posts. These tests were also unsuccessful, except for one in which the dominant male oscillated longer near the lure treated with female urine than that treated with the mixture of ketones. The results of the tests that were carried out during daytime were not reproducible and practically useless.

Fortunately, one week before the final target date for handing in this thesis, a Rhinocam day/night camera (RIK Rhino Surveillance, Somerset West, South Africa) was made available for this research. This camera made it possible to produce videos of the reactions of the two caracal to various lures during the night. Two cotton fleece cloth squares (15 cm × 15 cm), treated as before with urine and synthetic VOC mixtures were place about 2 m apart in the middle of the animals' enclosure at about 18:00 and were left there until 08:00 the next morning. The following tests were carried out:

1. Ketones (0.5 mg each) *vs.* female urine.

   The ketones dissolved in DCM (2.5 ml) were spread as uniformly onto a square (15 cm × 15 cm) of cotton fleece cloth using a Pasteur pipette. The solvent was allowed to evaporate

at 22 °C. Female urine (2.5 ml) was applied in the same manner to a similar cloth square. The lures were transported to Giraffe House in glass containers.

2. Cyclopentadecanone (0.3 mg in 2.5 ml DCM) *vs.* female urine (2.5 ml).

3. Mixtures of two different groups of the miscellaneous compounds listed in Table 3.1 *vs.* each other (2 mg each in 2.5 ml DCM).

4. Synthetic C_{13}–C_{16} ketones. Using cyclopenadecanone as external standard and the data in Tables 5.1 and 5.2, it was possible to determine the average concentration of cyclopentadecanone per millilitre of caracal urine. The exceptionally high values determined in the analyses of samples UE1 and UE3 were excluded from the calculations, which gave an average concentration of *ca.* 1 mg/ml urine. A macrocyclic ketone solution containing cyclotridecanone (1.5 mg), cyclotetradecanone (7.7 mg), cyclopentadecanone (16.5 mg), cyclohexadecanone (4.8 mg) in DCM was prepared and diluted to obtain test solutions with concentrations of these ketones that, intentionally, did not correspond to those of any urine samples analysed. These test solutions were made up in DCM (2.5 ml) and were applied to a cloth square as before.

5. Synthetic C_{13}–C_{16} ketones (as in experiment 4) in admixture with squalene (69 mg in 2.5 ml DCM) *vs.* male urine (2.5 ml) from another source.

The videos, which contained excellent information, were downloaded and analysed in the laboratory.

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CHAPTER 3

CHEMICAL CHARACTERISATION OF THE URINARY VOCS OF THE CARACAL

3.1 Introduction

In previous projects carried out by LECUS on the chemical characterisation of the urine of the cheetah (*Acinonyx jubatus*) and the Bengal tiger (*Panthera tigris*) (Burger *et al*., 2006 and 2008, respectively), the best results with regard to sensitivity and reproducibility were obtained by headspace analysis using a SEP (Burger *et al*., 2011), followed by GC-MS analysis of the trapped (enriched) organic material. In the present study, GC-MS analyses were first carried out on a GC-LRMS instrument, after which a limited number of urine samples were also analysed on a GC-HRMS instrument and other MS instruments for the identification of specific compounds or compound classes.

The GC-MS analyses of the VOCs present in the headspace gas of caracal urine were carried out on capillary columns with different polarities, which made it possible to select total ion chromatograms (TICs) in which different compound classes were optimally separated for the interpretation of MS data. Relatively 'clean' mass spectra could therefore be obtained, even of constituents that were present in very low concentrations. Columns coated with the following phases were used: Zebron ZB-5MS (5% phenyldimethylpolysiloxane) (apolar), ZB-1701 (medium polar), and ZB-Wax (polar).

Examples of the TICs obtained in analyses of male and female caracal urine samples from geologically different areas on some of these columns installed in various instruments are depicted in Figs. 3.1–3.5.

3.2 Structural elucidation of caracal urinary VOCs

The following rationale was followed in the identification of the urinary VOCs. Prominent features in the mass spectra of the organic constituents present in the urine were interpreted against the background of other information, such as the comparison of the constituents' retention indices (RIs), mass spectral information published in monographs on the interpretation of the mass spectra of organic compounds, and mass spectra available in reference libraries (NBS, 1990; Adams, 2004; NIST, 2005), in order to decide which of the structures proposed by a library search could probably

be correct. If trace constituents had to be identified, the mass spectra of the unknowns were generally quite weak and structures were often put forward in library searches with correlation factors lower than 70%, which, in our experience was mostly of very little diagnostic value.

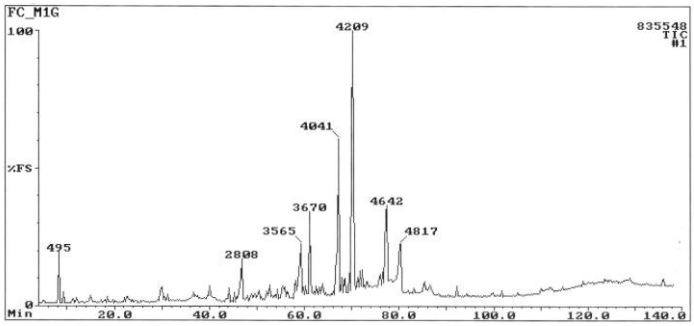


Fig. 3.1: TIC of VOCs enriched for 15 h from the headspace gas of male caracal urine at 23 °C using a SEP50. VOCs were analysed on a GC-LRMS instrument (Fisons MD 800) fitted with a ZB-5MS column (5% phenyldimethylpolysiloxane), temperature programmed from 40 to 280 °C at 2 °C/min.

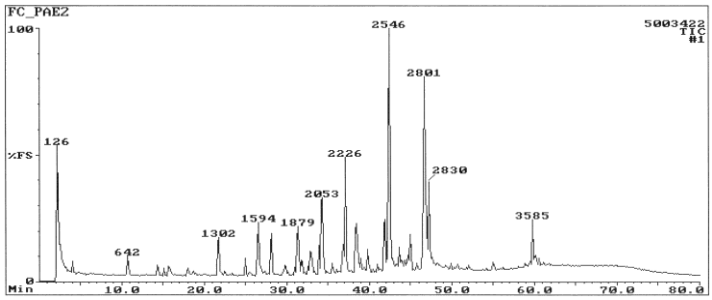


Fig. 3.2: TIC of VOCs extracted from male caracal urine with TBME and analysed on a GC-LRMS (Fisons MD 800) fitted with a ZB-5MS column, programmed from 40 to 280 °C at 2 °C/min.

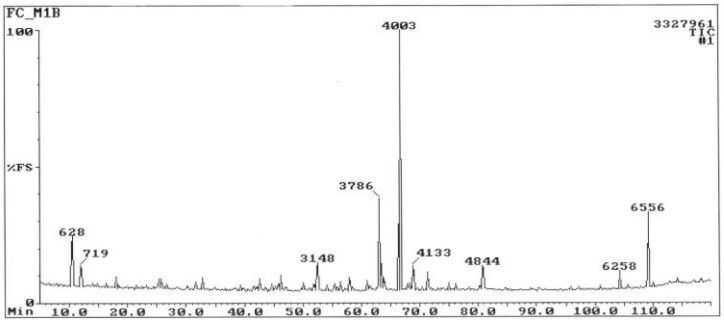


Fig. 3.3: TIC of VOCs enriched for 15 h from the headspace gas of male caracal urine at 23 °C using a SEP50. VOCs were analysed on a GC-LRMS (Fisons MD 800) fitted with a ZB-1701 column, programmed from 40 to 280 °C at 2 °C/min.

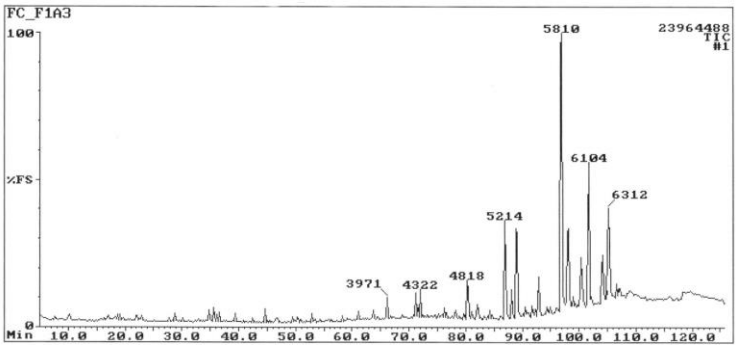


Fig. 3.4: TIC of VOCs enriched for 15 h from the headspace gas of female caracal urine at 23 °C using a SEP50. VOCs were analysed on a GC-LRMS (Fisons MD 800) fitted with a ZB-Wax column (100% polyethyleneglycol), programmed from 40 to 250 °C at 2 °C /min.

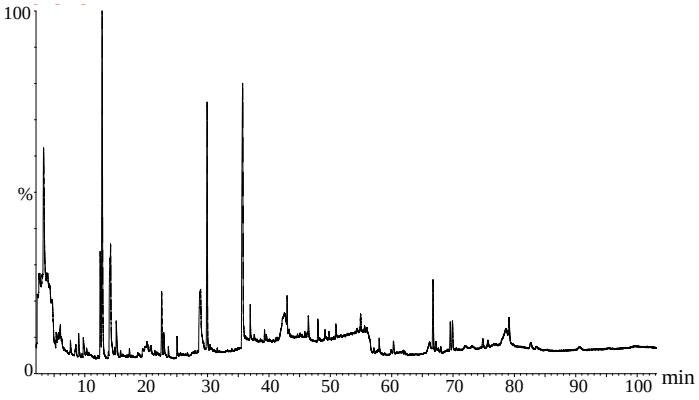


Fig. 3.5: TIC of VOCs enriched for 15 h from the headspace gas of female caracal urine at 23 °C using a SEP50. VOCs were analysed on a GC 8000 series gas chromatograph coupled to a GCT Premier Micromass MS Technologies GC-TOF-HRMS, on a ZB-5MS column, programmed from 40 to 280 °C at 2 °C /min. The broad fronting peaks at ca. 53 and 79 min are due to the overloading of the column with urea and sulphur (S_8), respectively.

Even results with correlation factors of 80% and higher were considered with circumspection and were not necessarily accepted as correct. In such cases, HRMS data and the presence or absence of typical ions in the experimental and library spectra were of great diagnostic value.

The RIs of the VOCs under examination were determined according to Van den Dool and Kratz (1963) using linear temperature programming. The results were compared with published RI data (e.g., NIST, 2005). RI differences of less than four units between the experimental and published values were accepted as evidence that the compound was probably correctly identified.

By employing an HRMS instrument in addition to the LR instrument, MS data were obtained that facilitated the interpretation of complex mass spectra by providing elemental compositions of the molecular ion and other key ions. If an unsaturated long-chain compound has a mass spectrum in which the heteroatom(s) of the functional group are retained in most of the abundant ions then the ions containing the heteroatom(s) can be extracted from the HRMS data and mass spectra containing only these ions can be constructed to provide information on the location of methyl branching for example (Burger, 2005: 231-278). The GC-TOF-HRMS instrument that was available during the later stages of the present investigation proved to be more sensitive than the quadrupole instruments in one important aspect; it provided invaluable information regarding the presence and composition of molecular ions that could previously not be detected in the mass spectra of some of the minor constituents of the samples that were analysed by LRMS. The high data acquisition rate of the TOF instrument also enabled deconvolution of certain overlapping peaks in the TIC. This made high-quality mass spectral information available and it was thus possible to identify compounds that would otherwise have remained unidentified or only tentatively identified. In high-resolution analyses with this instrument, mass differences of 1 mDa or less between the observed mass and the mass calculated for a specific ion were considered acceptable.

In this chapter, the term "constituent" is used to refer to an unidentified component of the urine. Once the constituent is tentatively identified or its compound class is established, it is referred to as a "compound". Finally, as far as practically possible, the identification of constituents was confirmed by GC-MS retention time comparison (co-injection) with either authentic, commercially available synthetic compounds, compounds that were available from previous research projects, or compounds that were synthesised during the course of the present investigation. The availability of a large library of synthetic reference compounds thus made it possible to substantiate the identification of about 86% of the VOCs beyond any doubt. The reference compounds were made up in DCM, either individually or as mixtures containing the reference compounds in relative concentrations corresponding to those of the compounds identified in the caracal urine samples. The identification of compounds was considered tentative or the compounds were classified as unidentified if the identification was based only on MS data.

As the data on which the identification of the urinary VOCs is based were obtained from GC-MS analyses carried out on GC columns with different polarities, and because some of the

compounds were only observed on one of the columns, it is impossible to list all of them in the order in which they were eluted from one specific column. They are therefore classified into different compound classes and subclasses according to their mass spectrometric properties, although this might not always be convenient from the viewpoint of the reader.

The mass spectra of representative examples of the VOCs present in caracal urine are given at the end of the chapter to avoid excessive fragmentation of the chapter. These mass spectra obtained from GC-LRMS and GC-HRMS are in their raw form, i.e. they were specifically not 'cleaned up'. The discussion of the mass spectra of a specific compound class usually commences with a typical example from a homologous series, if present in the urine, or with a comparison of the mass spectra of compounds with similar structures. Typically, such a discussion concludes with a statement that the constituent was tentatively identified. A final statement concerning the contribution of the calculation of the RIs of the urinary constituents and other diagnostic techniques to the unequivocal identification of constituents was, however, not repeated at the end of every discussion. More information is given in interesting cases and if a constituent was only tentatively identified.

A comparison of RIs of the urinary constituents with RIs from the literature, the availability of HR mass spectra, and information on retention time comparison (co-injection) of the natural compounds with authentic synthetic reference compounds can be found in Table 3.1, where the identified compounds are listed. The table is numbered 3.1 although it appears at the end of the chapter. Due to limited space, it was not possible to list the constituents in Table 3.1 under their systematic names; only the trivial names are used.

To a certain extent, the present research is a preliminary or exploratory investigation. The absolute configurations of the large number of chiral VOCs were not determined. However, if at a later stage bioassays show that some of the VOCs play a key role in the intraspecific communication of the caracal, the necessary GC analyses will be carried out on a wide range of available enantioselective columns and the natural enantiomers will be synthesised for field tests.

3.2.1 Aliphatic hydrocarbons

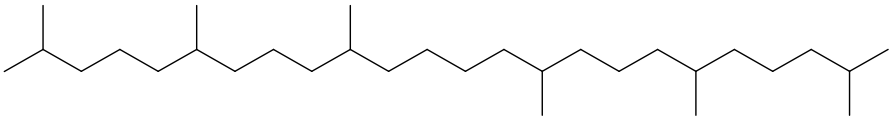
3.2.1.1 Alkanes

Conventional EI ionisation at 70 eV of unbranched hydrocarbons produces the characteristic mass spectra of these compounds (Budzikiewicz *et al.*, 1967: 50; Pavia *et al.*, 2001: 405–408). The

complete homologous series of unbranched alkanes from C_8 to C_{28} (constituents **3.1–3.21** in Table 3.1) was identified in caracal urine. The mass spectra of these compounds are all characterised by relatively abundant molecular ions and a regular series of fragment ion peaks separated by 14 atomic mass units (amu). These ion clusters occur with gradually decreasing abundance, and with the most abundant ion of each cluster corresponding to the general formula (C_nH_{2n+1}), i.e., at *m/z* 43, 57, 71, 85, …. (Millard and Shaw, 1966; McCarthy *et al*., 1968; Pavia *et al*., 2001: 405–408).

The mass spectrum of constituent **3.8** (Fig. 3.6), for example, exhibits this typical fragmentation pattern. The ion at *m/z* 212, which according to HRMS has the composition $C_{15}H_{32}$, can be accepted as the constituent's molecular ion. Its RI in the NIST compendium is 1500 and, as it is a reference standard for the determination of RIs, its experimentally determined RI is 1500. It co-eluted with an authentic synthetic sample of pentadecane. The other *n*-alkanes present in caracal urine were identified in a similar manner.

The molecular ion of constituent **3.22** is not detectable in its LR spectrum (Fig. 3.7), probably due to the presence of extensive branching in the molecule. However, a computerised library search suggested 2,6,10,15,19,23-hexamethyltetracosane (squalane) as a likely candidate structure with a correlation of 90%. The suggested structure was confirmed by co-elution of the natural material with a commercially available sample of squalane.



Squalane

It is interesting to note that, although squalane has such a low vapour pressure that it was used as a stationary phase in the early years of GC, it could nevertheless be detected in a SEP analysis of a dialysed sample of the urinary proteins of the caracal.

3.2.1.2 Unbranched alkenes

Alkenes mostly have distinct molecular ions because the double bonds of alkenes are capable of absorbing considerable energy (Pavia *et al*., 2001: 410). Hydrogen rearrangements are mostly not observed in saturated hydrocarbons (Millard and Shaw, 1966; Budzikiewicz *et al.*, 1967: 90), but they are prevalent in alkenes, resulting in the migration of radical sites along the chain. The mass

spectra of isomeric alkenes are therefore very similar (Beynon, 1960: 325–345), except for those alkenes with a tetrasubstituted double bond (Spiteller, 1966: 88, 97–98). Methods other than MS must therefore be used to determine the position of double bonds in many compounds belonging to different chemical classes.

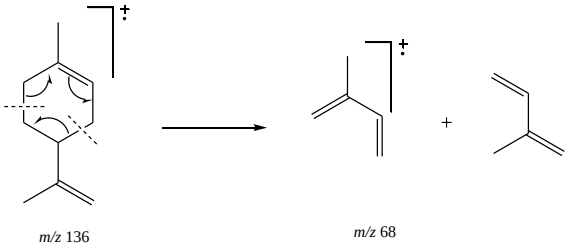
Due to the loss of a molecule of water, either thermally in a heated ion source or by fragmentation of the molecular ion, the mass spectra of certain aliphatic alcohols are often indistinguishable from those of the corresponding alkenes. This problem can be circumvented by RI determination and comparison with published data and/or by retention time comparison if an authentic reference sample is available.

The mass spectra of mono-olefins are dominated by ions having the general formula C_nH_{2n-1}, gradually declining in abundance with increasing mass. The molecular ion is more pronounced than in the spectra of the saturated analogues due to better stabilisation of the positive charge by removal of one of the π-electrons (Budzikiewicz *et al.*, 1967: 55; McLafferty, 1993: 230). The mass spectrum of constituent **3.25** (Fig. 3.8) is a typical example of the mass spectrum of a long-chain alkene. In addition to the ions of odd mass in this spectrum, it also contains $(C_nH_{2n})^+$ ions of even mass that are formed by the elimination of an olefin. This could be ascribed to the involvement of McLafferty rearrangements. However, there is evidence that more complex hydrogen migrations could be involved in the genesis of these ions of even mass (Budzikiewicz *et al.*, 1967: 57). Constituents **3.23–3.25** were identified as 1-undecene, 1-tetradecene and 1-pentadecene, respectively.

3.2.1.3 Branched and cyclic hydrocarbons

The mass spectra of constituents **3.26–3.28** contain relatively abundant ions at *m/z* 53, 68, 79, 93, 107, 121 and 136, which are typically present in the spectra of many isoprenoids. These constituents were presumed to be terpenoids. The mass spectrum of constituent **3.26** (Fig. 3.9), for example, contains an abundant ion at even mass *m/z* 136 ($C_{10}H_{16}$), which is typically present in the spectra of the monoterpenoids, albeit not always as the base peak. The terpenoids constitute such a large and diverse class of organic compounds that it is generally impossible to identify them exclusively on the basis of their mass spectral properties. However, in the present investigation, a computerised search of available library of mass spectra simplified the problem and suggested the monoterpene, 1-methyl-4-(1-methylethenyl)-cyclohexene (limonene), as a likely candidate. In the

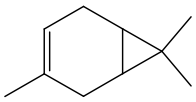
mass spectrum of limonene, the base peak at *m/z* 68 corresponds to the diene fragment arising from the retro-Diels–Alder reaction (Pavia *et al*., 2001: 412):



Limonene

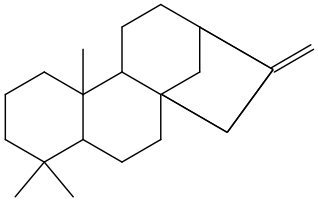
Limonene is a relatively stable and odorous terpene. It was found in about 50% of the available urine samples.

The mass spectrum of constituent **3.27** (Fig. 3.10) has its base peak at *m/z* 93 and other relatively abundant ions at *m/z* 71, 91, 121 and 136, suggesting that this constituent could also be a monoterpene. Based on the results of a library search and HR data, it was tentatively identified as 3,7,7-trimethylbicyclo[4.1.0]hept-3-ene (3-carene). However, this hypothesis was neither supported by the constituent's RI, nor by co-injection with (1*S*)-(+)-3-carene. 2-Carene has a similar mass spectrum, but is not commercially available, and its RI is not in agreement with the experimentally determined RI here.



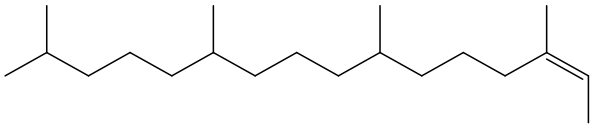
3-Carene

Constituent **3.28** was only detected in one urine sample that was analysed on the TOF-HRMS instrument (Fig. 3.11). This constituent was found to be a hydrocarbon with the molecular composition $C_{20}H_{32}$, which thus has five double bond equivalents, i.e., double bonds or rings. The constituent was presumed to be a polycyclic diterpene. A library search suggested that constituent **3.28** could be kaurene. Unfortunately, the RI of this constituent does not correspond with the RI of kaurene as reported in literature. Kaurene is not commercially available and this constituent remained unidentified.



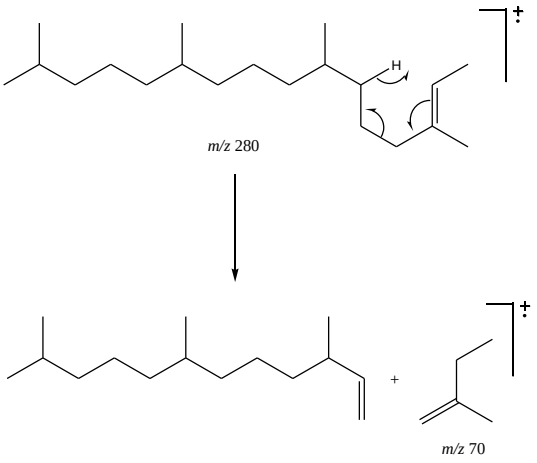
Kaurene

Constituents **3.29** and **3.30** have identical mass spectra (Fig. 3.12). They exhibit relatively abundant molecular ions at *m/z* 280 ($C_{20}H_{40}$), which indicates the presence of a cyclic structure or a double bond, probably formed by the loss of a molecule of water from the corresponding alcohol, as well as clusters of ions in the lower mass range of the spectrum, albeit not of gradually decreasing abundance as in the mass spectra of the unbranched *n*-alkanes. A computerised library search suggested 3,7,11,15-tetramethyl-2-hexadecene (2-phytene) as a likely candidate structure. These constituents were presumed to be the cis and trans isomers of this diterpene.



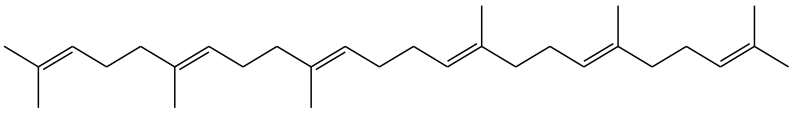
cis-2-Phytene

The mass spectra of these constituents have base peaks at *m/z* 70, i.e., at an even mass. This was accepted as an indication that this ion could be formed via a McLafferty rearrangement as follows (Budzikiewicz *et al*., 1967: 56):



Experimental RIs of the *cis*- and *trans*-phytene are 1786 and 1846, respectively. The literature RIs of *trans*-phytene and *cis*-isomer are not reported in the literature, and a commercially available mixture of isomers was not available for retention time comparison with the natural compounds.

Constituents **3.31** and **3.32** also have identical mass spectra (Fig. 3.13). Their experimental RIs are 2803 and 2821, respectively. The ion at *m/z* 69, which is the base peak in the spectra, is the base peak in the mass spectra of many long-chain polyisoprenoids. A library search suggested 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene (squalene) as the most likely basic structure of these two compounds and, most likely, they are squalene isomers. Their presence in caracal urine was confirmed by retention time comparison with an authentic reference sample. Although it is highly unlikely that these compounds and their saturated analogue, squalane, could be semiochemicals, they probably play a role in the controlled release of the VOCs present in caracal urine.



trans-Squalene

3.2.2 Aliphatic alcohols

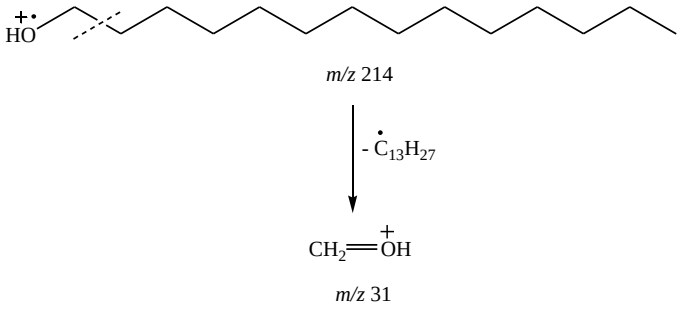
Polar compounds tend to elute as broad asymmetric peaks from apolar columns, in contrast to the sharp and therefore more prominent peaks with which they elute from polar columns. It was difficult to distinguish the peaks of very low concentrations of polar compounds from those of background impurities in urine samples when using an apolar column. As expected, a larger number of alcohols were thus observed in analyses carried out on the polar wax column than on the apolar polydimethylsiloxane column or the medium-polar OV1701 column.

3.2.2.1 Unbranched alcohols

The mass spectra of constituents **3.33–3.41** contain ion clusters at intervals of 14 amu, of which the series $(C_nH_{2n-1})^+$ (*m/z* 41, 55, 69, 83, ….), $(C_nH_{2n})^+$ (*m/z* 42, 56, 70, 84, ….) and $(C_nH_{2n+1})^+$ (43, 57, 71, 85, ….) are of relatively high abundance. It was initially thought that these constituents could be 1-alkenes. However, the experimental RIs of these compounds did not even remotely

correspond with published values (NIST). The mass spectra of long-chain primary alcohols and formates closely resemble those of the corresponding 1-alkenes. The molecular ions of alcohols result from the loss of an electron from the non-bonded electrons of oxygen. Due to the facile loss of a molecule of water from an alcohol or from its molecular ion, the abundance of the molecular ion in the mass spectra of primary and secondary alcohols is usually quite low, and it is generally entirely absent from the mass spectra of tertiary alcohols. If the molecular ions of these compounds are not detected, their spectra cannot easily be distinguished from those of the terminal alkenes, with the exception of the formates, the HRMS spectra of which have an ion at *m/z* 102 $(C_5H_{10}O_2)^+$, containing the two heteroatoms. It is possible to distinguish between primary, secondary and tertiary alcohols using chemical ionisation with NO as reactant gas (Burger, 2005: 852–863), although technically this is a rather daunting task.

In the case of a primary unbranched alcohol, the fragmentation of the alkene that is formed by the loss of a water molecule resembles that of terminal alkenes. The mass spectra of primary alcohols have an ion of moderate abundance, depending on the chain length, at *m/z* 31. This is formed from the molecular ion by the loss of an alkyl radical, in which the odd electron is stabilised more effectively than on an expelled hydrogen atom, as in the fragmentation shown below.

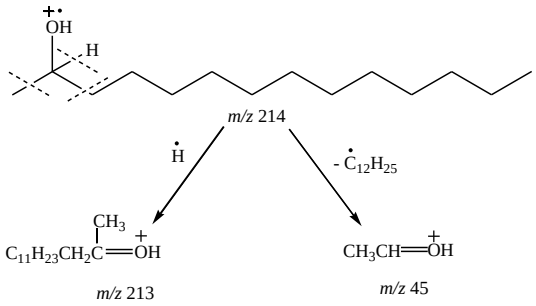


The mass spectrum of constituent **3.40** (Fig. 3.14) resembles that of constituent **3.25** (Fig. 3.8), except for the presence of the ion at *m/z* 31 in the spectrum of constituent **3.40** and differences in the higher regions of these spectra due to the different carbon chain lengths of the two compounds. Constituent **3.40** was identified as 1-tetradecanol and its mass spectrum is a typical example of the spectrum of a long-chain primary alcohol. Using RI determination and retention

time comparison with authentic standards, the primary unbranched alcohols were unambiguously identified as the C_8, C_9, C_{10}, C_{12}, C_{14} and C_{16} members of the homologous series of 1-alkanols.

To a certain extent, the mass spectra of the short-chain primary alcohols differ from those of their long-chain homologues. Taking their RIs and co-elution with synthetic standards into consideration, constituents **3.33**–**3.35** were identified as 1-pentanol, 1-hexanol, and 1-heptanol, respectively.

The mass spectrum of constituent **3.42** (Fig. 3.15) has its base peak at *m/z* 45. This ion is commonly observed in the EI spectra of 2-alkanols. Here it is formed by the loss of a neutral radical from the compound's molecular ion at *m/z* 214 $(C_{14}H_{30}O)^+$. The largest radical is preferentially lost because such a radical can be stabilised more readily in the longer chain by rearrangement or further decomposition (Budzikiewicz *et al*., 1967: 96).



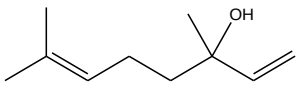
Thus the loss of $(C_{12}H_{25})$ radical from the molecular ion to yield the oxonium ion at *m/z* 45 (100%) is favoured over the loss of a methyl radical, yielding the ion at *m/z* 181 (1%). The identification of this constituent as 2-tetradecanol was confirmed in the usual manner by retention comparison with an authentic synthetic sample of the compound (co-elution).

3.2.2.2 Branched and cyclic alcohols

The mass spectrum of constituent **3.43** (Fig. 3.16) has a base peak at *m/z* 57. The ions at *m/z* 41, 43, 55, 69, 83 and 97 are typically present in the mass spectra of alkenes, and the ion at *m/z* 112 (C_8H_{16}) could therefore be the molecular ion of an octene. However, this compound's RI of 1499 cannot be reconciled with such a possibility. It was concluded that this constituent could be an alcohol, albeit not an unbranched 1-octanol. A library search suggested 2-ethyl-1-hexanol as a possibility with high correlation. This was confirmed by the constituent's RI of 1499, which is in full agreement with a literature RI of 1499 (NIST database) (both determined on a wax column).

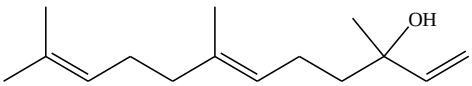
The base peak of the mass spectrum of constituent **3.44** (Fig. 3.17) occurs at *m/z* 71 (C_4H_7O). The ion at *m/z* 128 ($C_8H_{16}O$) appeared to be the compound's molecular ion. However, RI determinations did not support this possibility. Although it was considered unlikely that the ion at *m/z* 128 could be the molecular ion of an alcohol, a library search suggested 2,6-dimethylcyclohexanol as a possible structure. Seven different dimethyl-cyclohexanols, as well as a large number of acyclic unsaturated alcohols with the molecular formula $C_8H_{16}O$ exist. A comparison of the mass spectra of all seven of the dimethylcyclohexanols obtained from the NIST standard reference database revealed that only one of them, 2,6-dimethylcyclohexanol, has a detectable molecular ion. The experimentally determined RI of 1096 is in agreement with the NIST RI of 1098. However, the synthetic compound was not available and hence this constituent remained tentatively identified.

The mass spectrum of constituent **3.45** (Fig. 3.18) contains some of the prominent ions that are typically present in the spectra of unsaturated hydrocarbons. However, the ions at *m/z* 121 and especially *m/z* 136 are particularly conspicuous as they are present in the spectra of many monoterpenes and terpenols. Assuming the ion at *m/z* 154 to be the constituent's molecular ion, the ion at *m/z* 136 could be formed by the thermal loss of a molecule of water from the constituent's molecular ion, and the ion at *m/z* 121 could be formed by the loss of a molecule of water and a methyl radical from the molecular ion. Assuming that the constituent is an alcohol, the ion at *m/z* 71 could result from α-cleavage of a tertiary alcohol containing a double bond in one of its alkyl groups. 3,7-Dimethyl-1,6-octadien-3-ol (linalool) would meet these requirements. Constituent **3.45** was identified as linalool.



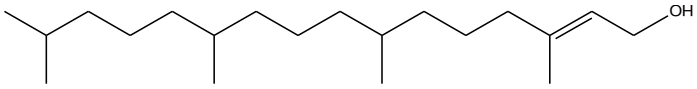
Linalool

A similar approach was followed in the identification of constituent **3.46**. This constituent is present in caracal urine in very low concentrations and it was not possible to obtain a 'clean' mass spectrum (Fig. 3.19). Nevertheless, a library search suggested nerolidol as a possibility, albeit with a correlation of only 77%. This constituent was eventually identified as nerolidol as it co-eluted with an authentic sample of nerolidol.



Nerolidol

Constituents **3.47** and **3.48** have identical mass spectra (Fig. 3.20). The spectra have fragment ions at *m/z* 43, 57, 68, 81, 95 and 123, but do not exhibit the ion clusters typically present in the spectra of unbranched alkanes or alkenes. The base peak in the spectrum occurs at *m/z* 68 $(C_5H_8)^+$ and the spectrum does not contain any oxygen-containing ions. An ion of very low abundance is present in the mass spectra at *m/z* 278 $(C_{20}H_{38})^+$. However, the RI of an unbranched C_{20} diene is expected to be in the order of 1961, while that of 1-icosanol is 2252, both determined on an apolar column, whereas the RIs of constituents **3.47** and **3.48** are 2114 and 2148, respectively, using the same type of column. It seemed logical to assume that these constituents could be branched long-chain alcohols and, in view of the presence of isoprenoids in caracal urine, the possibility that these constituents could be isomeric diterpenols, for example, *cis*- and *trans*-3,7,11,15-tetramethyl-2-hexadecen-1-ol, respectively. Using a polar wax column, from which alcohols elute as symmetric peaks, the RIs of the two constituents are 2572 and 2611. The NIST RIs of these compounds are 2570 and 2617, respectively. Retention time comparison with a commercially available mixture of these isomers proved this conclusion correct.

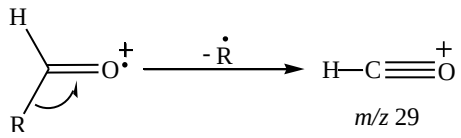


trans-Phytol

3.2.3 Aliphatic aldehydes

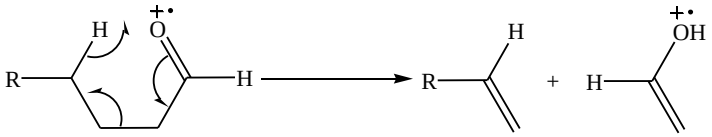
3.2.3.1 Unbranched alkanals

The molecular ion of aliphatic aldehydes is generally of low abundance, and decreases with increasing chain length. There is a paucity of diagnostic information in the higher mass ranges of the spectra of long-chain aldehydes, which complicates the interpretation of their mass spectra. Saturated aliphatic aldehydes undergo α- and β-cleavages, of which α-cleavage predominates for aldehydes in the lower molecular weight range and results in the production of the formyl ion at *m/z* 29 (Budzikiewicz *et al*., 1967: 130).



m/z 29

In most cases, aldehydes also exhibit β-cleavage accompanied by the rearrangement of a hydrogen atom to the oxygen-containing fragment, i.e., a McLafferty rearrangement takes place to produce an ion at *m/z* 44, for example. This ion is prominent and it can even be the base peak in aldehydes with four to seven carbon atoms containing a γ-hydrogen atom for transfer to the carbonyl oxygen (Budzikiewicz *et al*., 1967: 131).



m/z 44

With increasing molecular mass, the hydrocarbon ions of unbranched aldehydes make a predominant contribution to their spectra. Using ^{18}O labelling, it was shown that the ions at *m/z* at 43 or 57, which are the most abundant in the spectra of straight-chain aldehydes with eight or more carbon atoms, are alkyl fragments. Ions resulting from C–C cleavage and the loss of two hydrogen atoms (C_nH_{2n-1}) are also observed in aldehyde spectra (Gilpin and McLafferty, 1957).

The ion at *m/z* 44, together with those at *m/z* 31 and *m/z* 45, indicate the presence of oxygen in the molecule. In the mass spectra of the lower aldehydes, such as propanal for example, the largest proportion of the ions carrying the charge of the ion at *m/z* 29 contains oxygen, but in higher aldehydes the charge on this ion is carried entirely by $(C_2H_5)^+$. An $(M–18)^+$ ion is present in the spectra of particularly those aldehydes containing more than six carbon atoms, while the loss of 28 (CO) and 44 (CH_3CHO) is characteristic in the mass spectra of most aldehydes (Beynon, 1968: 210–213).

As in the mass spectra of the long-chain alkanes, alkenes and primary alcohols, the spectrum of constituent **3.53** (Fig. 3.21) also contains ion clusters. However, the clusters are broader. The most abundant ions in several of the clusters also do not occur at intervals of 14 amu. From previous experience, it is known that the ion at *m/z* 82 (C_6H_{10}) with a relative abundance of approximately 40% is typically present in the mass spectra of long-chain unbranched aldehydes.

In our laboratory (LECUS), this ion is often used to construct a selected ion chromatogram for the detection of aldehydes in TICs of complex mixtures. It is interesting that in the mass spectrum of constituent **3.53**, obtained on the GC-HRMS instrument, this ion is the base peak. The spectra obtained with both the LR and HR instruments do not contain an ion at *m/z* 156, the molecular ion of decanal. However, the spectrum of constituent **3.53** does contain an ion at *m/z* 138 $(C_{10}H_{18})^+$, formed by the loss of a molecule of water from the molecular ion, which is a common feature in the mass spectra of aliphatic aldehydes, as mentioned above. The spectrum also contains an ion at *m/z* 44, but in this spectrum it is not particularly abundant. The identification of constituent **3.53** as decanal was confirmed in the usual manner.

Constituents **3.49**, **3.50**, **3.51**, **3.52**, **3.54** and **3.55** were similarly identified as hexanal, heptanal, octanal, nonanal, dodecanal and octadecanal, respectively.

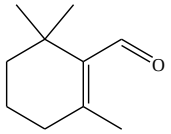
3.2.3.2 Alkenals and cyclic alkanals

The mass spectrum of constituent **3.56** (Fig. 3.22) has an ion at *m/z* 83 $(C_5H_7O)^+$, which is typically present in the mass spectra of 2-alkenals. Assuming that this constituent, which was identified only in one urine sample analysed on the HRMS instrument, is a long-chain aldehyde, the ion at *m/z* 136 $(C_{10}H_{16})^+$ is presumably formed by the loss of a molecule of water from the molecular ion. This constituent was tentatively identified as a 2-decenal. This constituent was compared with synthetic *trans*-2-decenal. The RIs of *cis*- and *trans*-2-decenal are 1259 and 1264 (NIST), respectively. Constituent **3.56** with an RI of 1259 was identified as *cis*-2-decenal.

Constituent **3.57**, on the other hand, was not present in the sample analysed on the HRMS instrument. The majority of the abundant ions observed in the mass spectra of constituent **3.56** are also present in the LR mass spectrum of constituent **3.57** (Fig. 3.23), with the exception of the $(M–H_2O)^+$ ion that is present in the latter spectrum at *m/z* at 150 $(C_{11}H_{18})^+$. This constituent was tentatively identified as a 2-undecenal. It has a RI of 1367 on an apolar column and, based on the NIST RIs of 1335 and 1369 of *cis*- and *trans*-2-undecenal, respectively, this constituent was identified as *cis*-2-undecenal. Final confirmation of its structure was obtained by retention time comparison with the commercially available synthetic compound.

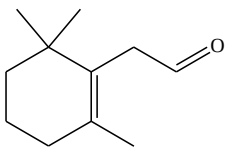
The LR mass spectrum of constituent **3.58** (Fig. 3.24) contains a regular series of abundant ions that could be ascribed to the loss of a series of alkyl radicals from an alcohol or a hydrocarbon. However, according to its HR mass spectrum, the molecular ion has the composition $(C_{10}H_{16}O)^+$,

hence the compound could be either an unsaturated or cyclic alcohol, or a carbonyl compound with one or more double bonds or rings. The ion at *m/z* 137 $(C_9H_{13}O)^+$ is the base peak in both the LR and HR spectra. In all the other prominent ions, down to *m/z* 81, a substantial part of the charge is carried by oxygen-containing species. This compound could thus not be an alcohol. The loss of a CHO moiety from the molecular ion to yield the ion at *m/z* 137 suggests that the compound could be an aldehyde. This possibility is supported by the results of a library search, which suggested 2,6,6-trimethylcyclohexene-1-carboxaldehyde as a possible structure with a correlation of 91%. Fortunately, this structure could be confirmed by retention time comparison with the commercially available synthetic aldehyde.



2,6,6-Trimethylcyclohexene-1-carboxaldehyde

Constituent **3.59** was only found in urine samples analysed on the HRMS instrument. According to the HR data, this constituent has the molecular composition $C_{11}H_{18}O$. As in the case of constituent **3.58**, the loss of a methyl radical is also responsible for the formation of the base peak of the spectrum (Fig. 3.25) at *m/z* 151 $(C_{10}H_{15}O)^+$. This could be an indication that this constituent also has several methyl substituents. However, the molecular ion and the ion at *m/z* 151 are the only oxygen-containing ions in the spectrum. It was concluded that this constituent could have a structure similar to that of constituent **3.58**, but with the additional CH_2 in a position where it facilitates the loss of the oxygen moiety, i.e., the aldehyde group. This would be the case if the aldehyde is involved in a McLafferty rearrangement in which acetaldehyde is expelled. The resulting putative hydrocarbon ion is present in the spectrum at *m/z* 122 (C_9H_{14}) in a low abundance of 14%. Unfortunately, accurate mass determination was only possible from about *m/z* 50, hence this acetaldehyde could not be detected with the HRMS instrument. The proposed structure was confirmed by retention time comparison with the commercially available compound.

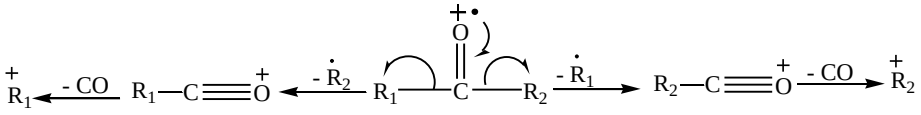


2,6,6-Trimethylcyclohexene-1-acetaldehyde

3.2.4 Aliphatic ketones

Ketones constitute the largest group of VOCs present in male and female caracal urine. As in the aldehydes, the mass spectrometric fragmentation of aliphatic ketones is largely driven by the carbonyl group, but the higher mass ranges of the spectra of ketones usually contain more diagnostic information than those of the aldehydes. Furthermore, the major fragmentations in the spectra of ketones do not result from simple bond rupture. Depending on the structure of an aliphatic ketone, several ions resulting from rearrangements may be present in its spectrum (Sharkey *et al*., 1956).

Aliphatic ketones commonly undergo α-cleavage, β-cleavage and McLafferty rearrangement processes. The α-cleavage of ketones on both sides adjacent to the carbonyl group yields two acylium ions and favours the loss of the larger alkyl group as the charge can be stabilised more effectively in the larger alkyl group. Further expulsion of CO results in the formation of the hydrocarbon ions (R_1^+ and R_2^+) as formulated below (Budzikiewicz *et al*., 1967: 134–138; MacLeod *et al*., 1967; Buchs *et al*., 1970).



α-Cleavage is more important in ketones than in aldehydes due to the higher stability of the acylium ions mentioned above. Thus, the ion at *m/z* 43 ($C_2H_3O^+$) is the base peak in the spectra of methyl ketones. The *m/z* 43 ion is also abundant when the charge is on the alkyl moiety (Budzikiewicz *et al.*, 1964: 6).

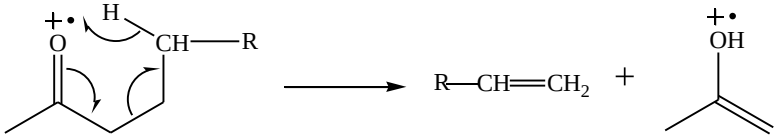
3.2.4.1 Unbranched alkanones

The mass spectra of two short-chain ketones **3.61** and **3.62**, as well as three long-chain unbranched isomeric C_9 ketones **3.69**, **3.70** and **3.71**, and a C_{15} ketone **3.80**, for some of which HR data are available, are discussed here as representatives of ketones belonging to three different ketone classes. In the HR mass spectrum of constituent **3.61**, 89% of the charge associated with the base peak at *m/z* 43 is carried by the acylium ion $(C_2H_3O)^+$ and 11% by the carbonium ion $(C_3H_7)^+$. The molecular ion is present at *m/z* 86 $(C_5H_{10}O)^+$ in the spectrum with an abundance of 84%. In the LR spectrum of this constituent (Fig. 3.26), the molecular ion is present with an abundance of only

18%. The ions at *m/z* 58 and 71 result from a McLafferty rearrangement (see example below) and the expulsion of a methyl radical, respectively. Constituent **3.61** was identified as 2-pentanone.

The acylium ion ($C_3H_5O^+$) is the base peak at *m/z* 57 in the HR spectrum of constituent **3.62**. It is formed by the loss of an ethyl group. The molecular ion at *m/z* 86 $(C_5H_{10}O)^+$ (40%) and a carbonium ion at *m/z* 29 in the LR mass spectrum of this compound (Fig. 3.27) indicated that the constituent could be the symmetrical ketone, 3-pentanone. The abundance of the molecular ion in the LR spectrum is only about 20%. As expected, an ion at *m/z* 58 is absent from the mass spectrum of this ketone as no γ-hydrogen is available for McLafferty rearrangement. Constituent **3.62** was identified as 3-pentanone.

In the HR mass spectrum of constituent **3.69**, the molecular ion $(C_9H_{18}O)^+$ is present with an abundance of 8%. In the LR spectrum of this constituent (Fig. 3.28), it is present with an abundance of 7%. Due to the availability of a γ-hydrogen, the product of a McLafferty rearrangement, $(C_3H_6O)^+$, is present at *m/z* 58 in the constituent's HR spectrum aspectrum, in which it is the base peak, whereas the ion at *m/z* 43 is the base peak in the LR spectrum. Constituent **3.69** was identified as 2-nonanone.



m/z 58

The molecular ion at *m/z* 142 (7%), a carbonium ion at *m/z* 57 and an ion at *m/z* 72, resulting from a McLafferty rearrangement in the LR mass spectrum of constituent **3.70** (Fig. 3.29), were interpreted as evidence in favour of this constituent being 3-nonanone.

The mass spectrum of constituent **3.71** (Fig. 3.30) has a base peak and molecular ion at *m/z* 43 and 142, respectively. In this spectrum, the McLafferty rearrangement product is present at *m/z* 86, which indicates that this constituent could be a 4-alkanone. The ions in the higher mass ranges of the mass spectra of this and the previous two nonanones are present in quite low abundances, and do not suggest the presence of carbon chain branching. The availability of synthetic reference samples facilitated unequivocal identification of these three ketones as 2-, 3- and 4-nonanone.

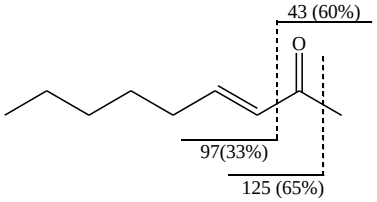
The base peak in the LR mass spectrum of the long-chain ketone **3.80** (Fig. 3.31) occurs at *m/z* 43. In this spectrum, the ion at *m/z* 58 has an abundance of 50%. The ion at *m/z* 71 (20%) is

formed by γ-cleavage, which is preferred to simple β- or δ-fission (Budzikiewicz *et al*., 1967: 138). δ-Fission, which results in the formation of an ion at *m/z* 85, is observed in the LR spectrum (7%), but is not present in the HR spectrum of this constituent. According to the HR data, the ion at *m/z* 59 (51%) has the composition C_3H_7O. The ion at *m/z* 226 $(C_{15}H_{30}O)^+$ (23%) was accepted as the compound's molecular ion. Constituent **3.80** was tentatively identified as a 2-pentadecanone. The typical loss of a molecule of water from the molecular ion of an aldehyde yields the ion at *m/z* 208 $(C_{15}H_{18})^+$, which is not present in the LR spectrum. The numerous carbonium ions and oxygen-containing ions in the higher mass range of the spectrum are all of quite low abundance. It was concluded that this constituent is the unbranched 2-pentadecanone.

Comparison of the RIs of the large group of 22 unbranched 2-, 3-, and 4-alkanones (**3.60–3.81)** with published data made a substantial contribution to the tentative identification of these ketones.

3.2.4.2 Unbranched alkenones

In contrast to the saturated aliphatic ketones, the α,β-alkenones exhibit a characteristic fragmentation pattern that is also observed in the presence of other substituents. α-Cleavage is the most common fragmentation process in acyclic α,β-alkenones (Shapiro and Djerassi, 1964; Budzikiewicz *et al*., 1967: 141). Typical α-cleavages in a long-chain 3-alken-2-one can be illustrated as follows:



Depending on the position of one or more double bonds and the presence of chain branching in the molecule, it may be difficult to recognise unsaturated aliphatic ketones in complex mixtures, mainly because the typical McLafferty rearrangement could be suppressed by a double bond in certain positions (Budzikiewicz *et al*., 1967: 141). In the case of constituents **3.82** and **3.83**, HR mass spectral information was not available. Fortunately, information resulting from a library search could be used as a starting point in the interpretation of their mass spectra. Constituent **3.82** was tentatively identified as 3-hepten-2-one. The compound's mass spectrum

(Fig. 3.32) and the corresponding library spectrum both have molecular ions at *m/z* 112 with an abundance of about 16%. A synthetic sample of 3-hepten-2-one with unknown configuration was available for retention time comparison. The RI of constituent **3.82** is 933. The RI of *cis*-3-hepten-2-one is not available in the literature, but the RI of the trans isomer is 937 (NIST). This constituent was identified as *trans*-3-hepten-2-one. Constituent **3.83** was tentatively identified as 3-nonen-2-one. The compound's molecular ion (1%) at *m/z* 140 is barely detectable in its mass spectrum (Fig. 3.33), but it has an abundance of 10% in the library spectrum. Retention time comparison with available synthetic reference material confirmed the identification of constituent **3.83** as *trans*-3-nonen-2-one.

3.2.4.3 Branched alkanones

The molecular ion in the mass spectrum of constituent **3.84** (Fig. 3.34) occurs at *m/z* 128 (5%) and its base peak at *m/z* 43. The ion at even mass *m/z* 72 (50%), produced by a McLafferty rearrangement, could be seen as an indication that the constituent is either a 3-alkanone or a 3-methyl-2-alkanone. A $(M–CH_3)^+$ ion is present in the spectrum at *m/z* 85 (10%), which could be evidence in favour of this constituent being a branched 2-alkanone, for example, 3-methyl-2-heptanone. However further branching in the alkyl moiety has to be considered. In fact, 3,4-dimethyl-2-hexanone has a mass spectrum that only differs from that of 3-methyl-2-heptanone in that the latter compound has a molecular ion that is somewhat more abundant than that of 3,4-dimethyl-2-hexanone. Constituent **3.84** was tentatively identified as 3-methyl-2-heptanone as its RI of 938 corresponds to the published RI of 937 (NIST).

The mass spectra of constituents **3.85** and **3.86** (Figs 3.35 and 3.36 respectively) are discussed here as examples of branched saturated aliphatic ketones. The mass spectra of these two constituents differ from that of 2-octanone (**3.67**, Fig. 3.37) only with respect to small differences in the relative abundances of the major diagnostic ions in the three spectra and the absence from the mass spectrum of constituent **3.85** of an ion at *m/z* 85, which is present in the spectra of the other two compounds. The LR spectra of both **3.85** and **3.86** have abundant ions at *m/z* 43 and 58, and they are therefore probably 2-alkanones. The assumption that the peak at *m/z* 128 is the molecular ion of these constituents is supported by the presence of peaks at *m/z* 110 $(M–H_2O)^+$ and *m/z* 113 $(M–CH_3)^+$ in these spectra, respectively. With the exception of the ions at *m/z* 85 and 99, the fragment ions that can theoretically be formed by simple carbon–carbon bond fission in the

spectra of long-chain 2-octanones are all present in the spectra, albeit in very low abundances in some cases. The absence of an ion at *m/z* 85 and 99 in the mass spectra of constituents **3.85** and **3.86**, respectively, was accepted as indication of the presence of methyl branching in these two constituents, i.e., they could be 5-methyl-2-heptanone and 6-methyl-2-heptanone, respectively. Despite a difference of 4 units between the NIST RI of 5-methyl-2-heptanone and the experimental RI of constituent 3.85, this constituent was tentatively identified as 5-methyl-2-heptanone. A synthetic sample was not available for retention time comparison which casts doubt on the structure suggested for this constituent. However, in the case of constituent **3.86**, the experimental RI corresponds with the NIST RI of 6-methyl-2-heptanone, a synthetic sample of which also co-eluted with this constituent.

A library search suggested 2-methyl-3-octanone as the most likely structure for constituent **3.87**. An ion at *m/z* 86 that could be ascribed to a putative McLafferty rearrangement is barely visible in the library spectrum. This ion is not present in the constituent's mass spectrum (Fig. 3.38). The mass spectrum of 2-methyl-3-octanone is remarkably similar to that of 2,3-octanedione. Unfortunately, HRMS data that could have solved this contradiction were not available. However, the NIST RI is identical to that of constituent **3.87**. This constituent remained only tentatively identified as 2-methyl-3-octanone.

The mass spectrum of constituent **3.88** (Fig. 3.39) contains ions at *m/z* 43, 58 and 71, which are normally present in the spectra of long-chain ketones. However, in this case, the spectrum contains very little diagnostic information beyond *m/z* 126. If, for some reason, such as chain branching, the enolic product of a putative McLafferty rearrangement (*m/z* 58) is less abundant than usual, and the charge is shared between the enolic product and the alkenic product (*m/z* 126), then the molecular mass of such a branched ketone could be 184. Due to the paucity of appropriate diagnostic mass spectrometric information in the higher mass ranges, the position of chain branching could not be reliably established. A library search suggested 2-dodecanone and 5,9-dimethyl-2-decanone as the most likely candidate structures. However, constituent **3.88** elutes with a shorter retention time than 2-dodecanone, which is also present in many of the available urine samples. The constituent thus has to be branched. In addition to small differences in the relative abundance of ions in its mass spectrum and the library spectrum, the absence of an $(M–H_2O)^+$ ion in the constituent's spectrum is the only, possibly significant, difference between these spectra. A synthetic reference compound was not available and its RI could not be found in the

literature. Constituent **3.88**, which was identified only in male urine, remained tentatively identified as 5,9-dimethyl-2-decanone.

Constituent **3.89** was present in a high concentration in one of the urine samples, which made it possible to obtain excellent LR and HR mass spectral data. The HR mass spectrum contains a typical McLafferty arrangement ion at *m/z* 58 and a series of abundant $(C_nH_{2n-1})^+$ ions at *m/z* 43, 57, 71 and 85, as well as another ion in this series at *m/z* 183. Neither the HR nor the LR mass spectrum (Fig. 3.40) contains a putative molecular ion. However, it is logical to accept that the ions at *m/z* 183 $(C_{12}H_{23}O)^+$ and 180 $(C_{13}H_{24})^+$ are formed by the loss of a methyl radical and a water molecule, respectively, and the compound could thus be a branched 2-tridecanone. The absence of other $(C_nH_{2n-1})^+$ ions in the higher mass range of the spectra led to the conclusion that this ketone, like the previous one, could be branched at more than one position. A library search suggested 6,10-dimethyl-2-undecanone as the most likely structure with a high correlation of 94%. A synthetic reference sample was available for retention time confirmation, which confirmed the structure proposed for this constituent.

3.2.4.4 Branched unsaturated ketones

The ion at *m/z* 43 is the base peak of the LR mass spectrum of constituent **3.90** (Fig. 3.41). This ion does not appear in its HR spectrum. Reliable HR data were not acquired below about *m/z* 50. The abundant ions at *m/z* 126 $(C_8H_{14}O)^+$, 111 $(C_7H_{11}O)^+$ and 108 $(C_8H_{12})^+$ in the HR mass spectrum of constituent **3.90** can be rationalised in terms of the compound being an octenal or octenone. The LR spectrum contains an abundant ion at *m/z* 58, which is probably the product of a McLafferty rearrangement. A library search suggested 6-methyl-5-hepten-2-one as a possible candidate structure. Retention time comparison with the synthetic analogue corroborated the proposed structure.

The LR mass spectrum of constituent **3.91** (Fig. 3.42) contains relatively little diagnostic information, except for the relatively stable ion with even mass at *m/z* 124. This is probably the constituent's molecular ion that loses a methyl radical to yield the compound's base peak at *m/z* 109. It was assumed that this compound contains at least one double bond. A library search suggested only one candidate structure that could reasonably qualify for further consideration: 6-methyl-3,5-heptadien-2-one. This structure and the compound's configuration were confirmed by retention time comparison with synthetic *trans*-6-methyl-3,5-heptadien-2-one.

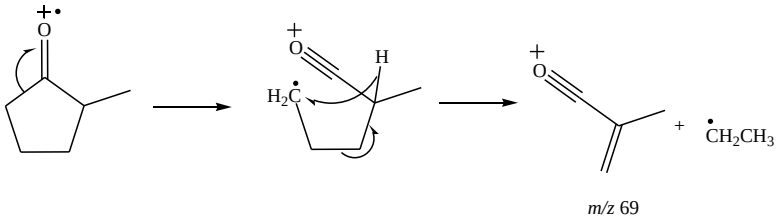
The ion at highest mass in the HR mass spectrum of constituent **3.92** occurs at *m/z* 194 $(C_{13}H_{22}O)^+$ (2%) and the base peak at *m/z* 69 $(C_5H_9)^+$. As HR data were only acquired for the mass range starting at *m/z* 53, the base peak at *m/z* 43 in the LR spectrum (Fig. 3.43) of this constituent does not appear in the HR mass spectrum. However, the abundance of this ion in the LR spectrum suggests that constituent **3.92** could probably be a methyl ketone. The prominent ion at *m/z* 69 and the other ions at, for example, *m/z* 93, 107, 121, 136, etc., that are present in the spectra of some of the terpenoids discussed above, suggested that this constituent could have a similar structure, although, containing 13 carbon atoms, it was thought unlikely that this constituent could be an isoprenoid. However, in our laboratory, several C_{13} and C_{18} compounds, for example, geranyl acetone and farnesyl acetone (Burger *et al*., 1978), with mass spectra containing these ions have already been identified in mammalian exocrine secretions. The identification of constituent **3.92** as *cis*-6,10-dimethyl-5,9-undecadien-2-one (geranyl acetone) was substantiated by retention time comparison with an available synthetic sample of this compound.

The urine constituent **3.93** was identified in the urine of only one male, whereas it was present in the urine of three females. Constituents **3.94** and **3.95** were each identified in only one female. With the exception of their different, relatively abundant, molecular ions, the mass spectra of these constituents contain the same prominent ions, albeit in varying abundances. Without information obtained from extensive studies on the mass spectrometric fragmentation of cyclic ketones, in which high-resolution and deuterium-labelling techniques were used during the early years of organic mass spectrometry (e.g., Beynon *et al.*, 1960; Seibel and Gäumann, 1963; Williams *et al.*, 1964), it would have been difficult to identify these methyl-substituted cycloalkanones exclusively on the basis of their LR mass spectra.

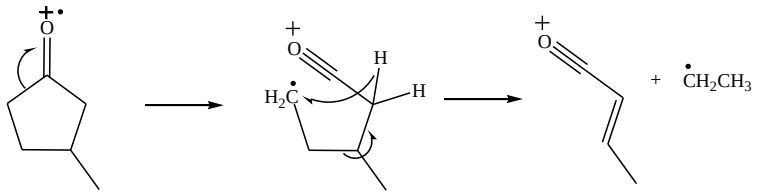
The identification of constituent **3.93** is discussed here to illustrate the subtle differences in the mass spectra of isomers of these methylcycloketones. Here, HRMS data are available in addition to this constituent's LR spectrum (Fig. 3.44). There are several conspicuous quantitative differences between the HR and LR spectra of this compound. The molecular ion at *m/z* 98 $(C_6H_{10}O)^+$, for example, is also the base peak in the HR spectrum of this constituent, whereas it is present at 34% in the LR spectrum. Nevertheless, all the prominent peaks are present in both spectra. A library search suggested 3-methyl-cyclopentanone as a likely candidate structure for this constituent with a correlation of 87%. 2-Methylcyclo-pentanone with a correlation of 85% is, however, a close contender. The library LR spectra of these two candidates contain the same ions,

the only difference being the presence of a barely visible $(M-18)^+$ ion in the spectrum of 2-methylcyclopentanone, which is absent from the spectrum of 3-methylcyclopentanone. The ions at *m/z* 55 and 69 are also much less abundant in this spectrum than in that of 3-methylcyclopentanone.

The peak at *m/z* 69 $(C_4H_5O)^+$ (80%) is formed by the expulsion of C_2H_5, in which the C-3 and C-5 hydrogens are largely involved (Seibel and Gräumann, 1963). In this spectrum, the ion at *m/z* 55 comprises of $(C_3H_3O)^+$ and $(C_4H_7)^+$ in a ratio of 94:6. The modes of formation of these ions have not been unequivocally established. However, the formation of the ion at *m/z* 69 in the spectra of 2- as well as 3-methylcyclopentanone can be rationalised by the loss of an ethyl radical as follows:



m/z 69



m/z 69

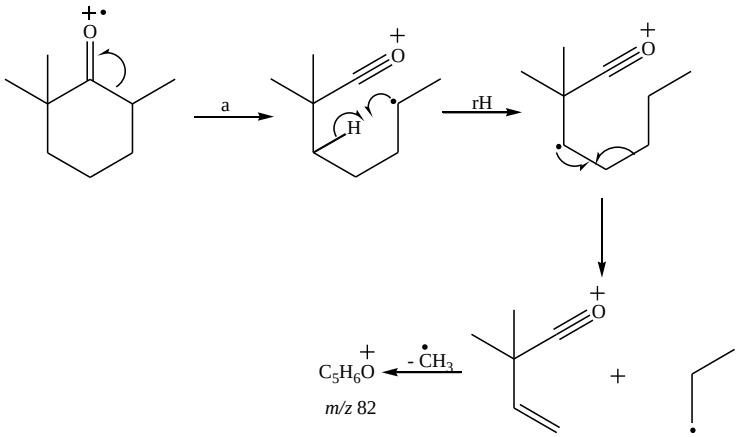
The *m/z* 69 ion could possibly be expected to be more abundant in the mass spectrum of 3-methylcyclopentanone than in that of the 2-methyl-substituted isomer. The charge of the *m/z* 42 ion is carried exclusively by the carbonium ion $(C_3H_6)^+$, while the ion at *m/z* 56 (60%) consists of the ions $(C_4H_8)^+$ and $(C_3H_4O)^+$ in a ratio of 1:2, according to the available HR data. Final confirmation of 3-methylcyclopentanone as the structure of constituent **3.93** was obtained by retention time comparison with the synthetic compound. The compound's RI and HR data also confirm this conclusion.

The base peak and molecular ion are present at *m/z* 69 and *m/z* 112, respectively, in the LR mass spectrum of constituent **3.94** (Fig. 3.45), as well as in its HR mass spectrum. In the library spectra of 2- ,3- and 4-methylcyclohexanone, the abundance of the *m/z* 69 ion is 50, 100 and 12%,

respectively. Furthermore, the base peak of 2-methylcyclohexanone occurs at *m/z* 68 and that of 4-methylcyclohexanone at *m/z* 55. Constituent **3.94** was tentatively identified as 3-methylcyclohexanone, mainly on the basis of comparison of its mass spectrum with the library spectra of these three isomeric methylcyclohexanones, and the correspondence between its experimental and published RIs. The proposed structure was confirmed by retention time comparison with synthetic 3-methylcyclohexanone.

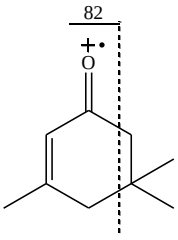
The molecular ion in the mass spectrum of constituent **3.95** (Fig. 3.46) occurs at *m/z* 126. This constituent could thus be a methylcycloheptanone isomer. The ion at *m/z* 69 is the base peak of the spectrum. The abundance of this ion in the library mass spectra of 2- ,3- and 4-methylcycloheptanone is 50, 100 and 33%, respectively. Apparently, a base peak at *m/z* 69 appears to be a typical feature of 3-methyl-substituted cycloalkanones. The assumption that constituent **3.95** is 3-methylcycloheptadecanone was not confirmed by comparison with synthetic material. This constituent remained tentatively identified.

The majority of the abundant ions present in the mass spectra of the methylcycloketones discussed above are also present in abundances higher than 40% in the mass spectrum of constituent **3.96**, (Fig. 3.47), except for the ion at *m/z* 98, which is present in this spectrum in an abundance of only 5%. The LR and HR mass spectra do not contain any ions between the ion at *m/z* 82 $(C_6H_{10})^+$ and the molecular ion at 140 $(C_9H_{16}O)^+$ (47% in the HR and 20% in the LR spectra). Constituent **3.96** was identified as 2,2,6-trimethylcyclohexanone, based on the results of a library search, the absence of ions in the higher mass ranges of its mass spectrum, and the presence in its spectrum of ions also present in the mass spectra of the methylcycloketones discussed above. The structure of constituent **3.96** was confirmed by retention time comparison with a synthetic reference compound. The base peak at *m/z* 82 is formed as follows (Cronje, 2010: 129):



2.4.5 Cyclic alkenones

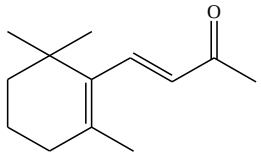
The base peak in the LR and HR mass spectra of constituent **3.97** (Fig. 3.48) occurs at *m/z* 82. The constituent's molecular ion at *m/z* 138 $(C_9H_{14}O)^+$ (16%) and a retention time difference of only about 5 min between this constituent and constituent 2,2,6-trimethylcyclohexanone (**3.96**) were initially interpreted as an indication that this compound could be a similarly substituted cyclohexanone derivative with an unsaturated substituent. These constituents both have a base peak at *m/z* 82. However, when an HR mass spectrum of constituent **3.98** became available, it appeared that in this case the *m/z* 82 ion is $(C_5H_6O)^+$. The ion at *m/z* 123 $(C_8H_{10}O)^+$ suggests that if the compound contains a cyclohexene ring, then the methyl could not be on C-4, as the mass spectrum does not contain an $(M–42)^+$ ion, expected in such an eventuality (Budzikiewicz *et al.*, 1967: 151). Retro-Diels-Alder-type reactions are common in the mass spectra of cyclohexenones (Budzikiewicz *et al.*, 1967: 153). If the results of a library search are accepted, specifically, that constituent **3.97** is 3,5,5-trimethyl-2-cyclohexenone (isophorone), then the formation of the compound's base peak at *m/z* 82 $(C_5H_6O)^+$ could be attributed to this type of fragmentation as follows:



Isophorone

Retention time comparison with commercially available isophorone confirmed that these assumptions are justified.

According to the HR mass spectrum of constituent **3.98**, the ions at highest mass *m/z* 192 and 177, which are also present in the constituent's LR spectrum (Fig. 3.49), have the composition $(C_{13}H_{20}O)^+$ and $(C_{12}H_{17}O)^+$, respectively. The loss of a molecule of water from the ion at *m/z* 177 yields the constituent's base peak and the presence of an ion at *m/z* 43 $(C_2H_3O)^+$ (63%) indicates the presence in the constituent of at least one oxygen atom, probably in an acyl moiety. A superficial evaluation of the repeated loss of C_2H_4 units starting from the *m/z* 177 ion can be followed in the HR spectrum and could be interpreted as an indication of the presence of an acyclic carbon chain. However, a plausible structure was only arrived at by carrying out a library search, which suggested 4-(2,6,6-trimethylcyclohex-1-en-1-yl)-3-buten-2-one (β-ionone) as the most likely structure of constituent **3.98**, with a correlation of 91%. This result was confirmed in the usual manner by retention time comparison with commercially available β-ionone.

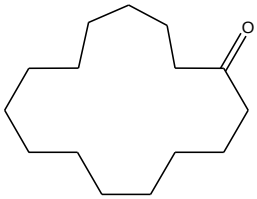


β-Ionone

3.2.4.6 Macrocyclic ketones

Male and female caracal urine contains the five constituents **3.99–3.103**, the mass spectra of which are practically identical in their lower mass ranges. One of these constituents, **3.101**, was present in relatively high concentrations in several urine samples, and was later also identified as a ligand of urinary protein of the caracal. The HR mass spectrum of this constituent has such an abundant molecular ion at *m/z* 224 $(C_{15}H_{28}O)^+$ (60%) (20% in the LR spectrum, Fig. 3.50) that it is unlikely that the compound could be a pentadecadienol or a cyclopentadecenol. The loss of a methyl radical yields an ion at *m/z* 209 $(C_{14}H_{25}O)^+$ (6%) and the loss of a molecule of water yields the ion at *m/z* 206 $(C_{15}H_{26})^+$ (13%). The constituent could be an unsaturated long-chain ketone, which would, however, also be difficult to reconcile with its abundant molecular ion. The constituent's LR mass spectrum (Fig. 3.50) has the typical appearance of that of a long-chain unbranched aliphatic compound. A library search suggested cyclopentadecanone as a likely structure. This was

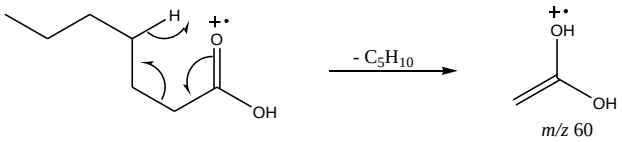
accepted as probably correct, despite the presence in its mass spectrum of the $(M–CH_3)^+$ ion. The $(M–CH_3)^+$ ion is also present in the spectra of the other members of this group of compounds. Constituent **3.101** was unambiguously identified as cyclopentadecanone by comparison of its mass spectrum and retention time with those of the commercially available synthetic compound. The C_{13}, C_{14}, C_{16} and C_{17} macrocyclic ketones, **3.99**, **3.100**, **3.102** and **3.103**, were likewise identified and, except for the C_{17} homologue, were synthesised from the corresponding alkanedioic ethyl esters (Prelog *et al.*, 1947).



Cyclopentadecanone

3.2.5 Aliphatic carboxylic acids

Male and female caracal urine contains a series of constituents, **3.104–3.121** that have mass spectra with a base peak, or at least an abundant ion, at *m/z* 60. This ion is typically present in the spectra of saturated carboxylic acids (fatty acids) that are not branched at C-2, and have a γ-hydrogen atom available for transfer in a McLafferty rearrangement that yields acetic acid as product (Budzikiewicz *et al.*, 1967: 214). It has been shown that the carbon atom of the carbonyl group is retained in the *m/z* 60 ion present in the mass spectra of these carboxylic acids (Happ and Steward, 1952).

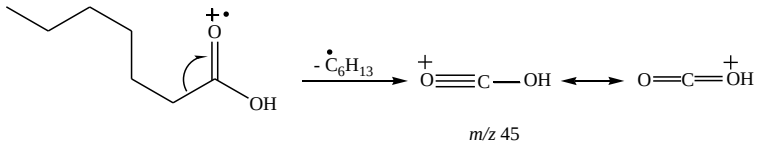


m/z 60

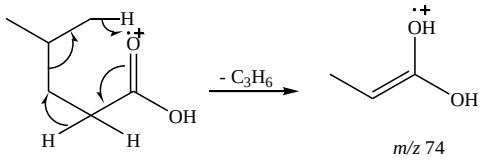
Butanoic acid and unbranched carboxylic acids with longer carbon chain lengths have spectra with an additional abundant ion at *m/z* 73. The *m/z* 60 ion carries an increasingly smaller percentage of the total charge with increasing chain length, while the abundance of the *m/z* 73 ion increases. In the mass spectrum of constituent **3.117** (Fig. 3.51) (tetradecanoic acid), the ions at

m/z 60 and 73 are equally abundant. In the spectrum of constituent **3.119** (hexadecanoic acid), the ion at *m/z* 73 is the base peak.

An ion at *m/z* 45 is present in the mass spectra of the lower carboxylic acids, the formation of which has been rationalised as follows (Budzikiewicz *et al*., 1967: 214):



Alkanoic acids containing a methyl group on C-4 have an ion at *m/z* 74 in an abundance that may be even higher than that of the *m/z* 60 ion. The following mechanism is considered the most probable for the formation of this ion (Rol, 1965; Budzikiewicz *et al.*, 1967: 215):



The somewhat enhanced ions at *m/z* 88 and 102 in the mass spectra of 5-methyl and 6-methyl-substituted acids can be explained in a similar way, possibly via larger ring transition states (Budzikiewicz *et al.*, 1967: 215).

The mass spectra of the higher members of the series of constituents under discussion have discernible molecular ions, but they do not have prominent or enhanced ions at *m/z* 74, 88 or 102. However, depending on their carbon chain lengths, they have ions at *m/z* 73, 129 and 185. The presence of these ions does not rule out the possibility of branching at the iso- and anteiso-positions in long-chain carboxylic acids.

The majority of the carboxylic acids present in caracal urine are unbranched and their mass spectra conformed to the above general remarks regarding the mass spectra of unbranched fatty acids. For obvious reasons, some of the ions that are typically present in the higher carboxylic acids are not present in their shorter chain homologues. Constituent **3.104** was identified as formic acid. It is interesting that formic acid, which is quite a strong acid, has recently been found in relatively high concentrations in the preorbital secretion of the bontebok, *Damaliscus pygargus*

pygargus (Wasilewski *et al.*, unpublished results). Acetic acid (**3.105**), propanoic acid (**3.106**) and the unbranched fatty acids from butanoic acid (**3.107**) to octadecanoic acid (**3.121**) were all identified in caracal urine and are listed in Table 3.1.

A few branched and unsaturated fatty acids were also identified in many of the male and female urine samples. The mass spectrum of constituent **3.122** (Fig. 3.52) has a barely detectable ion at *m/z* 186 ($C_{11}H_{22}O_2$) (1%), presumed to be the compound's molecular ion, an ion at *m/z* 60 ($C_2H_4O_2$) (43%) and the base peak at *m/z* 87 ($C_4H_7O_2$). The relatively low abundance of the *m/z* 60 ion was presumed to indicate that this acid could be branched at C-3. A library search (NIST) suggested 3-methyldecanoic acid as the most likely structure. The molecular composition of this constituent was confirmed by the HR spectral data information. Its RI could not be found in the literature and it remained only tentatively identified.

The LR mass spectrum of constituent **3.123** (Fig. 3.53) has prominent ions at *m/z* 74 and 87, and less prominent ions at *m/z* 129, 143 and 157. The ion at *m/z* 200 $(C_{12}H_{24}O_2)^+$ was presumed to be the compound's molecular ion. These ions are also present in the mass spectrum of methyl undecanoate, which contains an ion at *m/z* 169 $(M–OCH_3)^+$. The mass spectrum of constituent **3.123** does not contain an ion at *m/z* 169, hence this constituent cannot be methyl undecanoate. The base peak in this spectrum at *m/z* 74 $(C_3H_6O_2)^+$, which presumably results from a McLafferty rearrangement, was accepted as evidence in favour of this possibility and retention time comparison with a synthetic sample confirms that this conatituent is 2-methylundecanoic acid.

The mass spectra of constituents **3.124** and **3.125** (Figs 3.54 and 3.55, respectively) have molecular ions at *m/z* 242 and contain all the abundant ions present in the mass spectrum of pentadecanoic acid. They elute with slightly shorter retention times than pentadecanoic acid (constituent **3.118**). They are apparently branched pentadecanoic acids. The relative abundances of the *m/z* 60 and 73 ions in the mass spectra of these compounds are in line with the remarks above with regard to the relative abundances of these ions in the unbranched series of fatty acids. It was concluded that the other isomers, **3.124** and **3.125**, could be 13-methyltetradecanoic acid and 12-methyltetradecanoic acid, *iso*- and *anteiso*-pentadecanoic acid, respectively. The most conspicuous difference between the unbranched and iso-branched isomers is that the *m/z* 43 ion is the base peak in the spectrum of the *iso*-pentadecanoic acid. There are some differences in the higher mass range of these spectra, the most conspicuous of which is the prominence of the ion at *m/z* 185 $(M–57)^+$ in the spectrum of *anteiso*-pentadecanoic acid. If, as in the present case, the

spectra are recorded on the same mass spectrometer, it is possible to distinguish between these isomers on the basis of their mass spectra. If not, other diagnostic information is required. The isomers eluted in the order *iso*-, *anteiso*- and *n*-pentadecanoic acid from the apolar as well the polar wax column that were used in the present investigation. Unfortunately, no HR data are available for these two constituents, as they were not detected in the urine samples that were analysed on the GC-HRMS instrument. The identification of constituents **3.124** and **3.125** as *iso*- and *anteiso*-pentadecanoic acid, respectively, was corroborated by retention time comparison with available synthetic reference compounds.

The mass spectrum of constituent **3.126** (Fig. 3.56) contains the same prominent ions as the spectrum of constituent **3.119** (hexadecanoic acid) and it contains a prominent ion at *m/z* 199 $(M–57)^+$. This constituent eluted with a slightly shorter retention time than hexadecanoic acid, and it was presumed to be a branched hexadecanoic acid. Constituent **3.126** was unequivocally identified as 13-methylpentadecanoic acid (*anteiso*-hexadecanoic acid) as it co-eluted with the synthetic analogue.

The mass spectra of constituents **3.127–3.130** (Figs 3.57, 3.58, 3.59 and 3.60, respectively) have *m/z* 60 ions at relative abundances ranging from 18% to 22%. They were presumed to be long-chain fatty acids that undergo McLafferty rearrangement to yield acetic acid as a product. Accepting the ions at highest mass in the spectra of this group of constituents as their molecular ions, they could probably be alkenoic acids with the general molecular formula $C_nH_{2n-2}O_2$. A library search suggested a 9-hexadecenoic acid as the most likely structure for constituent **3.127** with a correlation of 82%. The presence in the spectrum of the compound's molecular ion at *m/z* 254 (5%) and an $(M–18)^+$ ion at *m/z* 236 (8%) supports this assumption. Library searches of the mass spectra of other members of this group of constituents did not produce unequivocal results, but their spectra contained ions that could be assigned to the molecular and $(M-18)^+$ ions of 9-hexadecenoic acid, 9-heptadecenoic acid and 9-octadecenoic acid, respectively. As the double bonds in natural unsaturated long-chain fatty acids commonly have cis configuration, and the trans isomer is expected to elute with a longer retention time than the cis isomer, constituents **3.127** and **3.128** are *cis*- and *trans*-hexadec-9-enoic acid, respectively. This conclusion was supported by their RIs and a commercially available reference sample of the cis isomer co-eluted with constituent **3.127**. Constituents **3.129** and **3.130** were tentatively identified as *cis*-heptadec-9-enoic acid and *cis*-octadec-9-enoic acid, respectively. The RI of *cis*-heptadec-9-enoic acid could

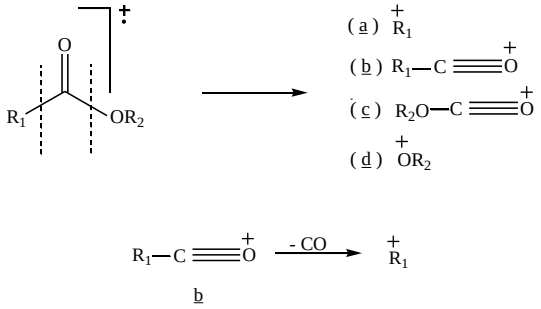
not be found in the literature. The published and experimental RIs of *cis*-9-octadecenoic acid (oleic acid) are 3168 and 3172, respectively.

The ion at highest mass in the spectrum of constituent **3.131** (Fig. 3.61) occurs at *m/z* 280 (6%) and an $(M–18)^+$ ion is present in the spectrum at *m/z* 262 (2%). This constituent was tentatively identified as *cis,cis*-octadeca-9,12-dienoic acid (Linoleic acid), and this was later confirmed by its retention index and by retention time comparison with a commercially available reference standard.

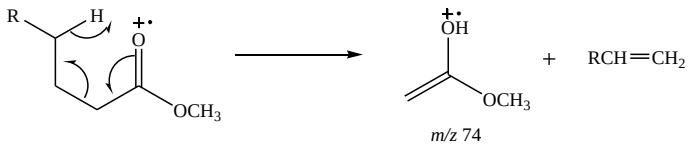
3.2.6 Aliphatic esters

During the first few decades after the invention of GC, fatty acids eluted from most of the then available packed columns with strongly tailing peaks. This made it difficult to detect or quantify less prominent peaks in complex chromatograms. For this reason, the fatty acids were mostly converted into methyl esters that elute as sharp peaks from all stationary phases. The methyl esters of the fatty acids have therefore been studied extensively, for example by Ryhage and Stenhagen (1959a, 1959b, 1959c and 1959d). Except for proteins, long-chain esters are probably the most widely studied natural product class.

The cleavage of a bond adjacent to the carbonyl group of an ester can occur in four ways, to give the ions a, b, c and d in the scheme below. The ion R_1^+ may not be a primary product as it can arise from the ion b by the loss of carbon monoxide.



The ions a and d are normally of low abundance in the spectra of unbranched long-chain esters. However, the ion d could be diagnostically useful. The base peak of unbranched methyl esters arises from β-cleavage with transfer of a γ-hydrogen atom in a McLafferty rearrangement to produce an ion at *m/z* 74 (Budzikiewicz *et al*., 1967: 176):

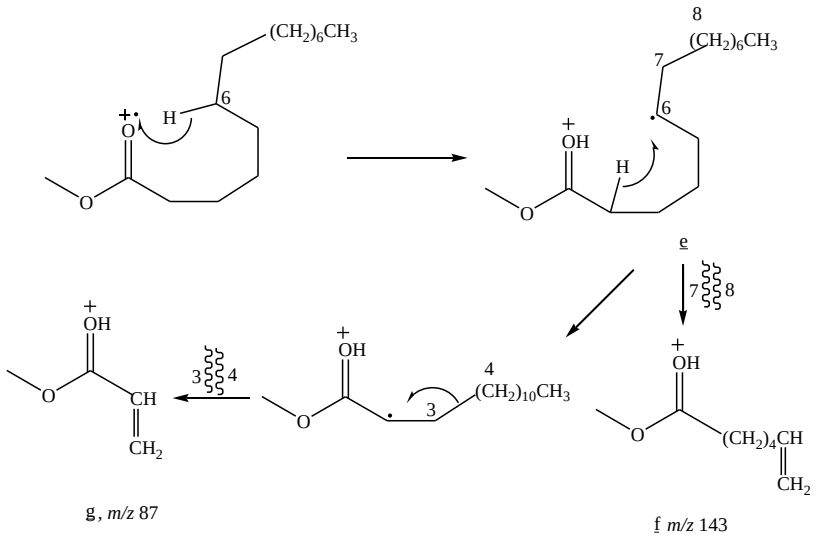


Base peaks are also present at *m/z* 88 and 102 in the spectra of ethyl and propyl esters, respectively.

Constituent **3.132** was only identified in an HRMS analysis of the urine of one female. The mass spectrum of this constituent (Fig. 3.62) does not contain an ion of even mass that could be the molecular ion of the constituent. However, according to the available HR data, the composition of the ions at *m/z* 75 ($C_3H_7O_2$) and *m/z* 87 ($C_4H_7O_2$) suggests that the constituent could be an ester. The constituent's RI and retention time comparison with synthetic reference esters proved that this constituent is propyl propanoate. Similar arguments and supporting information led to the unequivocal identification of constituents **3.133–3.135** as propyl butanoate, butyl propanoate and butyl butanoate, respectively.

The mass spectra of constituents **3.136–3.140**, for example that of constituent **3.138** (Fig. 3.63), have a base peak at *m/z* 74 ($C_3H_6O_2$) and a relatively abundant ion at *m/z* 87 ($C_4H_7O_2$) (32–47%). These two ions are the most prominent features of the mass spectra of the methyl esters of long-chain fatty acids. The base peak at *m/z* 74 is formed by a McLafferty rearrangement as shown above.

As in the mass spectra of the carboxylic acids, the spectra of methyl esters contain a series of ions with the general formula $[(CH_2)_nCOOCH_3]^+$ with n = 2, 3, 4, 5, etc. In the spectra of long-chain methyl esters, the ions with n = 2, 6, 10, etc. are particularly abundant, with the ion at *m/z* 87 being the second most abundant ion in the spectra. The genesis of these ions must be energetically favoured and can be rationalised in terms of hydrogen radical transfer from C-6 to yield the radical cation e, which, upon homolysis of the 7–8 bond, yields the ion f (*m/z* 143). Alternatively, e may undergo hydrogen radical transfer from C-2 to C-6 with associated homolysis of the 3–4 bond to afford g (*m/z* 87) (Budzikiewicz *et al*., 1967: 179).

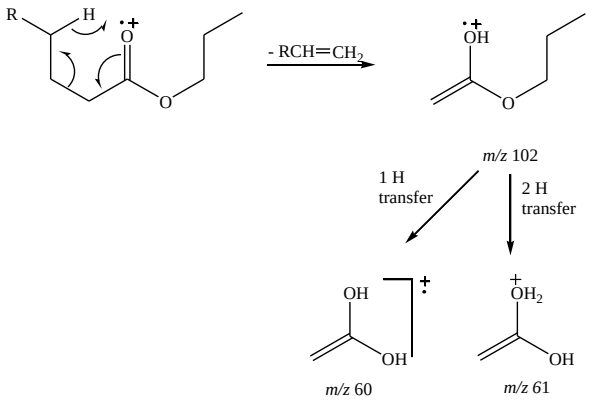


g, *m/z* 87 f *m/z* 143

Constituents **3.136–3.140** were identified as the methyl esters of the C_8–C_{12} fatty acids. The molecular ions of the esters are not detectable in the LR mass spectra of short-chain esters of carboxylic acids containing up to nine carbon atoms. In esters with longer carbon chains, the abundance of the molecular ion increases with increasing chain length. In the LR mass spectrum of methyl decanoate **3.138** (Fig. 3.63), for example, it is present in an abundance of only 1%, and in the spectrum of methyl dodecanoate with an abundance of 2%. The molecular ions of these esters are only marginally more abundant in their HR spectra than in their LR mass spectra, in contrast to the large differences in the abundance of molecular ions that were observed between the LR and HR mass spectra of several ketones. The mass spectra of constituents **3.141** and **3.142** (Fig 3.64) both contain a base peak at *m/z* 88, while the second most abundant ion occurs at *m/z* 101, i.e., a difference of 13 amu, as in the spectra of the methyl esters discussed above. A logical conclusion was that these two constituents are ethyl esters. The ions at highest even mass *m/z* 200 ($C_{12}H_{24}O_2$) (3%) and 228 ($C_{14}H_{28}O_2$) (4%), respectively, were presumed to be these constituents' molecular ions. Constituents **3.141** and **3.142** were identified as unbranched ethyl decanoate and dodecanoate, respectively.

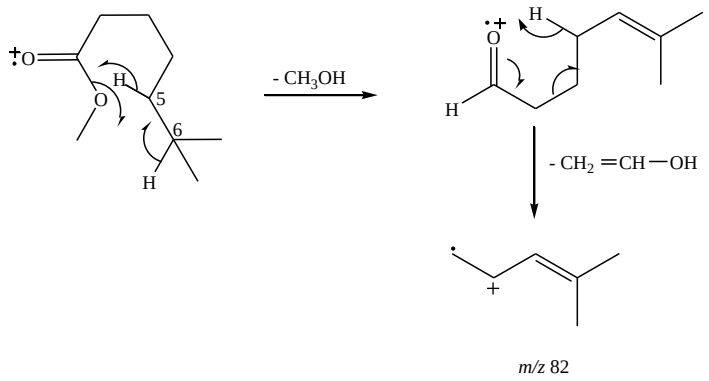
The mass spectrum of constituent **3.143** (Fig. 3.65) contains ions of even mass at *m/z* 60 (44%) and 102 ($C_5H_{10}O_2$) (40%), a series of $(C_nH_{2n-1}O_2)^+$ ions from *m/z* 129 to 185 that are normally present in the spectra of fatty acids and their esters, as well as the base peak at *m/z* 43. The ion at highest mass occurs at *m/z* 228 ($C_{14}H_{28}O_2$) (18%). Except for the high abundance of the *m/z* 43 ion and the even-mass ion at *m/z* 102, this constituent appeared to be a branched fatty

acid. A library search suggested isopropyl tetradecanoate as a possible candidate structure. The library spectrum also contains ions at *m/z* 60 and 102. A fairly abundant rearrangement ion at *m/z* 60 occurs in the spectra of esters when there are four or more carbon atoms in the acid chain and two or more in the alcohol moiety. A hydrogen is first transferred from the γ-position in the acid chain (McLafferty rearrangement), followed by the transfer of a hydrogen from the alcohol moiety, as follows (Budzikiewicz *et al*., 1967: 188):



Constituent **3.143** was identified as isopropyl tetradecanoate. The identification of the above esters was substantiated by their RIs and by retention time comparison with authentic synthetic materials.

The mass spectrum of constituent **3.144** (Fig. 3.66) contains ions at *m/z* 74 and 87 that are typically present in the spectra of methyl esters. However, this constituent's RI cannot be reconciled with it being an unbranched methyl ester. The spectrum also has an unexpected even-mass ion at *m/z* 82 (45%) that could not be ascribed to the incomplete gas chromatographic separation of two constituents. There are a few examples of the presence of $(M-76)^+$ or $(M-90)^+$ ions in the mass spectra of 6-methyl-substituted long-chain methyl esters, the formation of which could be facilitated by the activation of a hydrogen atom attached to C-6 of the alkyl chain (Meyerson and Leitch, 1966). Although the mass spectrum of this constituent does not have a detectable molecular ion, the presence of the *m/z* 82 ion in the spectrum under discussion could be rationalised if it is accepted that this constituent is methyl 6-methylheptanoate (methyl *iso*-octanoate), in which case the formation of the *m/z* 82 ion can be formulated as follows (Budzikiewicz *et al*., 1967: 182):



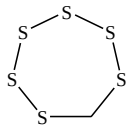
3.2.7 Sulphur and sulphur-containing compounds

Caracal urine does not have an odour that can be described as particularly unpleasant to the human nose; it does not have a rotten-egg odour, for example. It was therefore not surprising that it does not contain high concentrations of the malodorous compounds that are present in the urine tiger, *Panthera tigris* (Burger *et al*., 2008) and lion, *Panthera leo* (McLean *et al*., 2007; Smith *et al*., 1989).

The presence of a sulphur atom in a compound can be inferred from the ratio of the two sulphur isotopes, ^{32}S (95.6%) and ^{34}S (4.4%). Some sulphur-containing compounds show fragmentation patterns comparable to those of their oxygen-containing analogues and their molecular ions are usually more abundant than those of the latter because of the lower electronegativity of the sulphur atom. The TICs of caracal urine samples contain the two peaks of the sulphur allotropes, S_6 and S_8, (**3.145** and **3.146**, respectively), which are not observed in analyses carried out on GCs equipped with a flame ionisation detector. The base peak and molecular ion in the spectrum of S_6 (Fig. 3.67) occurs at *m/z* 192. The abundances of the ions at *m/z* 64, 96, 128, 160 and 192 reflect the relative stability of the S_3 to S_6 rings that can be formed. A similar situation is observed in the mass spectrum of constituent **3.146** (Fig. 3.68), which was identified as S_8.

The isotope ratio of the molecular ions of constituent **3.147** (Fig. 3.69) at *m/z* 206 and 208 indicates that this constituent contains six sulphur atoms. This was confirmed by its HR spectrum, according to which it is CH_2S_6. The base peak of the mass spectrum at *m/z* 142 has the composition CH_2S_4. The other abundant ions in the spectrum consist of a CH_2 plus 2–5 sulphur atoms, and are probably ring structures. This constituent was tentatively identified as hexathiepane. The

prominent peaks at *m/z* 174, 142, 110 and 78 are due to the loss of successive sulphur atoms from the molecular ion at *m/z* 206.

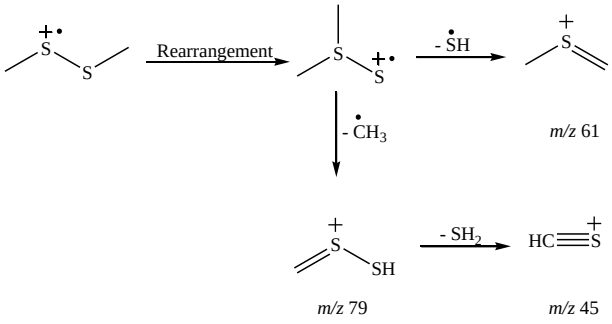


Hexathiepane

The mass spectrum of constituent **3.148** (Fig.3.70) has a prominent ion at *m/z* 94 $(C_2H_6O_2S)^+$, which loses a methyl radical to yield the base peak at *m/z* 79 $(CH_3O_2S)^+$. A library search suggested dimethyl sulphone as a likely candidate structure. The identification of this constituent was substantiated in the usual manner.

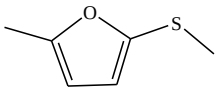
Dimethyl sulphone

As far as the most abundant ions are concerned, the mass spectrum of constituent **3.149** (Fig. 3.71) resembles that of dimethyl sulphone. However, the availability of HR data showed that, instead of only two oxygens atoms, this constituent contains an extra sulphur atom. It was identified as dimethyl disulphide. The formation of the ions at *m/z* 45, 61 and 79 can be rationalised as follows:



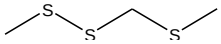
Constituents **3.150–3.152** were likewise tentatively identified as the dimethyl polysulphides, dimethyl trisulphide, dimethyl tetrasulphide and dimethyl pentasulphide, respectively.

Constituent **3.153** was tentatively identified as 2-methyl-5-(methylthio)-furan. The base peak of its mass spectrum (Fig. 3.72) at *m/z* 128 $(C_6H_8OS)^+$ is this constituent's molecular ion and the ion at *m/z* 113 is formed by the loss of CH_3.



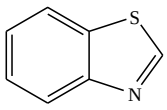
2-methyl-5-(methylthio)-furan

According to the available HRMS data, constituent **3.154** could be either C_3N_4OS or $C_3H_8S_3$. However, the molecular ion (Fig. 3.73) must lose CH_3S to yield the ion at *m/z* 93 $(C_2H_5S_2)^+$, which is not feasible if the parent ion contains only one S atom. The base peak at *m/z* 61 $(C_2H_5S)^+$ cannot be formed from the ion at *m/z* 79 $(CH_3S_2)^+$; it is probably formed from the molecular ion. A library search suggested methyl (methylthio)methyl disulphide as by far the most likely candidate structure. A synthetic reference was not available and this constituent remained tentatively identified.



Methyl(methylthio)methyl disulphide

The base peak in the HR mass spectrum of constituent **3.155** (Fig. 3.74) at *m/z* 135 (C_7H_5NS) is also the constituent's molecular ion. This suggests that this constituent could be an aromatic compound with C-, N- and S-containing substituents or a condensed ring, probably with the C and N in neighbouring positions, which would facilitate the loss of HCN from the molecular ion to yield the ion at *m/z* 108 (38%) $(C_6H_4S)^+$. The constituent was tentatively identified as benzothiazole. This structure was confirmed by co-elution with the synthetic compound.



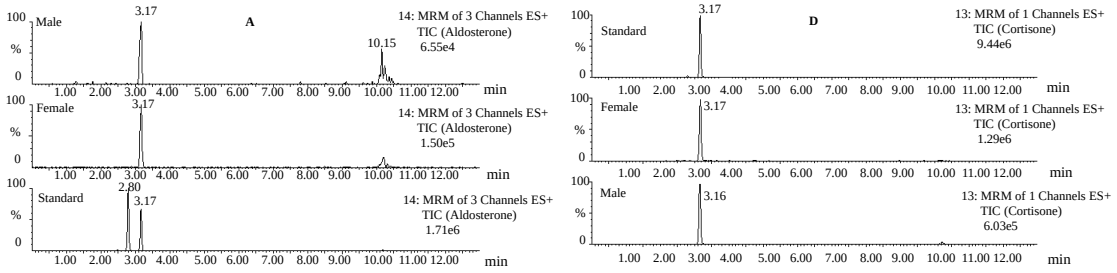
Benzothiazole

3.2.8 Steroids

GC-MS analysis revealed the presence of cholesterol in extracts of caracal urine. At LECUS, several years ago, the then available dual purpose LR/HRMS instrumentation was not helpful in attempts to identify steroids in mammalian secretions and lizard epidermal secretions (e.g., Louw *et al*., 2007a). Later, fortunately, a Waters API Xevo triple quadrupole mass spectrometer became available at Stellenbosch University, and a very successful analytical protocol was developed for the analysis of steroids (Storbeck *et al*., 2013). UPLC interfaced with MS/MS, with use of the electrospray probe in the positive ionisation mode (ESI^+), was chosen for the analysis of urinary steroids extracted from caracal urine, for the following reasons: ease of sample preparation, short analysis time and higher sensitivity of this method, and no necessity for analyte derivatisation. The presence of various steroids was detected using the instrument in the MRM mode (Schloms *et al.*, 2012; Storbeck *et al*., 2013). Identification was based on the ratio's of two MRM transitions for each compound in accurate mass determinations of parent ion and daughter ion, and by comparison of the retention times of the analytes with those of 31 reference standards, as shown in Fig. 3.75.

The steroids identified in male and female urine are listed in Table 3.1. The MS data of the steroids, including the parent ions, daughter ions, cone and collision voltages, are listed in Tables 3.2 and 3.3.

Androstenedione and testosterone were not found in the female urine samples that were analysed. However, it is possible that they were present at concentrations below the detection limit of the mass spectrometer. The male urine contained all five of the identified steroids. Cortisone, aldosterone and cortisol were present in somewhat higher concentrations in the male than in the female urine.



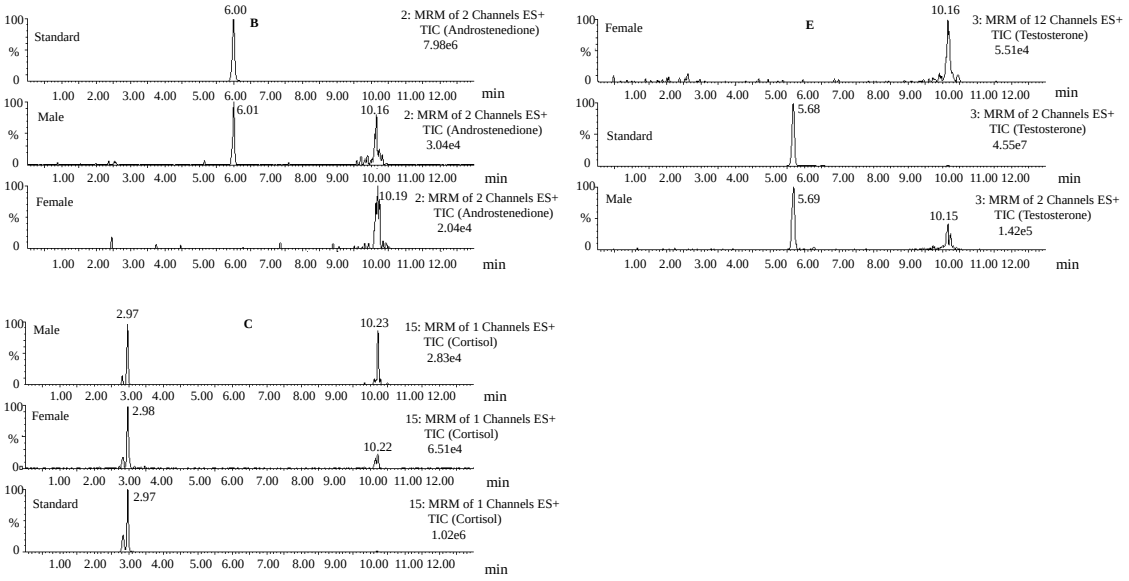


Fig. 3.75: Comparison of the UPLC-MS/MS chromatograms of steroids identified in male and female caracal urine with those of reference standards: A, aldosterone; B, androstenedione; C, cortisol; D, cortisone and E, Testosterone. The broad peak at 10 min in the chromatograms resulted from the presence of non-steroidal constituents.

Table 3.2: MS data pertinent to the identification of the urinary steroids

Compound	Retention time (min)		Parent ion	Daughter ion	Collision Voltage
	Female	Male			
Androstenedione	-	6.01	287.2	96.9	15
			287.2	108.8	15
Testosterone	-	5.69	289.2	97.2	22
			289.2	109	22
Aldosterone	3.17	3.17	361.4	97	32
			361.4	315.1	20
			361.4	343.2	18
Cortisol	2.97	2.98	363	121	20
Cortisone	3.17	3.16	361.2	163	30

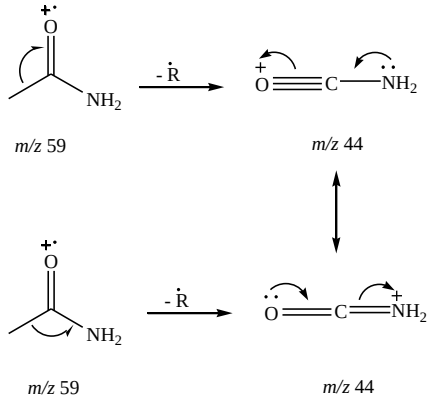
Table 3.3: Quantitation of the urinary steroids

No	Steroids	Retention time (min)		Concentration (ppm)	
		Female	Male	Female	Male
3.156	Androstenedione	-	6.01	-	0.004
3.157	Testosterone	-	5.69	-	0.003
3.158	Aldosterone	3.17	3.17	0.04	0.09
3.159	Cortisol	2.97	2.98	0.03	0.06
3.160	Cortisone	3.17	3.16	0.06	0.14

3.2.9 Miscellaneous unclassified compounds

To avoid further fragmentation of Chapter 3 by subdividing the 35 constituents discussed here into a large number of compound classes and subclasses, they are listed in Table 3.1, in alphabetical order. They are, however, discussed here in a more or less logical order.

The mass spectrum of constituent **3.161** (Fig. 3.76) contains only two prominent ions. Assuming that the ion at *m/z* 59 is the constituent's molecular ion, the constituent contains an uneven number of nitrogen atoms. An ion at *m/z* 44 is typically present in the spectra of short-chain aliphatic amides (Gilpin, 1959). The *m/z* 44 results from the expulsion of a methyl radical from the molecular ion of acetamide as follows (Budzikiewicz *et al*., 1967: 336):

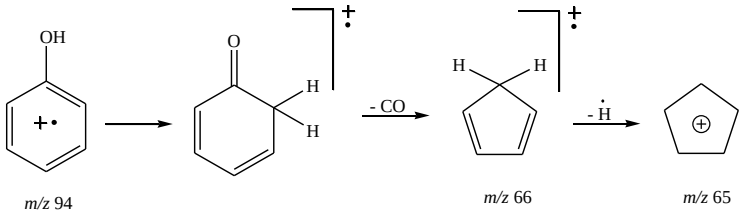


If a carbon chain with a γ-hydrogen is available, a McLafferty rearrangement predominates and the *m/z* 44 ion is much less abundant.

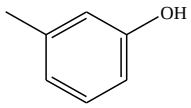
As the mass spectrum of constituent **3.173** (Fig. 3.77) has remarkably many even-numbered ions, this constituent was considered to be a compound containing an uneven number of nitrogen atoms. It was only found in one sample of female urine and no HR data were available. However, the ion at *m/z* 59 could be the nitrogen-containing product of a McLafferty rearrangement, which could then be accepted as evidence that this constituent is a long-chain, probably unbranched, aliphatic amide, and that the ion at *m/z* 255 is its molecular ion. The results of a library search concurred with these observations and constituent **3.173** was tentatively identified as hexadecanamide.

The ion at *m/z* 94 in the mass spectrum of constituent **3.181** (Fig. 3.78) is the base peak in the spectrum, as well as the constituent's molecular ion. The constituent was identified as phenol.

The formation of the ions at *m/z* 65 and 66 can be rationalised as follows (Beynon *et al*., 1959; Budzikiewicz *et al*., 1967: 116).



The general appearance of the mass spectrum of constituent **3.178** (Fig. 3.79) suggests that it is probably an aromatic compound. According to the available HR data, it has the molecular formula C_7H_8O. It was presumed to be a cresol isomer. The only other relatively abundant ions are present at *m/z* 77 (C_6H_5) and 79 (C_6H_7) in approximately the same relative abundances in all the cresol isomers. Retention time comparison with an authentic sample of the cresol isomers was thus used to identify this constituent as *m*-cresol (3-methylphenol). The mass spectra of the cresols exhibit a very abundant M-1 ion, which is presumably due to the formation of a hydroxyl tropylium ion (Budzikiewicz *et al*., 1967: 117).



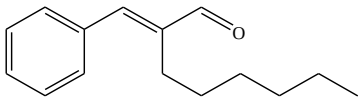
m-Cresol

Having abundant M^+ and $(M-1)^+$ ions at *m/z* 106 $(C_7H_6O)^+$ and 105 $(C_7H_5O)^+$, and an ion at *m/z* 77 $(C_6H_5)^+$ (phenyl), the mass spectrum of constituent **3.165** (Fig. 3.80) has features similar to those of *m*-cresol, i.e., an aromatic ring with a substituent that cannot take part in the formation of a tropylium ion $(C_7H_7)^+$. This constituent was identified as benzaldehyde.

A similar situation was encountered in the spectrum of constituent **3.175** (Fig. 3.81). In this case, however, the spectrum contains neither an ion at *m/z* 77, nor a tropylium ion at *m/z* 91. HR data were unfortunately not available, but if the ion at 122 is accepted as the constituent's molecular ion, then this constituent could be a hydroxybenzaldehyde. A library search suggested the *o*-, *m*- and *p*-isomers with almost the same confidence. The library spectrum of *p*-hydroxybenzaldehyde, however, contains ions at *m/z* 39 and 76 that are absent or of very low abundance in the spectrum of the *o*-isomer. The library spectrum of *o*-hydroxybenzaldehyde has

an ion at *m/z* 104 (20%) that is present in the spectrum under discussion, but it is absent or of very low abundance in the spectra of the *m*- and *p*-isomers. The ion at *m/z* 104 is probably formed by the elimination of a molecule of water. Retention time comparison with authentic reference compounds confirmed this constituent to be 2-hydroxybenzaldehyde (salicylaldehyde).

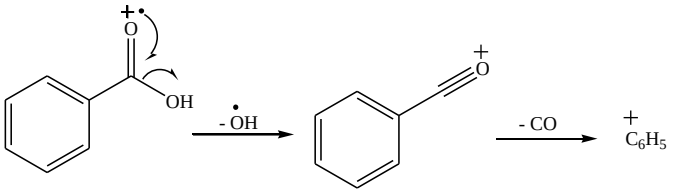
Constituent **3.174** was only found in a male urine sample that was analysed on the HR mass spectrometer. The general appearance of its mass spectrum (Fig. 3.82), combined with the relatively high abundances of the ions at *m/z* 91 $(C_7H_7)^+$ and the molecular ion at *m/z* 216 (61 %) $(C_{15}H_{20}O)^+$, indicated that this constituent could be an aromatic compound. The loss of a series of fragments (C_nH_{2n-1}) gives rise to the ions at *m/z* 173, $(C_{12}H_{13}O)^+$, 159 $(C_{11}H_{10}O)^+$ and 145 $(C_{10}H_9O)^+$. Apparently the compound contains an aromatic moiety and an unbranched alkyl chain with at least five carbon atoms. With the exception of the molecular ion and the ion at *m/z* 145 $(C_{10}H_9O)^+$, all the other abundant ions apparently contain a substituted benzene ring. Despite the availability of HRMS data, it was not possible to suggest a plausible structure without carrying out a library search, which suggested α-hexylcinnamaldehyde (2-phenylmethyleneoctanal) as a possible structure. The natural compound co-eluted with commercially available α-hexylcinnamaldehyde.



α-Hexylcinnamaldehyde

Having three ions at *m/z* 122, 105 and 77 with abundances higher than 50%, the LR mass spectrum of constituent **3.166** (Fig. 3.83) has the general appearance of that of an aromatic compound. If the ion at highest mass at *m/z* 122 is accepted as the constituent's molecular ion, the formation of the ion at *m/z* 105 can be ascribed to the loss of a hydroxyl radical. Constituent **3.166** was eluted from the apolar column as a broad peak, exhibiting the strong fronting that is typically observed in analyses of carboxylic acids on apolar columns. Unfortunately the constituent was not found in the samples that were analysed on the HR

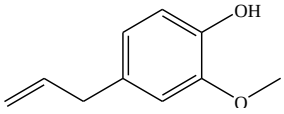
instrument. However, it was identified as benzoic acid. The expulsion of a hydroxyl radical and



m/z 122 *m/z* 105 *m/z* 77

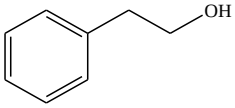
the formation of the ion at *m/z* 77 are formulated as follows (Budzikiewicz *et al*., 1967: 219):

Constituent **3.171** was identified only in urine samples analysed on the HR mass spectrometer. The molecular ion of the constituent at *m/z* 164 $(C_{10}H_{12}O_2)^+$ is also the base peak of the spectrum (Fig. 3.84). The ion at *m/z* 91 is of relatively low abundance. It was hypothesised that, although this constituent could not be an aromatic compound of the type that would fragment in a simple manner to produce a tropylium ion, the aromatic ring could nevertheless contain a suitable hydrocarbon substituent. The molecular ion loses a methyl radical to yield the ion at *m/z* 149 $(C_9H_9O_2)^+$. The expulsion of a vinyl group and CH_3O from the molecular ion yields the ions at *m/z* 137 $(C_8H_9O_2)^+$ and 133 $(C_9H_9)^+$, respectively. A library search suggested 2-methoxy-4-(2-propenyl)-phenol (eugenol) as a possible candidate structure. This identification was confirmed in the usual manner.



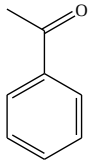
Eugenol

The base peak of the mass spectrum of constituent **3.182** (Fig. 3.85) occurs at *m/z* 91. As mentioned above, this ion is a common feature in the mass spectra of mono-substituted aromatic compounds from which a tropylium ion $(C_7H_7)^+$ can be formed. The molecular formula of the constituent under discussion is $C_8H_{10}O$ and the presence of an ion at *m/z* 91 thus indicates that it contains a phenyl group substituted with a C_2H_5O moiety. The identification of this constituent as 2-phenylethanol was confirmed in the usual manner.



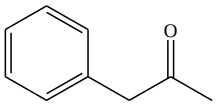
2-Phenylethanol

According to the available HR data, the mass spectrum of constituent **3.162** (Fig. 3.86) contains three prominent ions at *m/z* 120, 105 and 77, ascribed to $C_8H_8O^+$, $C_7H_5O^+$ and $C_6H_5^+$, respectively. Apparently this is an aromatic compound that does not have a substituent that can participate in the formation of a tropylium ion at *m/z* 91. Thus the molecular ion of this aromatic compound loses a methyl radical to yield the ion at *m/z* 105. This ion then loses CO, or, otherwise, the molecular ion loses CH_3CO, to yield the ion at *m/z* 77 (C_6H_5). This constituent was identified as 1-phenylethanone (acetophenone).



Acetophenone

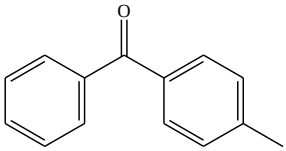
Unfortunately, the urine samples analysed on the HR mass spectrometer did not contain constituent **3.183**. The base peak of the mass spectrum of this constituent (Fig. 3.87) occurs at *m/z* 43, the spectrum has an abundant ion at *m/z* 91, and the ion at highest mass at *m/z* 134 could be its molecular ion. The high abundance of the ion at *m/z* 43 was tentatively interpreted as an indication of the presence of an acetyl moiety in the molecule. Of the possible structures that can be formulated for this constituent, 1-phenyl-2-propanone appeared to be the most likely.



1-Phenyl-2-propanone

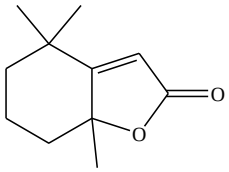
Constituent **3.177** was identified only in one sample of male urine, analysed on the HR mass spectrometer. According to the composition of the constituent's molecular ion at 196 ($C_{14}H_{12}O^+$) (67%), it contains nine double bond equivalents, i.e., double bonds and/or rings. The spectrum (Fig. 3.88) has an ion at *m/z* 91 (14%) $(C_7H_7)^+$ and an ion at *m/z* 181 (13%) $(C_{13}H_9O)^+$, which indicates the presence of at least one methyl-substituted benzene ring. Of the limited number of possible structures, a methyl-substituted benzophenone appeared to be the most likely structure. A library search revealed that the base peak of 2-methylbenzophenone occurs at *m/z*

196 (the molecular ion), whereas the base peaks of 3-methyl- and 4-methylbenzophenone are both at *m/z* 119. It was concluded that constituent **3.177** is not 2-methylbenzophenone. The LR library spectra of the other two isomers differ in that the molecular ion of 3-methylbenzophenone is more abundant that that of the 4-methyl isomer. However, the difference between the mass spectra of these two isomers is so subtle that it would be risky to reach a conclusion on the comparison of a HR mass spectrum obtained on a TOF instrument with LR spectra. This constituent co-eluted with a synthetic sample of 4-methylbenzophenone.



4-Methylbenzophenone

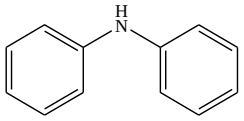
Constituent **3.189** was present in male as well as female urine. Its LR mass spectrum is depicted in Fig. 3.89. According to the available HR data, the ion at *m/z* 180 $(C_{11}H_{16}O_2)^+$ (30%) could be accepted as the constituent's molecular ion, while its base peak occurs at *m/z* 111 $(C_6H_7O_2)^+$. In addition to several ions present in low abundance, abundant ions are present at *m/z* 109 $(C_8H_{13})^+$ (34%) and 137 $(C_9H_{13}O)^+$ (40%). The LR mass spectrum contains an ion at *m/*z 43 (85%). The latter could not be detected with the HR instrument. A library search suggested only one structure with a mass spectrum matching that of the natural product reasonably well (80%): 4,4,7a-trimethyl-5,6,7,7a-tetrahydro-2(4H)-benzofuranone. A reference compound was not available and constituent **3.189** remained tentatively identified.



4,4,7a-Trimethyl-5,6,7,7a-tetrahydro-2(4H)-benzofuranone

In the mass range for which reliable HR data were obtained, the mass spectrum of constituent **3.187** (Fig. 3.90) contains only three ions, at *m/z* 57 $(C_3H_7N)^+$, 58 $(C_3H_8N)^+$ and 59 $(C_3H_9N)^+$. This constituent was identified as trimethylamine and was found only in one female urine sample.

Constituent **3.170** was found only in a sample of female urine analysed on the HR mass spectrometer. According to the HR data (only reliable data above *m/z* 97), the ion at *m/z* 169 $(C_{12}H_{11}N)^+$ could be the constituent's molecular ion. The HR mass spectrum (Fig. 3.91) contains three ions at *m/z* 168 $(C_{12}H_{10}N)^+$ (63%), 167 (34%) and 166 (3%) that could be ascribed to the consecutive loss of hydrogen atoms. The ions at *m/z* 142 $(C_{11}H_{10})^+$ and 141 $(C_{11}H_9)^+$, although present in low abundance, represent significant (M-HCN) and (M-H_2CN) rearrangement ions (Budzikiewicz *et al*., 1967: 135). The identification of this constituent as diphenylamine was confirmed by retention time comparison with a synthetic sample of the compound.



Diphenylamine

The base peak at *m/z* 79 in the mass spectrum of constituent **3.185** (Fig. 3.92) is also its molecular ion. This constituent could be an aromatic compound containing one nitrogen atom. The loss of hydrogen cyanide yields the only other abundant ion in the spectrum. This mode of fractionation is typical for certain aromatic heterocyclic compounds (Jennings and Boggs, 1964). The constituent was identified as pyridine.

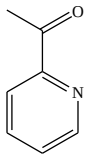
Similarly, constituent **3.179** (mass spectrum 3.93) was identified as a methyl-substituted pyridine. The ions at *m/z* 39 and 78 in the mass spectrum of 2-methylpyridine are of much lower relative abundance than those in the mass spectra of 3- and 4-methylpyridine, and it was concluded that this constituent could not be 2-methylpyridine. The mass spectra of 3- and 4-methylpyridine are practically identical. This constituent was identified as 3-methylpyridine by retention time comparison with an authentic reference sample.

The base peak of the mass spectrum of constituent **3.169** (Fig. 3.94) at *m/z* 107 $(C_7H_9N)^+$ was tentatively accepted as this constituent's molecular ion. Alkyl-substituted pyridines appeared to be the most likely candidate structures for this constituent. Comparison of the constituent's HR spectrum with LR library spectra clearly shows that this constituent is neither 3- nor 4-ethylpyridine, as the loss of a methyl fragment from the parent ion is strongly favoured in these two ethylpyridines. This is not the case, however, in 2-ethylpyridine, where the M-1 peak is the base peak. The constituent was tentatively identified as 2,6-dimethylpyridine based on the similar

relative abundances of the ions at *m/z* 65 $(C_5H_5)^+$, 66 $(C_5H_6)^+$ and 79 $(C_6H_7)^+$ in the spectrum of constituent **3.169** and those in the library spectrum of 2,6-dimethylpyridine, *viz*., 16:23:9% and 18:21:8%, respectively, although this is rather weak evidence, seeing that an HR spectrum is here compared with LR reference spectra. The constituent was identified as 2,6-dimethylpyridine based on retention time comparison with a synthetic sample.

The base peak in the mass spectrum of constituent **3.190** (Fig. 3.95) was tentatively accepted as this constituent's molecular ion. A library search suggested 2,3,6-, 2,4,6- and 2,3,5-trimethylpyridine as possible structures for this constituent with practically identical correlations. It is thus impossible to distinguish between the various trimethylpyridine isomers on mass spectrometric evidence alone. As a starting point 2,4,6-trimethylpyridine was selected for retention time comparison. The RIs of the component in the urine and published for 2,4,6-trimethylpyridine are 1369 and 1365, respectively and this structure was confirmed by retention time comparison with the synthetic compound.

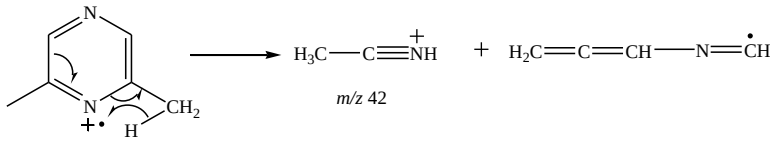
If the ion at *m/z* 121 in the mass spectrum of constituent **3.163** (Fig. 3.96) is its molecular ion, the constituent contains an uneven number of nitrogen atoms. Unfortunately no HRMS data are available as this constituent was not present in the urine samples that were analysed on the HR instrument. However, it seems reasonable to presume that the ions at *m/z* 93 and 78 could be formed by the loss of CO and CH_3CO, respectively, from the molecular ion. The base peak appears at *m/*z 79. Constituent **3.163** could thus be an acetylpyridine. α-Cleavage with hydrogen rearrangement is a favoured process in pyridines having a C_2 unit in position 2 (Spiteller, 1966). In the case of 2-acetylpyridine, ketene is eliminated, which yields the abundant ion at *m/z* 79. This ion does not occur in the spectra of 3- and 4-acetylpyridine (Spiteller, 1966). The constituent's RI of 1594 is close enough to the NIST RI of 1590 to accept this structure as probably correct. Co-elution of the natural compound and the commercially available synthetic analogue proved this assumption correct.



2-Acetylpyridine

The general appearance of the mass spectrum of constituent **3.164** (Fig. 3.97) and the relative abundances of the most abundant ions in the spectrum are remarkably similar to that of constituent **3.162**. This constituent appears to be a methyl-substituted 2-acetylpyridine. This assumption is supported by the available HRMS data. A library search also suggested 2-acetyl-6-methylpyridine as a likely candidate structure. Although, the constituent's experimental RI of 1102 differs slightly from the NIST RI of 1107. This constituent remained tentatively identified.

In the mass spectrum of constituent **3.168** (Fig. 3.98), the molecular ion and base peak occurs at even mass, *m/z* 108 $(C_6H_8N_2)^+$. The molecular ions of simple pyrazines can be misleading if HR data are not available. The only indication that constituent **3.168** could be a pyrazine is the relatively abundant ion at *m/z* 42 $(C_2H_4N)^+$. The mass spectra of several polyalkylated pyrazines have been discussed by Biemann (1962: 132, 184–186). The genesis of the ions in the spectra of polyalkylated pyrazines cannot be explained by simple one-bond fragmentations and the elimination of neutral fragments, with the exception of a hardly detectable M-1 ion in the spectra of some of them. In the alkylated pyrazines, all the side chains are attached to carbon atoms adjacent to nitrogen, which influences the fragmentation (β and γ) of the side chains (Budzikiewicz *et al*., 1967: 583). The pyrazines found in caracal urine are, however, all polymethyl-substituted, and the fragmentation of side chains, for example, McLafferty rearrangements, does not need be considered here. The molecular ion of 2,5-dimethylpyrazine has a very low abundance of only about 8%. The ion at *m/z* 42 (protonated acetonitrile) is the base peak in its spectrum. The formation of this ion can be rationalised as follows:

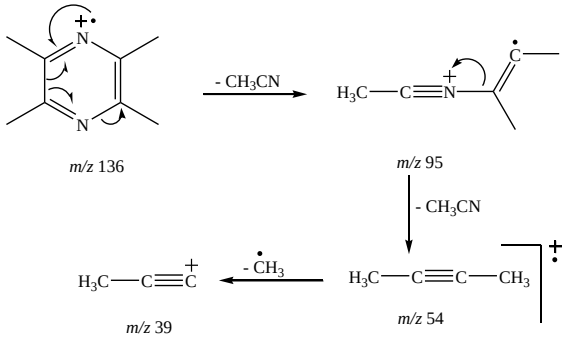


m/z 108

The ion at *m/z* 67 is the base peak in the spectrum of 2,3-dimethylpyrazine. Constituent **3.168** (Fig. 3.98) was tentatively identified as 2,6-dimethylpyrazine. The identification of this and the other pyrazines was largely based on the results of library searches and retention time comparison with synthetic reference compounds. Nevertheless, care has to be taken as there are only subtle differences between the spectra of some dimethylpyrimidines and those of the dimethylpyridines.

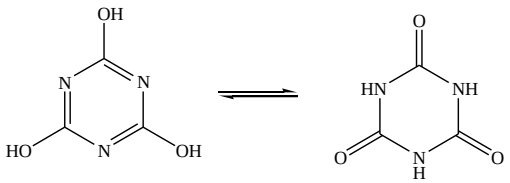
The ion at *m/z* 42 $(C_2H_4N)^+$ is the base peak in the mass spectrum of constituent **3.188** (Fig. 3.99). This compound's molecular ion at *m/z* 122 $(C_7H_{10}N_2)^+$ could indicate that this constituent could be trimethylpyrazine or an ethylmethylpyrazine. Following the same approach as above, it was identified as trimethylpyrazine.

The diagnostic ion at *m/z* 42 also occurs in the mass spectrum of constituent **3.186** (Fig. 3.100). HR data were not available for this constituent, but if the ion at *m/z* 136 is accepted as the constituent's molecular ion, the compound could be a pyrazine with at least one methyl group as substituent. If the pyrazine ring carries only methyl groups, as in the previous case, then the formation of the other prominent peaks in its spectrum at *m/z* 39, 54 (base peak) and 95 can be rationalised as follows (Budzikiewicz *et al*., 1967: 583):



This constituent was identified as tetramethylpyrazine.

The base peak of the mass spectrum of constituent **3.167** (Fig. 3.101), which is also its molecular ion, occurs at *m/z* 129. According to the available HR data, its molecular formula is $C_3H_3N_3O_3$. A library search suggested triazine-2,4,6-triol (cyanuric acid) as a likely candidate structure, albeit with a correlation of only 60%. However the constituent co-eluted with synthetic cyanuric acid. This compound is only slightly soluble in water and it can exist in two tautomeric forms, formulated below. The keto form predominates in solid cyanuric acid and the enol form in solution.

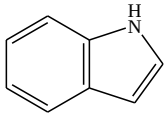


Enol form Keto form

Cyanuric acid

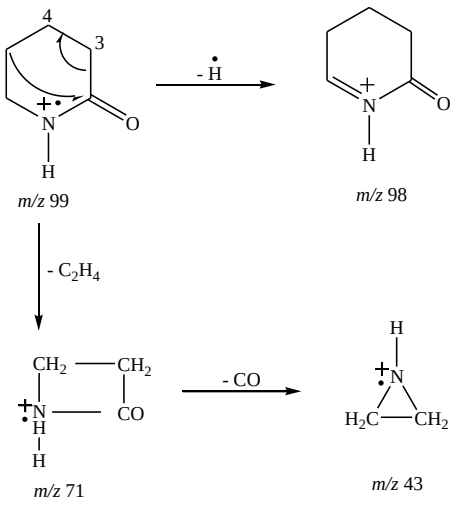
The mass spectrum of constituent **3.191** (Fig. 3.102) contains only two abundant ions and, according to the available HR data, its molecular composition at *m/z* 60 is $(CH_4N_2O)^+$. The expulsion of NH_2 yields the ion at *m/z* 44 $(CH_2NO)^+$. This constituent was identified as urea, a compound that is excreted by most terrestrial vertebrates (Brown *et al*., 1975). The ions at *m/z* 43 and 42 in its mass spectrum are formed by successive losses of hydrogen atoms from the ion at *m/z* 44 (Chen and Isa, 1998).

The LR mass spectrum of constituent **3.176** is depicted in Fig. 3.103 If the ion at *m/z* 117 $(C_8H_7N)^+$, which is also the base peak, is accepted as the constituent's molecular ion, then the typical appearance of the spectrum suggests that this constituent could be a heterocyclic aromatic compound. The expulsion of HCN from the molecular ion could then yield the ion at *m/z* 90 $(C_7H_6)^+$ (48%). The compound was tentatively identified as indole, although it was suggested with a relatively low correlation in a library search. The identification was nonetheless confirmed, in the usual manner.

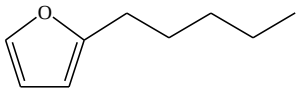


Indole

The base peak at *m/z* 99 in the LR spectrum of constituent **3.184** (Fig. 3.104) is commonly present in the spectra of δ-lactones. However, in this case, although the base peak *m/z* 99 ion is isobaric with the base peak of δ-lactones, it has the composition $(C_5H_9NO)^+$. The LR and HR spectra contain ions of relatively low abundance at *m/z* 98 $(C_5H_8NO)^+$, 71 $(C_3H_5NO)^+$, 70 $(C_4H_6O)^+$, 55 $(C_3H_3O)^+$ and 43 $(C_2H_5N)^+$. Credible HR data were not available for the ions in the mass range below *m/z* 43. The stability of the molecular ion of this constituent suggests that it could be a cyclic compound, for example, a 2- or 4-piperidone. Budzikiewicz *et al*. (1967: 353) has remarked that the fragmentation of small-ring saturated lactams could be very complicated. A library search suggested only one structure of which the mass spectrum correlated reasonably well with that of constituent **3.184**, namely, δ-valerolactam (2-piperidone). The following fragmentation of this compound has been suggested by Duffield *et al.* (1964) and Budzikiewicz *et al*. (1967: 356). The identification of this constituent was confirmed by retention time comparison with synthetic piperidone.

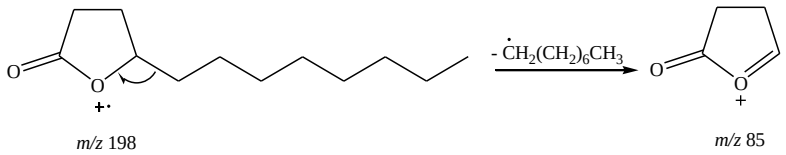


If the ion at *m/z* 138 $(C_9H_{14}0)^+$ (13%) is accepted as the molecular ion in the mass spectrum of constituent **3.180** (Fig. 3.105), the stability of the base peak at *m/z* 81 $(C_5H_5O)^+$ could probably indicate that this constituent could have a cyclic structure in which the oxygen is included in the ring, as in a substituted furan, for example. This constituent was unequivocally identified as 2-pentylfuran.



2-Pentylfuran

Unbranched γ-lactones can readily be located in complex mixtures and distinguished from δ-lactones. This is because γ-lactones are characterised by the presence of an extraordinarily abundant base peak at *m/z* 85 in their mass spectra, whereas the spectra of δ-lactones have a somewhat less abundant, but nevertheless prominent, base peak at *m/z* 99. Probably due to the stability of the *m/z* 85 ion, the rest of the spectra of long-chain γ-lactones contain very little diagnostic information and their molecular ions are often hardly detectable. The mass spectrum of constituent **3.172** (Fig. 3.106) is an example in point.



m/z 198 *m/z* 85

This constituent was identified as γ-dodecalactone (4-octylbutyrolactone, dodecan-4-olide, or 5-octyl-4,5-dihydrofuran-2(3*H*)-one) by retention time comparison with the synthetic analogue.

Referring to Table 3.1, the components 3-carene, kaurene, methyl(methylthio)methyl disulphide and 4,4’7a-trimethyl-5,6,7,7a-tetrahydro-2(4H)-benzofuranone probably were incorrectly identified. The published RIs values of some compounds was not available and was not provided in the table. The RIs of 2-butanone and trimethylamine could not be determined as these compounds elute before the first alkane in the homologues series.

Table 3.1: Compounds identified in male and female caracal urine[a]

No	Compound	Urine[b]		Identification			
		Male	Female	HRMS[c]	Rt[d]	Exp RI[e]	Lit RI[f]
Alkanes							
3.1	Octane	1	1		✓	800	
3.2	Nonane	3	2	✓	✓	900	
3.3	Decane	5	3	✓	✓	1000	
3.4	Undecane	6	3	✓	✓	1100	
3.5	Dodecane	7	4	✓	✓	1200	
3.6	Tridecane	4	2	✓	✓	1300	
3.7	Tetradecane	6	4	✓	✓	1400	
3.8	Pentadecane	6	6	✓	✓	1500	
3.9	Hexadecane	5	4	✓	✓	1600	
3.10	Heptadecane	4	4	✓	✓	1700	
3.11	Octadecane	2	3		✓	1800	
3.12	Nonadecane	4	2		✓	1900	
3.13	Icosane	1	2		✓	2000	
3.14	Heneicosane	2	1		✓	2100	
3.15	Docosane	2	2		✓	2200	
3.16	Tricosane	2	2		✓	2300	
3.17	Tetracosane	2	2		✓	2400	
3.18	Pentacosane	2	2		✓	2500	
3.19	Hexacosane	2	2		✓	2600	
3.20	Heptacosane	2	1		✓	2700	
3.21	Octacosane	2	2		✓	2800	
3.22	Squalane	2	1		✓	2658	2660
Alkenes							
3.23	1-Undecene	2	0			1087	1091
3.24	1-Tetradecene	1	0		✓	1391	1389
3.25	1-Pentadecene	6	0		✓	1492	1494
3.26	Limonene	5	5	✓	✓	1024	1027
3.27	3-Carene	1	0	✓		1092	1011
3.28	Kaurene	0	1	✓		1980	2034
3.29	*cis*-2-Phytene	7	3	✓		1786	
3.30	*trans*-2-Phytene	4	2	✓		1846	
3.31	Squalene (*isomer 1*)	4	2		✓	2803	2812
3.32	Squalene (*isomer 2*)	4	2		✓	2821	2830
Alkanols							
3.33	1-Pentanol	1	1	✓	✓	1254[w]	1255
3.34	1-Hexanol	0	1		✓	1361[w]	1360
3.35	1-Heptanol	0	1		✓	1463[w]	1462
3.36	1-Octanol	2	2		✓	1563[w]	1560
3.37	1-Nonanol	3	2		✓	1667[w]	1668
3.38	1-Decanol	0	2		✓	1767[w]	1767
3.39	1-Dodecanol	2	2		✓	1973[w]	1970
3.40	1-Tetradecanol	2	2		✓	2178[w]	2174
3.41	1-Hexadecanol	0	1		✓	2384[w]	2382
3.42	2-Tetradecanol	0	2		✓	2030[w]	2033
3.43	2-Ethyl-1-hexanol	3	6	✓	✓	1499[w]	1499

Table 3.1: *Contd.*

3.44	2,6-Dimethylcyclohexanol	1	3	✓		1096	1098
3.45	Linalool	0	2		✓	1558 [w]	1558
3.46	Nerolidol	6	3		✓	2046 [w]	2046
3.47	*cis*-Phytol	5	2	✓	✓	2572 [w]	2570
3.48	*trans*-Phytol	5	2	✓	✓	2611 [w]	2617
Aliphatic aldehydes							
3.49	Hexanal	2	2	✓	✓	801	802
3.50	Heptanal	1	2	✓	✓	904	902
3.51	Octanal	2	4	✓	✓	1002	1001
3.52	Nonanal	7	3	✓	✓	1105	1102
3.53	Decanal	7	6	✓	✓	1202	1200
3.54	Dodecanal	0	1		✓	1409	1412
3.55	Octadecanal	1	1		✓	1996	1999
3.56	*cis*-2-Decenal	0	1	✓	✓	1259	1259
3.57	*trans*-2-Undecenal	0	1	✓	✓	1367	1369
3.58	2,6,6-Trimethyl-1-cyclohexene-1-carboxaldehyde	7	7	✓	✓	1213	1216
3.59	2,6,6-Trimethyl-1-cyclohexene-1-acetaldehyde	1	1	✓	✓	1249	1251
Aliphatic ketones							
3.60	2-Butanone	3	2		✓		
3.61	2-Pentanone	6	3	✓	✓	706	703
3.62	3-Pentanone	4	1	✓	✓	709	707
3.63	2-Hexanone	4	1		✓	796	795
3.64	3-Hexanone	5	2	✓	✓	791	791
3.65	2-Heptanone	6	3		✓	887	890
3.66	4-Heptanone	6	1	✓	✓	868	871
3.67	2-Octanone	3	1		✓	988	992
3.68	3-Octanone	2	0		✓	986	985
3.69	2-Nonanone	7	5	✓	✓	1091	1091
3.70	3-Nonanone	5	0		✓	1086	1089
3.71	4-Nonanone	2	0		✓	1030	1030
3.72	2-Decanone	4	2		✓	1192	1192
3.73	3-Decanone	1	0		✓	1191	1195
3.74	2-Undecanone	5	3		✓	1291	1294
3.75	3-Undecanone	1	0			1284	1283
3.76	4-Undecanone	1	0		✓	1210	1208
3.77	2-Dodecanone	4	2		✓	1397	1397
3.78	3-Dodecanone	3	2		✓	1386	1387
3.79	2-Tridecanone	6	2	✓	✓	1489	1491
3.80	2-Pentadecanone	2	0	✓	✓	1695	1694
3.81	2-Heptadecanone	2	0		✓	1894	1897
3.82	*trans*-3-Hepten-2-one	0	3		✓	933	937
3.83	*trans*-3-Nonen-2-one	2	2		✓	1505 [w]	1508
3.84	3-Methyl-2-heptanone	3	0			938	937
3.85	5-Methyl-2-heptanone	2	0			1252 [w]	1256
3.86	6-Methyl-2-heptanone	3	2		✓	954	956
3.87	2-Methyl-3-octanone	3	3			985	985
3.88	5,9-Dimethyl-2-decanone	4	0			1643 [w]	
3.89	*trans*-6,10-Dimethyl-2-undecanone	4	2		✓	1663 [w]	1661
3.90	6-Methyl-5-hepten-2-one	2	2	✓	✓	1314 [w]	1313
3.91	*trans*-6-Methyl-3,5-heptadien-2-one	5	2		✓	1585 [w]	1582
3.92	*trans*-6,10-Dimethyl-5,9-undecadien-2-one	1	6	✓	✓	1851 [w]	1850
3.93	3-Methylcyclopentanone	0	3	✓	✓	847	847

Table 3.1: *Contd.*

3.94	3-Methylcyclohexanone	0	1	✓	✓	935	931
3.95	3-Methylcycloheptanone	6	1			1316 [w]	
3.96	2,2,6-Trimethylcyclohexanone	0	5	✓	✓	1033	1035
3.97	Isophorone	2	2	✓	✓	1136	1138
3.98	β-Ionone	2	3	✓	✓	1483	1485
3.99	Cyclotridecanone[g]	2	1		✓	1579	
3.100	Cyclotetradecanone[g]	2	1		✓	1679	
3.101	Cyclopentadecanone	9	6	✓	✓	1782	
3.102	Cyclohexadecanone	4	4		✓	1862	
3.103	Cycloheptadecanone	4	3	✓		1942	
Aliphatic acids							
3.104	Formic acid	2	1		✓	1540 [w]	1543
3.105	Acetic acid	5	1		✓	1459 [w]	1460
3.106	Propanoic acid	0	1	✓	✓	1551 [w]	1550
3.107	Butanoic acid	0	1	✓	✓	1637 [w]	1637
3.108	Pentanoic acid	0	2	✓	✓	1747 [w]	1746
3.109	Hexanoic acid	1	1	✓	✓	1859 [w]	1860
3.110	Heptanoic acid	0	1		✓	1967 [w]	1971
3.111	Octanoic acid	2	2		✓	2069 [w]	2070
3.112	Nonanoic acid	2	2		✓	2184 [w]	2180
3.113	Decanoic acid	3	2		✓	2291 [w]	2294
3.114	Undecanoic acid	3	3		✓	2399 [w]	2400
3.115	Dodecanoic acid	6	3	✓	✓	2499 [w]	2502
3.116	Tridecanoic acid	4	3	✓	✓	2605 [w]	2603
3.117	Tetradecanoic acid	7	4	✓	✓	2717 [w]	2716
3.118	Pentadecanoic acid	4	3		✓	2820 [w]	2819
3.119	Hexadecanoic acid	4	4		✓	2926 [w]	2928
3.120	Heptadecanoic acid	3	2			2018	2022
3.121	Octadecanoic acid	4	3		✓	3139 [w]	3136
3.122	3-Methyldecanoic acid	5	3	✓		2309 [w]	
3.123	2-Methylundecanoic acid	4	2	✓	✓	2412 [w]	
3.124	*iso*-Pentadecanoic acid	1	1		✓	2772 [w]	2776
3.125	*anteiso*-Pentadecanoic acid	1	1		✓	2781 [w]	
3.126	*anteiso*-Hexadecanoic acid	0	1		✓	2893 [w]	
3.127	*cis*-9-Hexadecenoic acid	5	2		✓	2905 [w]	2908
3.128	*trans*-9-Hexadecenoic acid	1	1		✓	2953 [w]	2957
3.129	*cis*-9-Heptadecenoic acid	1	1			2003	
3.130	*cis*-9-Octadecenoic acid	4	3		✓	3168 [w]	3172
3.131	*cis,cis*-9,12-Octadecadienoic	1	2		✓	3173 [w]	3176
Aliphatic esters							
3.132	Propyl propanoate	0	1	✓	✓	1056 [w]	1059
3.133	Propyl butanoate	0	1	✓	✓	1125 [w]	1123
3.134	Butyl propanoate	0	1	✓	✓	1147 [w]	1143
3.135	Butyl butanoate	0	1	✓	✓	1124 [w]	1225
3.136	Methyl octanoate	2	2		✓	1393 [w]	1394
3.137	Methyl nonanoate	3	4	✓	✓	1495 [w]	1496
3.138	Methyl decanoate	2	2		✓	1595 [w]	1599
3.139	Methyl undecanoate	0	1	✓	✓	1698 [w]	1694
3.140	Methyl dodecanoate	2	1	✓	✓	1800 [w]	1800
3.141	Ethyl decanoate	3	3	✓	✓	1842 [w]	1840
3.142	Ethyl dodecanoate	0	1	✓	✓	1639 [w]	1635
3.143	Isopropyl tetradecanoate	5	3	✓	✓	2037 [w]	2041
3.144	Methyl 6-methylheptanoate	2	0			1041	

Table 3.1: *Contd.*

Sulphur and sulphur compounds							
3.145	Sulphur S_6	0	1	✓	✓	1500	1499
3.146	Sulphur S_8	7	5	✓	✓	2009	2004
3.147	Hexathiepane (CH_2S_6)	2	1	✓		1696	1697
3.148	Dimethyl sulfone	2	1	✓	✓	1903 [w]	1904
3.149	Dimethyldisulfide	1	4	✓	✓	1087 [w]	1086
3.150	Dimethyltrisulfide	1	3	✓		950	950
3.151	Dimethyltetrasulfide	3	3	✓		1200	1203
3.152	Dimethylpentasulfide	2	1	✓		1436	
3.153	2-Methyl-5-(methylthio)-furan	4	1	✓		1346 [w]	
3.154	Methyl(methylthio)methyl disulphide	0	1	✓		1111	1134
3.155	Benzothiazole	4	5	✓	✓	1946 [w]	1947
Steroids[h] (see Table 3.2)							
3.156	Androstenedione	1[e]	1[e]	✓	✓		
3.157	Testosterone	1	1	✓	✓		
3.158	Aldosterone	1	1	✓	✓		
3.159	Cortisol	1	1	✓	✓		
3.160	Cortisone	1	1	✓	✓		
		1	1				
Miscellaneous compounds							
3.161	Acetamide	0	2		✓	1776 [w]	1775
3.162	Acetophenone	8	3	✓	✓	1640 [w]	1638
3.163	2-Acetylpyridine	0	1		✓	1594 [w]	1590
3.164	2-Acetyl-6-methylpyridine	0	1	✓		1102	1107
3.165	Benzaldehyde	5	4	✓	✓	1513 [w]	1515
3.166	Benzoic acid	1	1	✓	✓	2437 [w]	2433
3.167	Cyanuric acid	2	0	✓	✓	2591 [w]	
3.168	2,6-Dimethylpyrazine	2	3	✓	✓	1329 [w]	1328
3.169	2,6-Dimethylpyridine	0	1		✓	1242	1241
3.170	Diphenylamine	0	1	✓	✓	2597 [w]	2595
3.171	Eugenol	1	0	✓	✓	2170	2167
3.172	γ-Dodecalactone	3	0	✓	✓	2356 [w]	2353
3.173	Hexadecanamide	0	1			2191	2186
3.174	α-Hexylcinnamaldehyde	?	?		✓	2344 [w]	
3.175	Salicylaldehyde	2	4	✓	✓	1687 [w]	1689
3.176	Indole	4	2	✓	✓	2448 [w]	2448
3.177	4-Methylbenzophenone	1	0	✓	✓	2781 [w]	
3.178	3-Methylphenol (*m*-Cresol)	3	1	✓	✓	2094	2097
3.179	3-Methylpyridine	0	1		✓	1296 [w]	1292
3.180	2-Pentylfuran	2	1	✓	✓	1229 [w]	1230
3.181	Phenol	4	5	✓	✓	2014 [w]	2011
3.182	2-Phenylethanol	5	5	✓	✓	1908 [w]	1904
3.183	1-Phenyl-2-propanone	3	0		✓	1709 [w]	1710
3.184	2-Piperidone	6	1	✓	✓	1172	1174
3.185	Pyridine	0	1		✓	1183 [w]	1185
3.186	Tetramethylpyrazine	0	2		✓	1464 [w]	1466
3.187	Trimethylamine	0	1	✓	✓		
3.188	Trimethylpyrazine	2	4	✓	✓	1398 [w]	1397
3.189	4,4'7a-Trimethyl-5,6,7,7a-tetrahydro-2(4H)-benzofuranone`	2	2	✓		1508	1525
3.190	2,4,6-Trimethylpyridine	0	1		✓	1369 [w]	1365
3.191	Urea	3	1	✓	✓		

Table 3.1: *Contd.*

[a]Trivial chemical nomenclature was used in this thesis to avoid the problem of having to fit long IUPAC names into tables.
[b]Number of samples in which compounds were identified (out of 11 male and 7 female urine samples, respectively).
[c]HRMS data available.
[d]Confirmation of the structure by co-injection with an authentic reference compound.
[e]Retention index of the natural constituent.
[f]Retention index from the literature.
[g]The C_{13} and C_{14} macrocyclic ketones were identified only in denatured urinary protein. This information is taken into consideration in counting the number of urine samples containing macrocyclic ketones.
[h]The steroids were determined only in the urine of one male and one female.
Retention indexes were determined on different phases to circumvent the problem of peak broadening and peak fronting on inappropriate columns. All RIs were determined on an apolar column (DB 5 equivalent), except those designated with [w] in this list.

3.2.10 Supporting information obtained by GC-HRMS

GC-HRMS analyses were carried out for the determination of the molecular composition of constituents and to obtain information that could facilitate the interpretation of the mass spectra. The relevant HRMS information for benzaldehyde, for example, is given in Table 3.4. The HR data from which the molecular formulae of other constituents were derived are summarised in Table 3.5.

Table 3.4: High resolution data for benzaldehyde

Mass	RA[a]	Calc. Mass	mDa	Formula[b]
49.0073	1.02	49.0078	-0.5	C_4 H
50.0147	13.74	50.0157	-1	C_4 H_2
51.0226	28.16	51.0235	-0.9	C_4 H_3
52.03	7.28	52.0313	-1.3	C_4 H_4
63.0233	2.16	63.0235	-0.2	C_5 H_3
74.0157	6.5	74.0157	0	C_6 H_2
		74.019	-3.3	C_3 H_6 S
75.0236	2.54	75.0235	0.1	C_6 H_3
		75.0268	-3.2	C_3 H_7 S
76.0315	3.73	76.0313	0.2	C_6 H_4
		76.0347	-3.2	C_3 H_8 S
77.0384	94.39	77.0391	-0.7	C_6 H_5
		77.0351	3.3	C H_5 N_2 O_2
78.0453	13.37	78.047	-1.7	C_6 H_6
		78.0429	2.4	C H_6 N_2 O_2
105.0329	97.85	105.0327	0.2	C_5 H_3 N_3
		105.034	-1.1	C_7 H_5 O
		105.03	2.9	C_2 H_5 N_2 O_3
		105.0361	-3.2	C_2 H_7 N_3 S

Table 3.4: *Contd.*

		105.0287	4.2	$H_3 N_5 O_2$
		105.0374	-4.5	$C_4 H_9 O S$
106.0416	100	106.0419	-0.3	$C_7 H_6 O$
		106.0405	1.1	$C_5 H_4 N_3$
		106.0439	-2.3	$C_2 H_8 N_3 S$
107.0458	8.27	107.0457	0.1	$C_2 H_7 N_2 O_3$
		107.0443	1.5	$H_5 N_5 O_2$
		107.0483	-2.5	$C_5 H_5 N_3$
		107.0497	-3.9	$C_7 H_7 O$
		107.0405	5.3	$C_3 H_9 N O S$

[a]Relative abundance
[b]Other possible molecular formulae are given as illustration of the accuracy of the high resolution measurements.

Table 3.5: List of volatile components obtained by GC-HRMS

No	Compound	Molecular formula	Molecular mass		
			Measured	Calculated	Mass difference (mDa)
3.3	Decane	$C_{10}H_{22}$	142.1703	142.1722	-1.9
3.4	Undecane	$C_{11}H_{24}$	156.1864	156.1878	-1.4
3.5	Dodecane	$C_{12}H_{26}$	170.2070	170.2035	3.5
3.6	Tridecane	$C_{13}H_{28}$	184.2196	184.2191	0.5
3.7	Tetradecane	$C_{14}H_{30}$	198.2358	198.2348	1.0
3.8	Pentadecane	$C_{15}H_{32}$	212.2515	212.2504	1.1
3.9	Hexadecane	$C_{16}H_{34}$	226.2661	226.2661	0.0
3.10	Heptadecane	$C_{17}H_{36}$	240.2818	240.2817	0.1
3.26	Limonene	$C_{10}H_{16}$	136.1259	136.1252	0.7
3.27	3-Carene	$C_{10}H_{16}$	136.1249	136.1252	-0.3
3.28	Kaurene	$C_{20}H_{32}$	272.2520	272.2504	1.6
3.29	*cis*-Phytene	$C_{20}H_{40}$	280.3148	280.3130	0.9
3.30	*trans*-Phytene	$C_{20}H_{40}$	280.3139	280.3130	0.9
3.33	1-Pentanol	$C_5H_{12}O$	88.0786	88.0783	0.3
3.43	2-Ethyl-1-hexanol	$C_8H_{18}O$	130.1242	130.1252	-1.0
3.44	2,6-Dimethyl-cylohexanol	$C_8H_{16}O$	128..1200	128.1201	-0.1
3.47	Phytol	$C_{20}H_{40}O$	296.2979	296.2974	0.5
3.49	Hexanal	$C_6H_{12}O$	100.0785	100.0783	0.2
3.50	Heptanal	$C_7H_{14}O$	114.1037	114.1045	-0.8
3.51	Octanal	$C_8H_{16}O$	128.1094	128.1096	-0.2
3.52	Nonanal	$C_9H_{18}O$	142.1254	142.1252	0.2
3.53	Decanal	$C_{10}H_{20}O$	156.1411	156.1409	0.2
3.56	*cis*-2-Decenal	$C_{10}H_{18}O$	154.1283	154.1252	3.1
3.57	*trans*-2-Undecenal	$C_{11}H_{20}O$	168.1367	168.1409	-4.2
3.58	2,6,6-Trimethyl-1-cyclohexene-1-carboxaldehyde	$C_{10}H_{16}O$	152.1202	152.1201	0.1
3.59	2,6,6-Trimethyl-1-cyclohexene-1-acetaldehyde	$C_{11}H_{18}O$	166.1361	166.1358	0.3
3.61	2-Pentanone	$C_{15}H_{10}O$	86.0730	86.0732	-0.2
3.62	3-Pentanone	$C_{15}H_{10}O$	86.0736	86.0732	0.4
3.64	3-Hexanone	$C_6H_{12}O$	100.0889	100.0888	0.1
3.66	4-Heptanone	$C_7H_{14}O$	114.1039	114.1045	-0.6
3.79	2-Tridecanone	$C_{13}H_{26}O$	198.1977	198.1984	-0.7

Table 3.5: *Contd.*

3.80	2-Pentadecanone	$C_{15}H_{30}O$	226.2292	226.2297	-0.5
3.82	*trans*-3-Hepten-2-one	$C_7H_{12}O$	112.0903	112.0888	1.5
3.86	6-Methyl-2-heptanone	$C_8H_{16}O$	128.1232	128.1201	3.1
3.89	6,10-Dimethyl-2-undecanone	$C_{13}H_{26}O$	198.1889	198.1878	1.1
3.90	6-Methyl-5-hepten-2-one	$C_8H_{14}O$	126.1048	126.1045	0.3
3.92	*trans*-6,10-Dimethyl-5,9-undecadien-2-one	$C_{13}H_{22}O$	194.1630	194.1671	-4.1
3.93	3-Methylcyclopentanone	$C_6H_{10}O$	98.0727	98.0732	-0.5
3.94	3-Methylcyclohexanone	$C_7H_{12}O$	112.0895	112.0888	0.7
3.96	2,2,6-Trimethyl-cyclohexanone	$C_9H_{16}O$	140.1205	140.1201	0.4
3.97	Isophorone	$C_9H_{14}O$	138.1043	138.1045	-0.2
3.98	β-Ionone	$C_{13}H_{20}O$	192.1523	192.1514	0.9
3.101	Cyclopentadecanone	$C_{15}H_{28}O$	224.2141	224.2140	0.1
3.103	Cycloheptadecanone	$C_{16}H_{30}O$	252.2458	252.2453	0.5
3.105	Acetic acid	$C_2H_4O_2$	60.0221	60.0211	1.0
3.106	Propanoic acid	$C_3H_6O_2$	74.0362	74.0390	1.1
3.107	Butanoic acid	$C_4H_8O_2$	88.0520	88.0524	-0.4
3.115	Dodecanoic acid	$C_{12}H_{24}O_2$	200.1776	200.1777	-0.1
3.116	Tridecanoic acid	$C_{13}H_{26}O_2$	214.1932	214.1933	-0.1
3.117	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.2091	228.2089	0.2
3.122	3-Methyldecanoic acid	$C_{11}H_{22}O_2$	186.1624	186.1620	0.4
3.123	2-Methylundecanoic acid	$C_{12}H_{24}O_2$	200.1775	200.1776	-0.1
3.135	Butyl butanoate	$C_8H_{16}O_2$	144.1050	144.1045	0.5
3.137	Methyl nonanoate	$C_{10}H_{20}O_2$	172.1452	172.1463	-1.1
3.140	Methyl dodecanoate	$C_{12}H_{24}O_2$	186.1576	186.1620	-4.4
3.141	Ethyl decanoate	$C_{12}H_{24}O_2$	200.1776	2000.1776	0.0
3.142	Ethyl dodecanoate	$C_{14}H_{28}O_2$	228.2086	228.2089	-0.3
3.143	Isopropyl tetradecanoate	$C_{17}H_{34}O_2$	270.2089	270.2089	0.0
3.145	Sulphur S_6	S_6	191.8329	191.8324	0.5
3.146	Sulfur S_8	S_8	255.7780	255.7766	1.4
3.147	Hexathiepane	CH_2S_6	205.8482	205.8481	0.1
3.148	Dimethylsulfone	$C_2H_6O_2S$	94.0086	94.0089	-0.3
3.149	Dimethyldisulfide	$C_2H_6S_2$	93.9920	93.9911	0.9
3.150	Dimethyltrisulfide	$C_2H_6S_3$	125.9634	125.9632	0.2
3.151	Dimethyltetrasulfide	$C_2H_6S_4$	157.9284	157.9278	0.6
3.152	Dimethylpentasulfide	$C_2H_6S_5$	189.9073	189.9073	0.0
3.153	2-Methyl-5-(methylthio)-furan	C_6H_8OS	128.03	128.0296	0.4
3.154	Disulfide, methyl (methylthio)methyl	$C_3H_8S_5$	139.9791	139.9793	-0.2
3.155	Benzothiazole	C_7H_5NS	135.0145	135.0143	0.2
3.162	Acetophenone	C_8H_8O	120.0581	120.0575	0.6
3.164	2-Acetyl-6-methylpyridine	C_8H_9NO	135.0681	135.0684	-0.3
3.165	Benzaldehyde	C_7H_6O	106.0415	106.0419	-0.4
3.167	Cyanuric acid	$C_3H_3N_3O_3$	129.0168	129.0174	-0.6

Table 3.5: *Contd.*

3.168	2,6-Dimethylpyrazine	$C_6H_8N_2$	108.0686	108.0687	-0.1
3.169	2,6-Dimethylpyridine	C_7H_9N	107.0739	107.0735	0.4
3.170	Diphenylamine	$C_{12}H_{11}N$	169.0897	169.0891	0.6
3.171	Eugenol	$C_{10}H_{12}O$	164.0836	164.0837	-0.1
3.174	α-Hexylcinnamaldehyde	$C_{15}H_{20}O$	216.1515	216.1514	0.1
3.175	Salicylaldehyde	$C_7H_6O_2$	122.0361	122.0368	-0.7
3.176	Indole	C_8H_7N	117.0576	117.0578	-0.2
3.178	3-methyl-phenol(*m*-cresol)	C_7H_8O	108.0571	108.0575	-0.4
3.180	2-Pentylfuran	$C_9H_{14}O$	138.1042	138.1045	-0.3
3.181	Phenol	C_6H_6O	94.0418	94.0419	-0.1
3.182	2-Phenylethanol	$C_8H_{10}O$	122.0730	122.0732	-0.2
3.184	2-Piperidone	C_5H_9NO	99.0682	99.0684	-0.2
3.187	Trimethylamine	C_3H_9N	59.0734	59.0735	-0.1
3.188	Trimethylpyrazine	$C_7H_{10}N_2$	122.0841	122.0844	-0.3
3.189	4,4´7a-Trimethyl-5,6,7,7a-tetrahydro-2(4H)-benzofuranone	$C_{11}H_{16}O_2$	180.1148	180.1150	-0.2
3.191	Urea	CH_4N_2O	60.0315	60.0324	-0.9

3.3. References

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3.4 Mass spectra of representative compounds

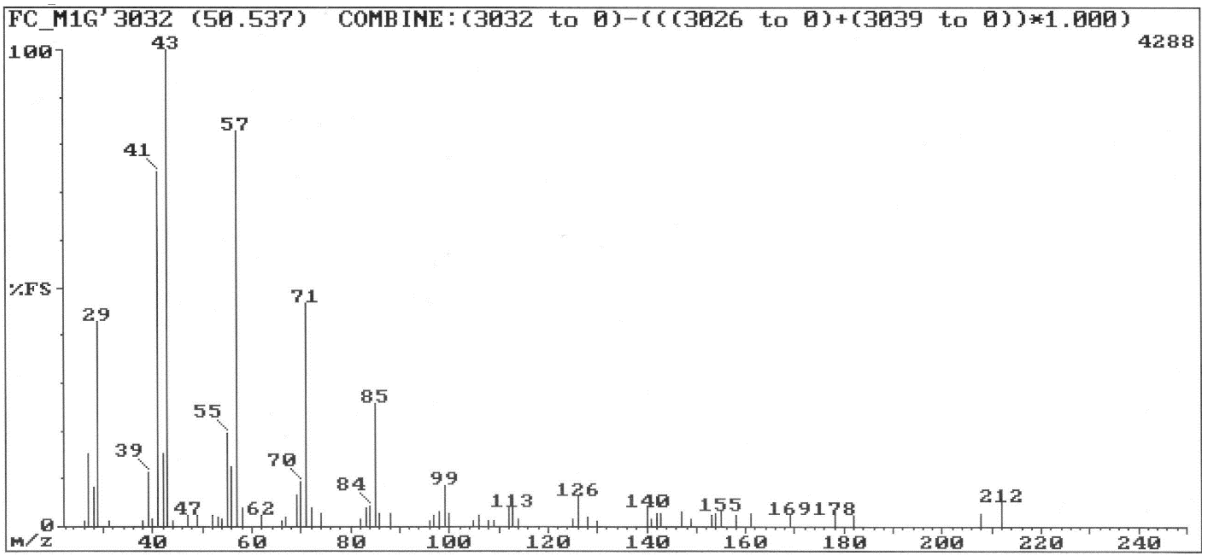


Fig. 3.6: EI-mass spectrum of pentadecane (apolar column).

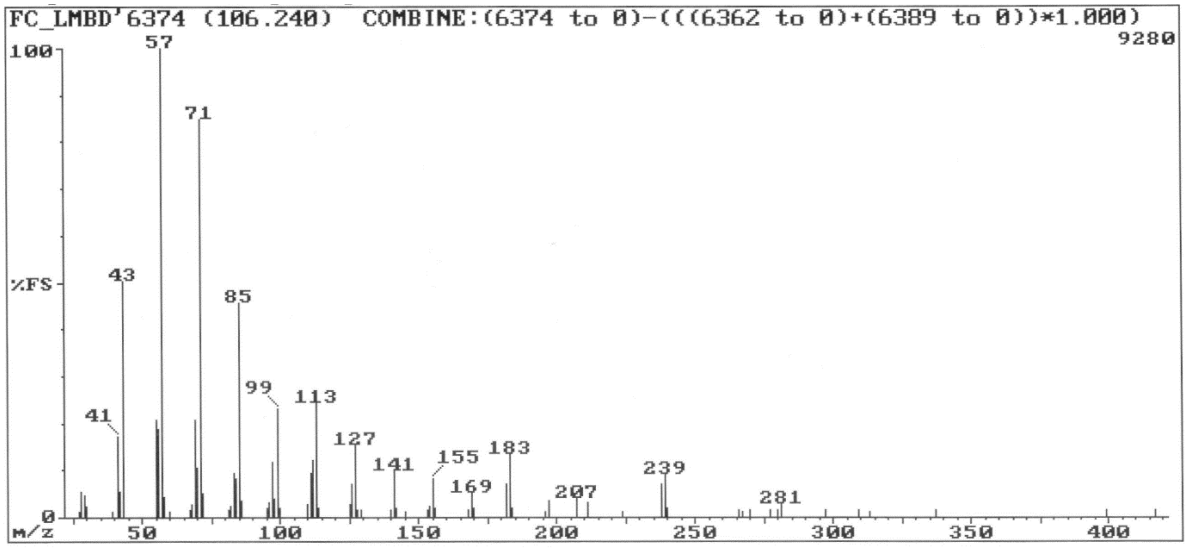


Fig. 3.7: EI-mass spectrum of squalane (apolar column).

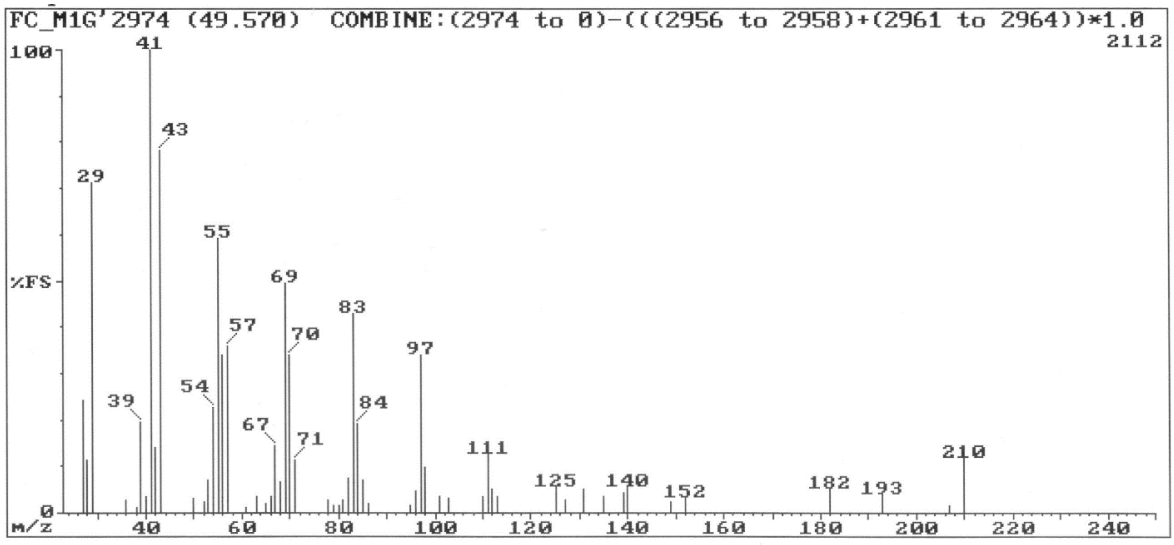


Fig. 3.8: EI-mass spectrum of 1-pentadecene (apolar column).

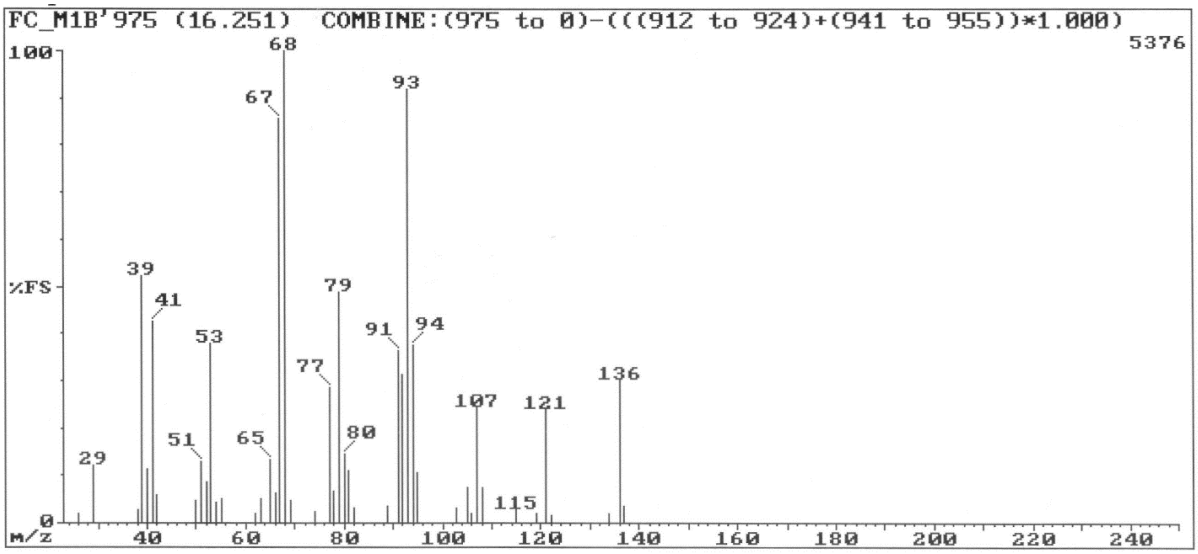


Fig. 3.9: EI-mass spectrum of limonene (1701column).

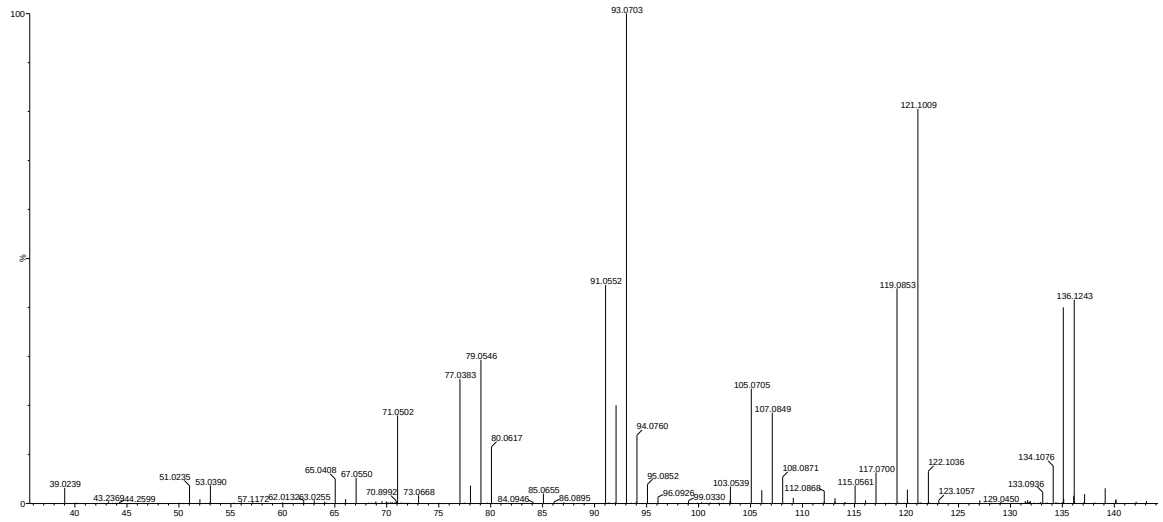


Fig. 3.10: EI-mass spectrum of 3-carene (apolar column).

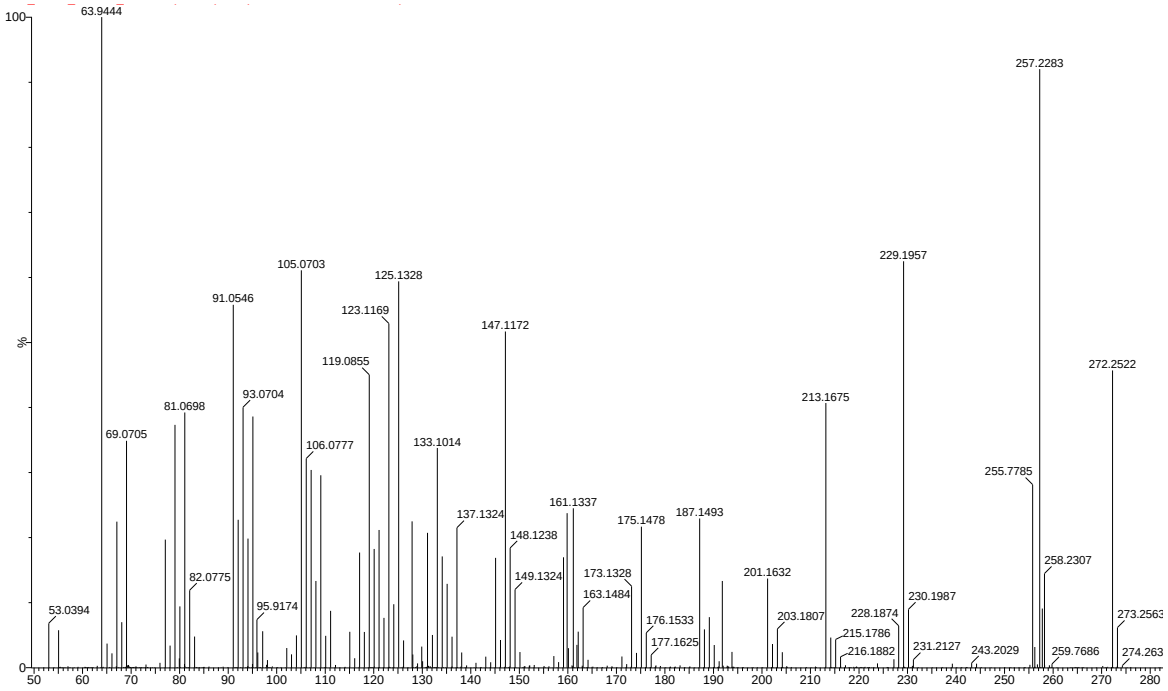


Fig. 3.11: EI-mass spectrum of kaurene (apolar column).

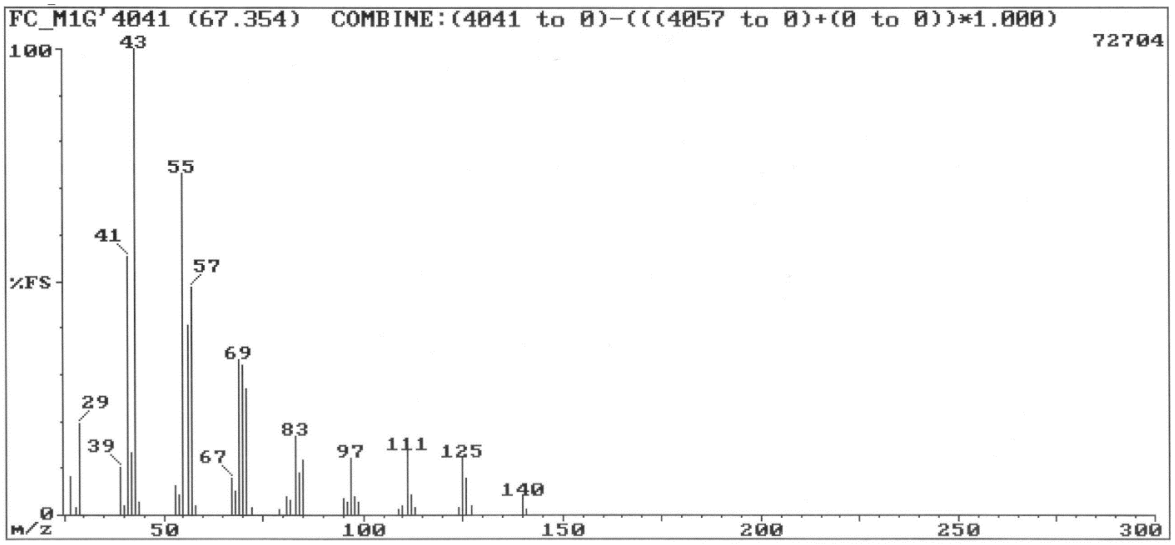


Fig. 3.12: EI-mass spectrum of *cis*-phytene (apolar column).

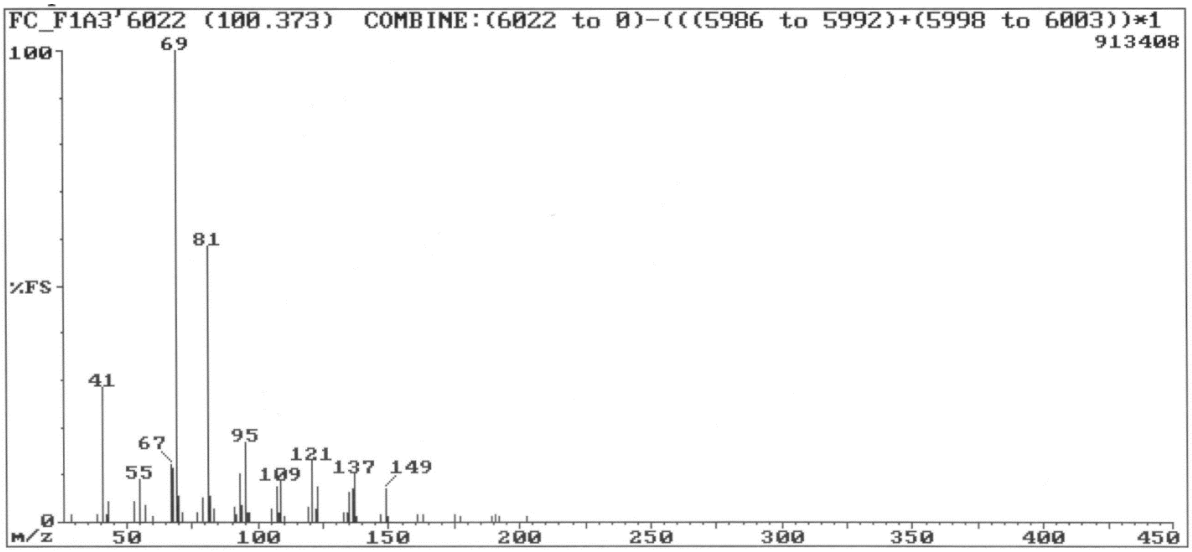


Fig. 3.13: EI-mass spectrum of squalene (apolar column).

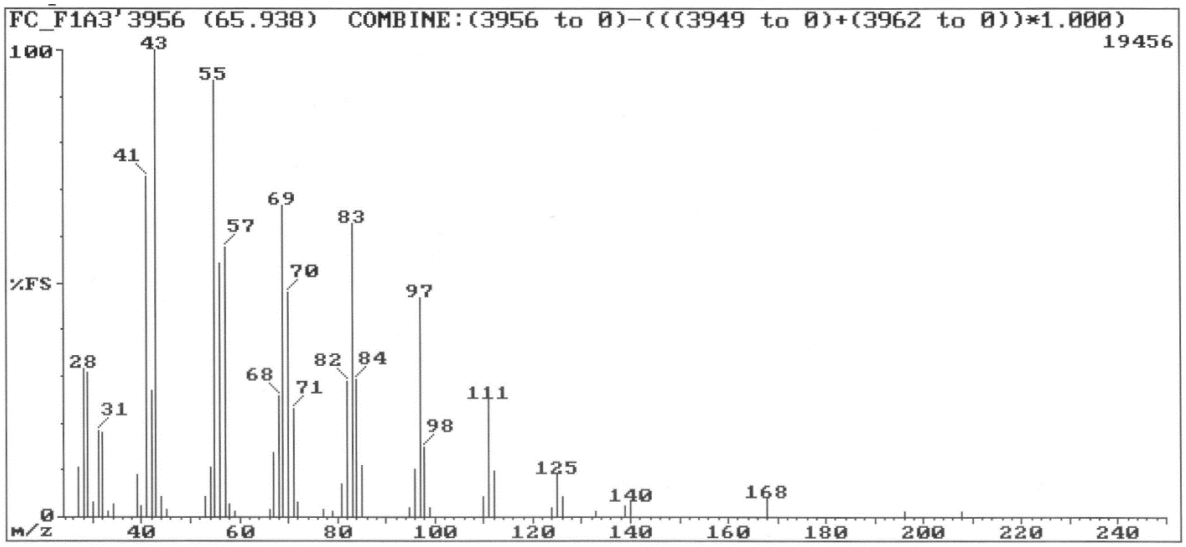


Fig. 3.14: EI-mass spectrum of 1-tetradecanol (wax column).

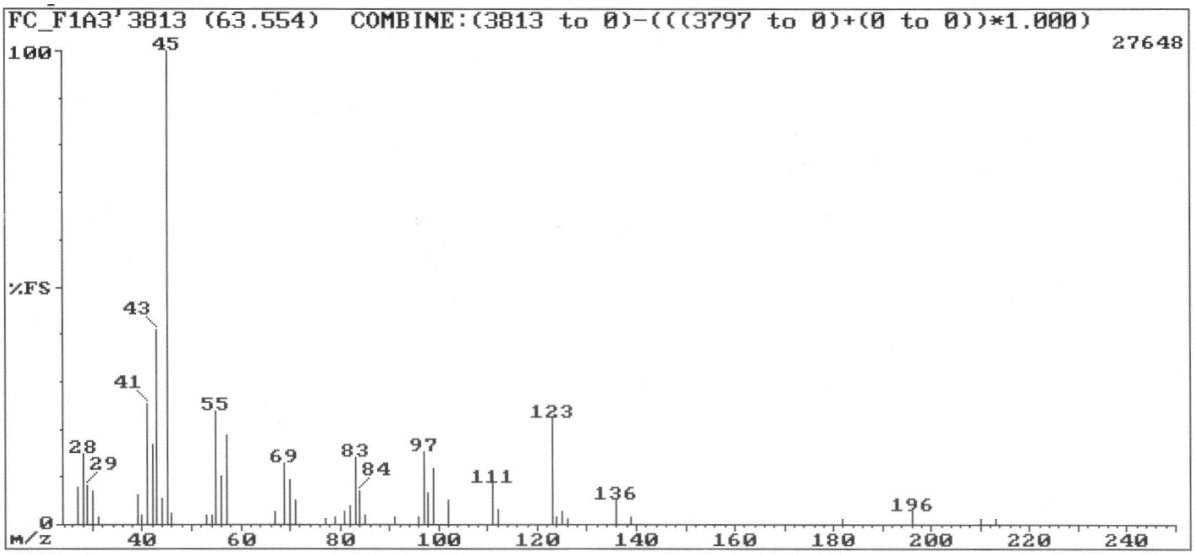


Fig. 3.15: EI-mass spectrum of 2-tetradecanol (wax column).

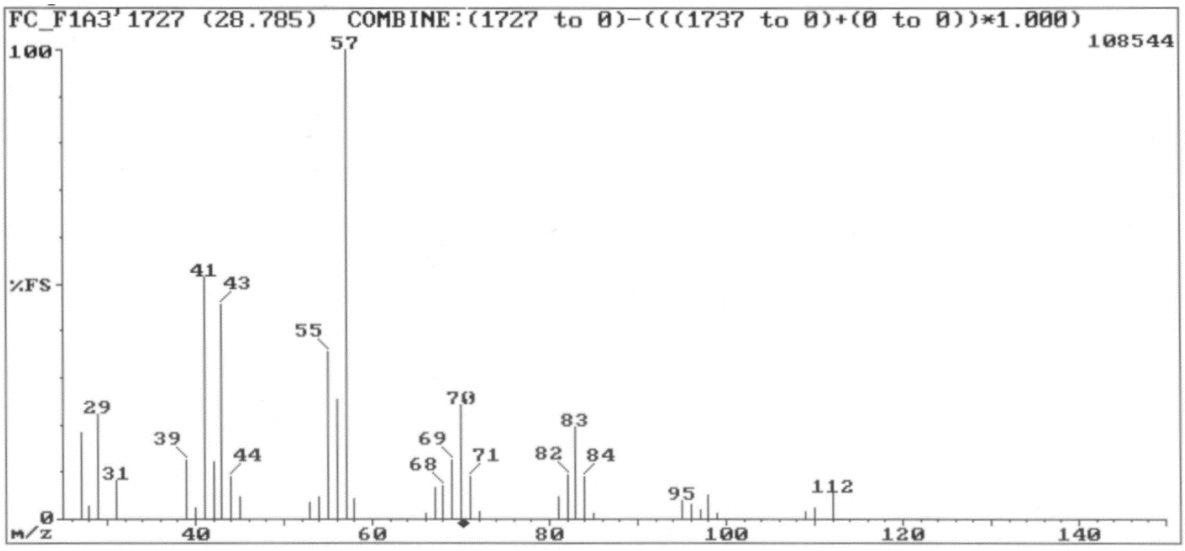


Fig. 3.16: EI-mass spectrum of 2-ethyl-1-hexanol (wax column).

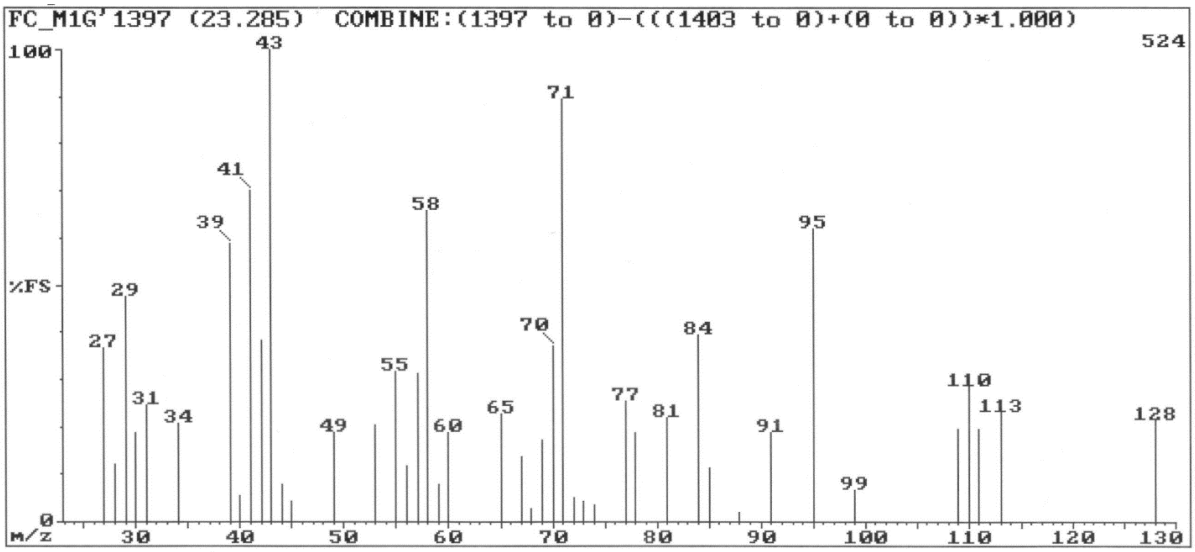


Fig. 3.17: EI-mass spectrum of 2,6-dimethylcyclohexanol (apolar column).

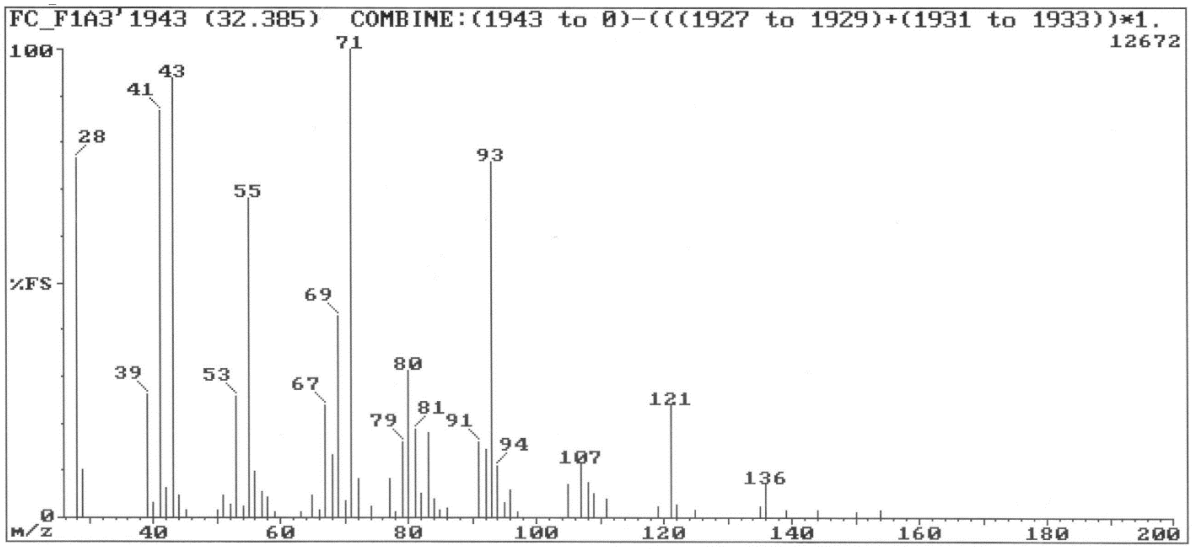


Fig. 3.18: EI-mass spectrum of linalool (wax column).

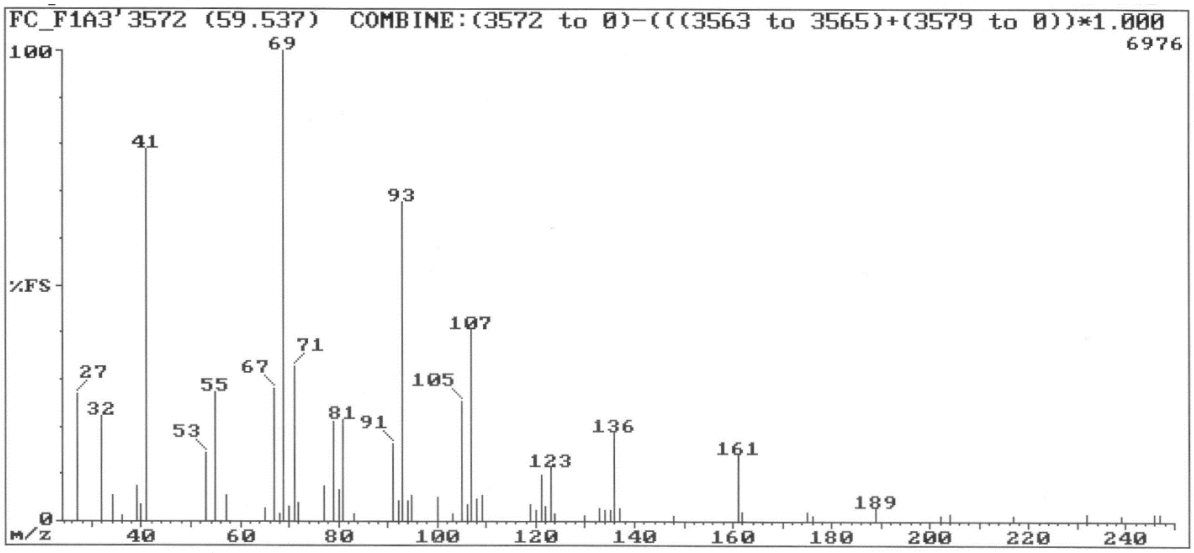


Fig. 3.19: EI-mass spectrum of nerolidol (wax column).

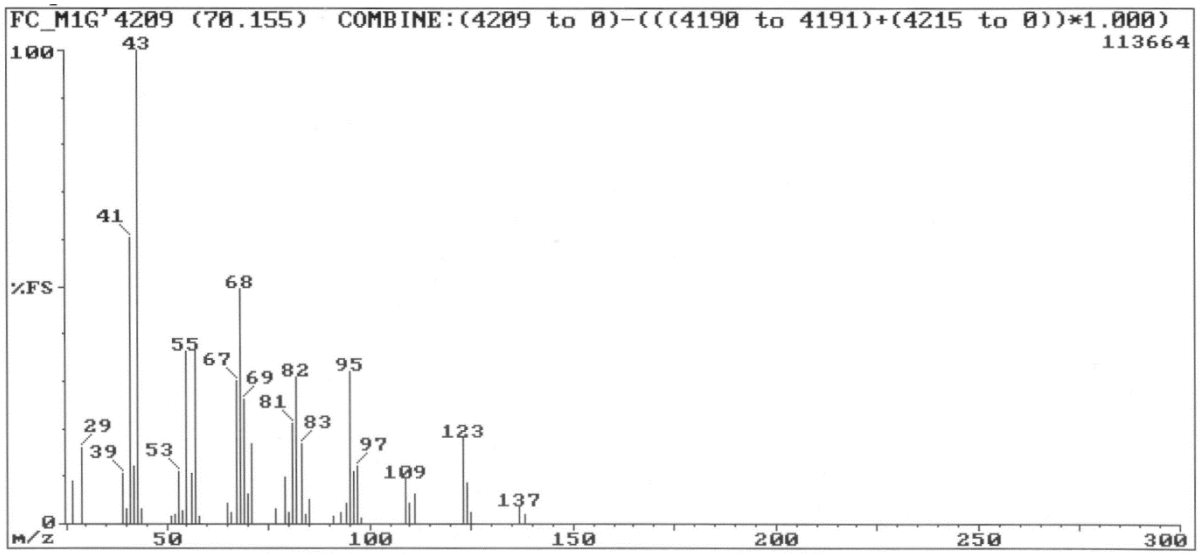


Fig. 3.20: EI-mass spectrum of *cis*-phytol (apolar column).

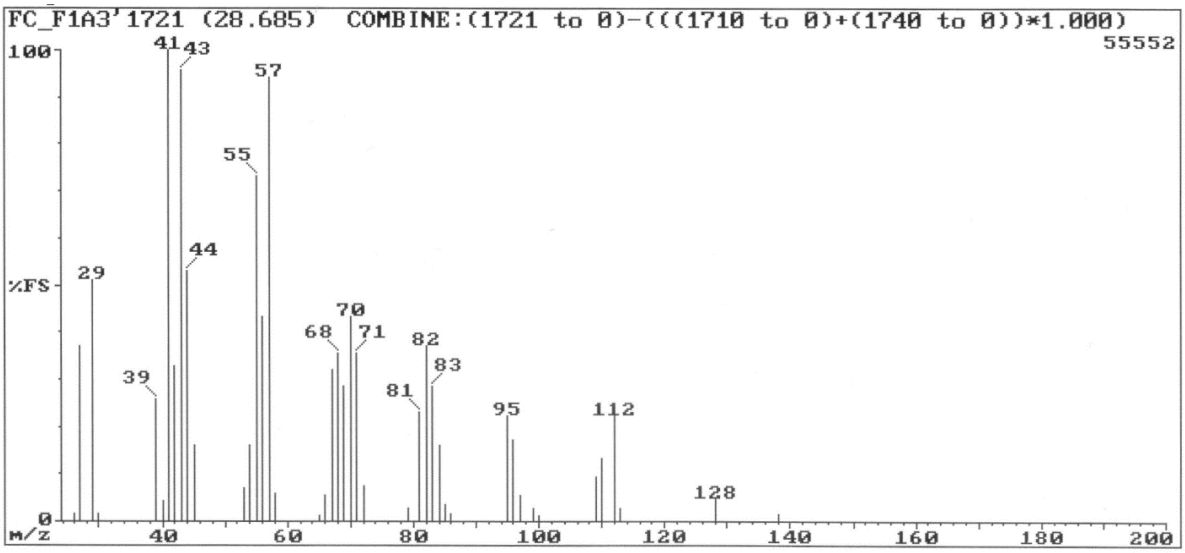


Fig. 3.21: EI-mass spectrum of decanal (wax column).

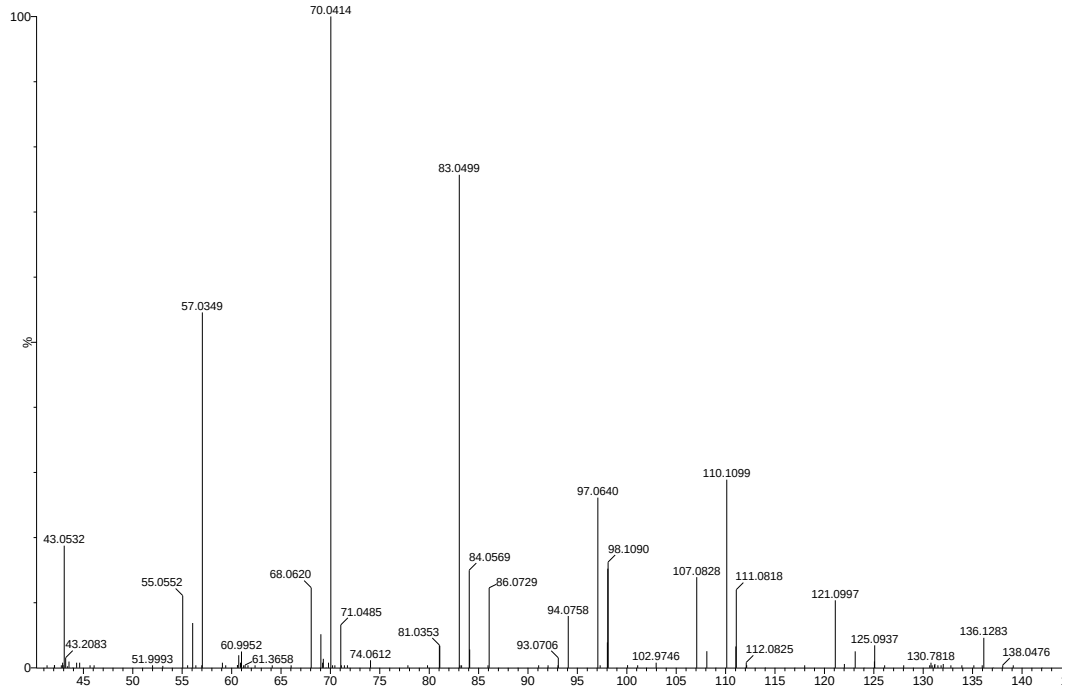


Fig. 3.22: EI-mass spectrum of *cis*-2-decenal (apolar column).

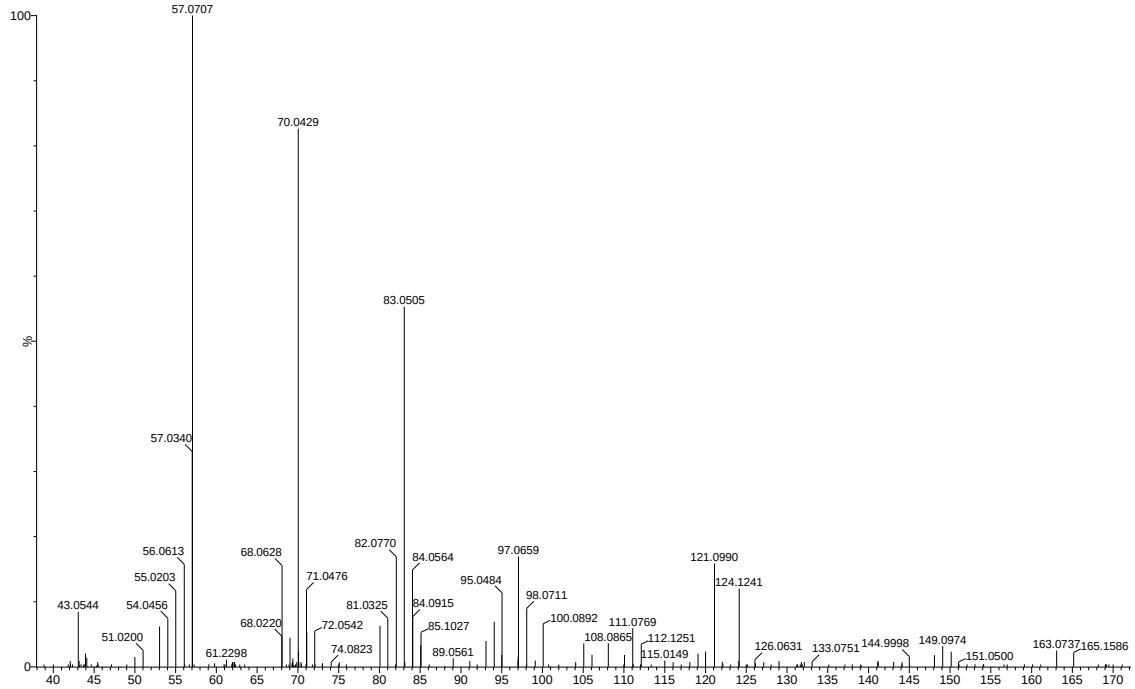


Fig. 3.23: EI-mass spectrum of *trans*-2-undecenal (apolar column).

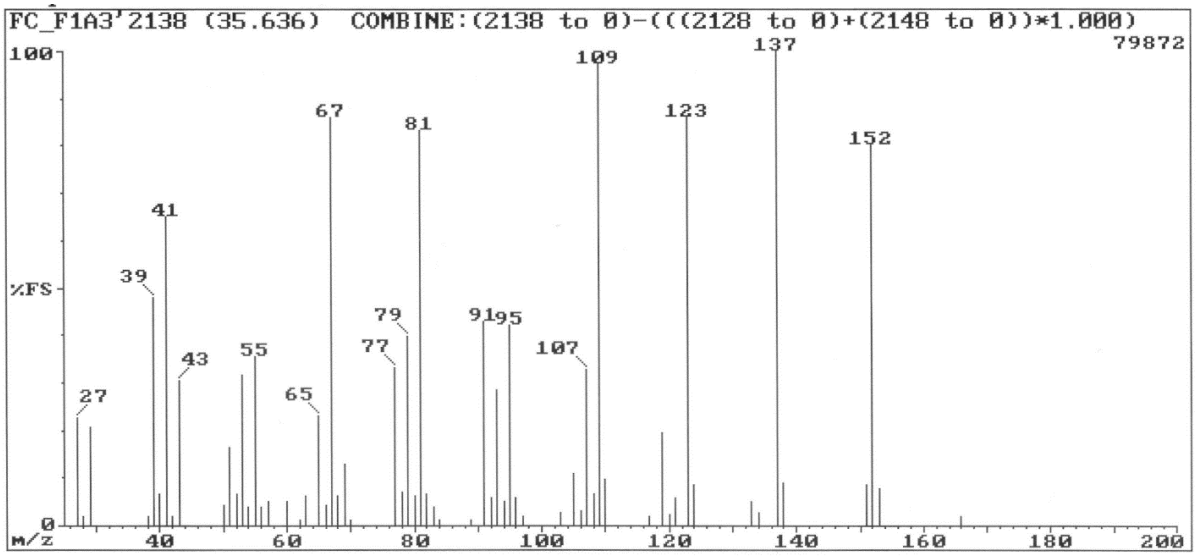


Fig. 3.24: EI-mass spectrum of 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (wax column).

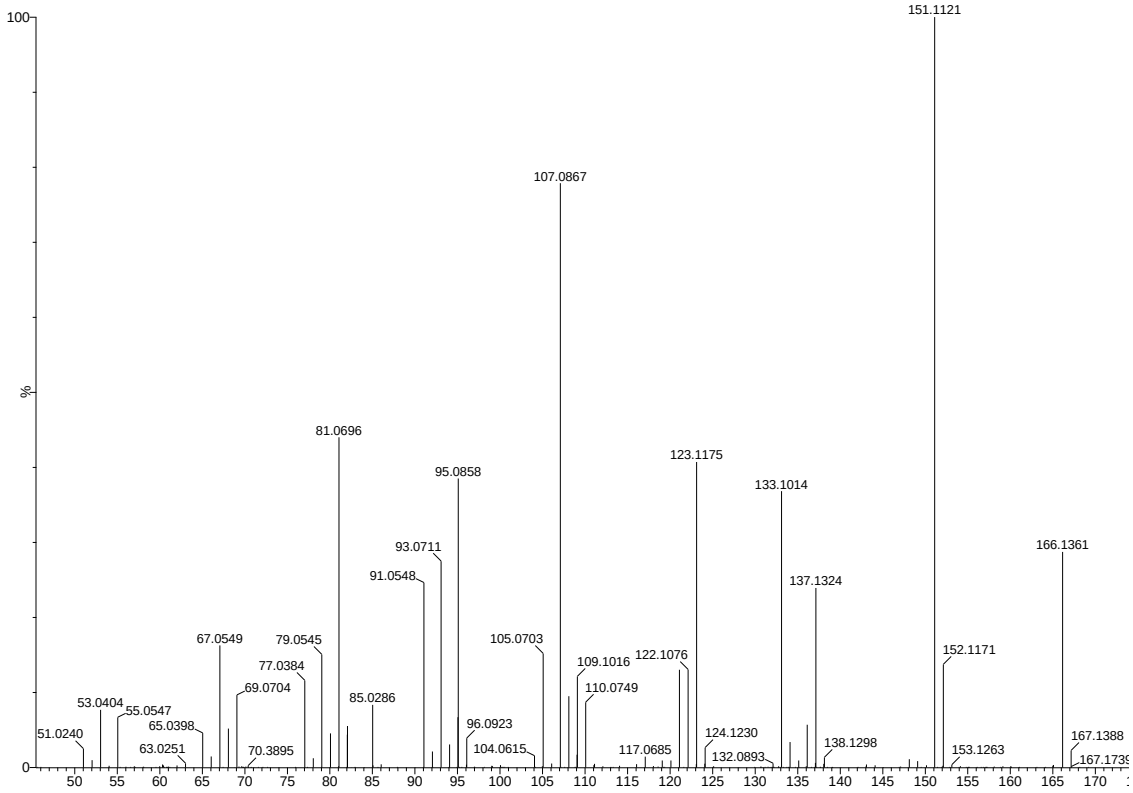


Fig. 3.25: EI-mass spectrum of 2,6,6-trimethyl-1-cyclohexene-1-acetaldehyde (apolar column).

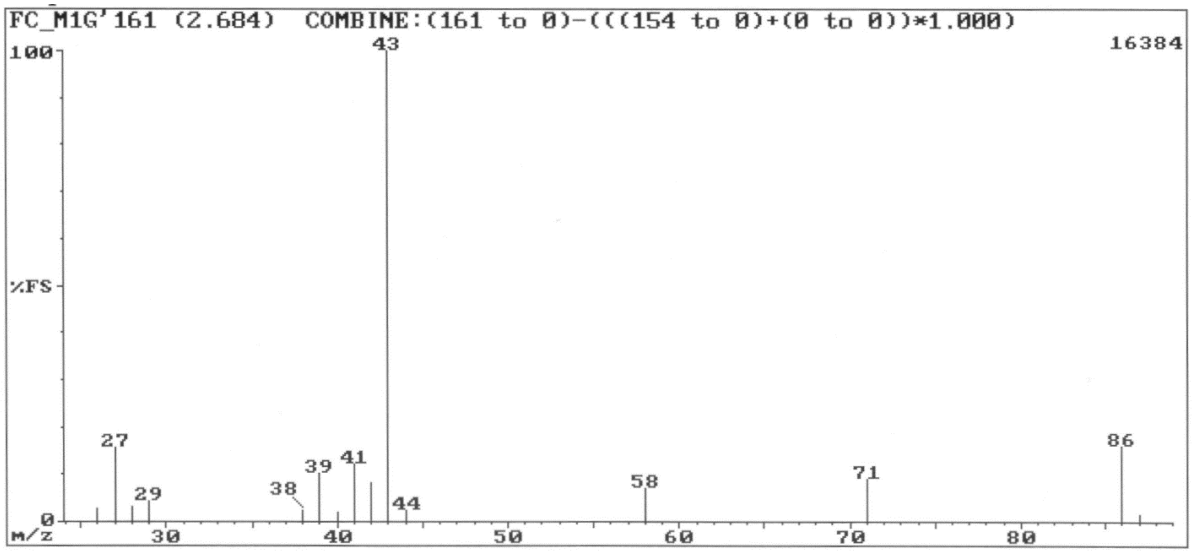


Fig. 3.26: EI-mass spectrum of 2-pentanone (apolar column).

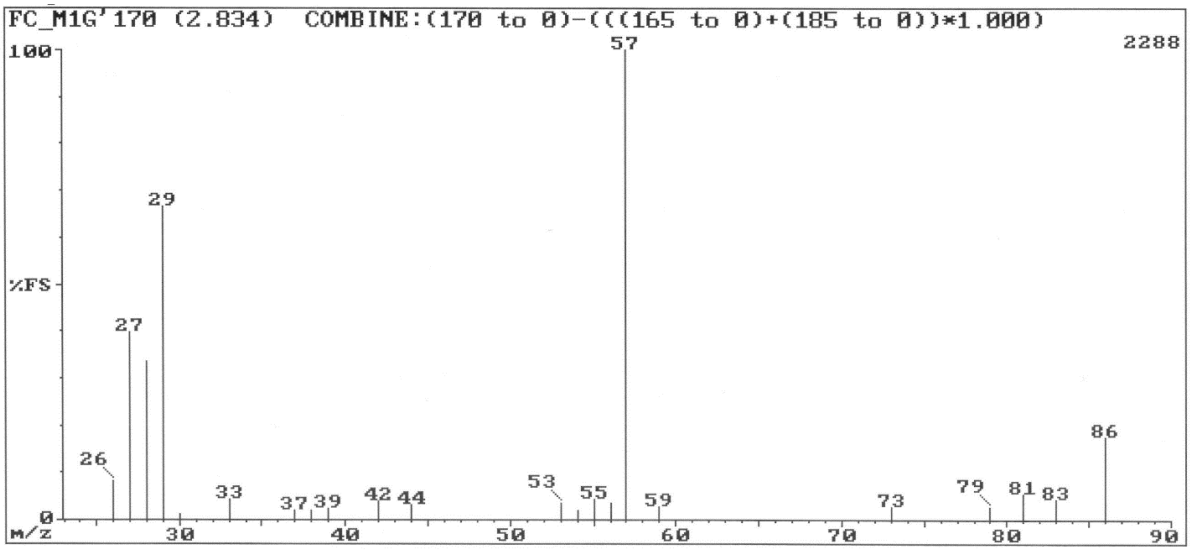


Fig. 3.27: EI-mass spectrum of 3-pentanone (apolar column).

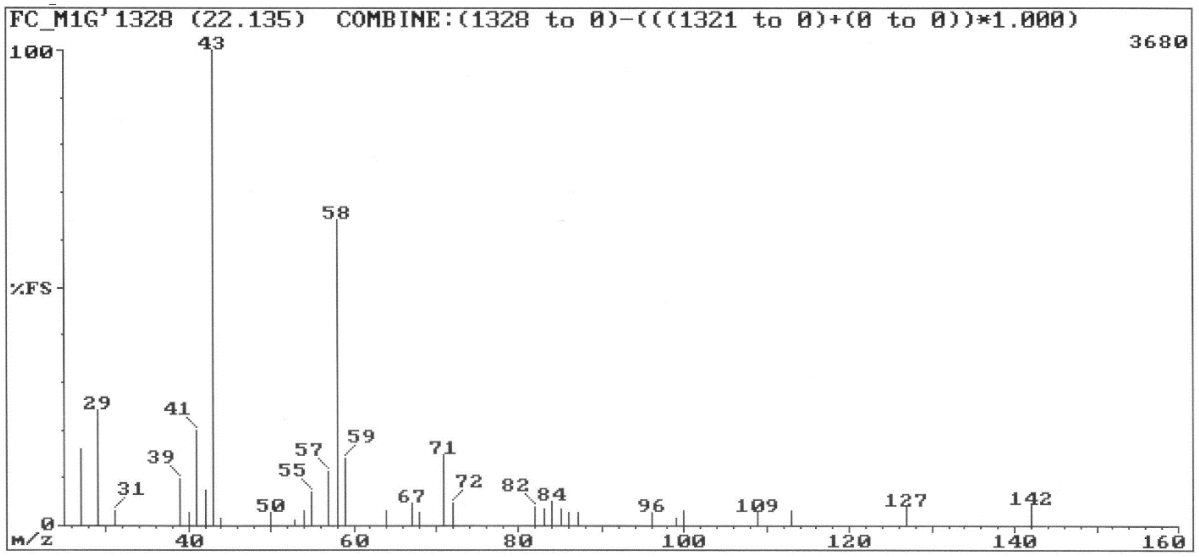


Fig. 3.28: EI-mass spectrum of 2-nonanone (apolar column).

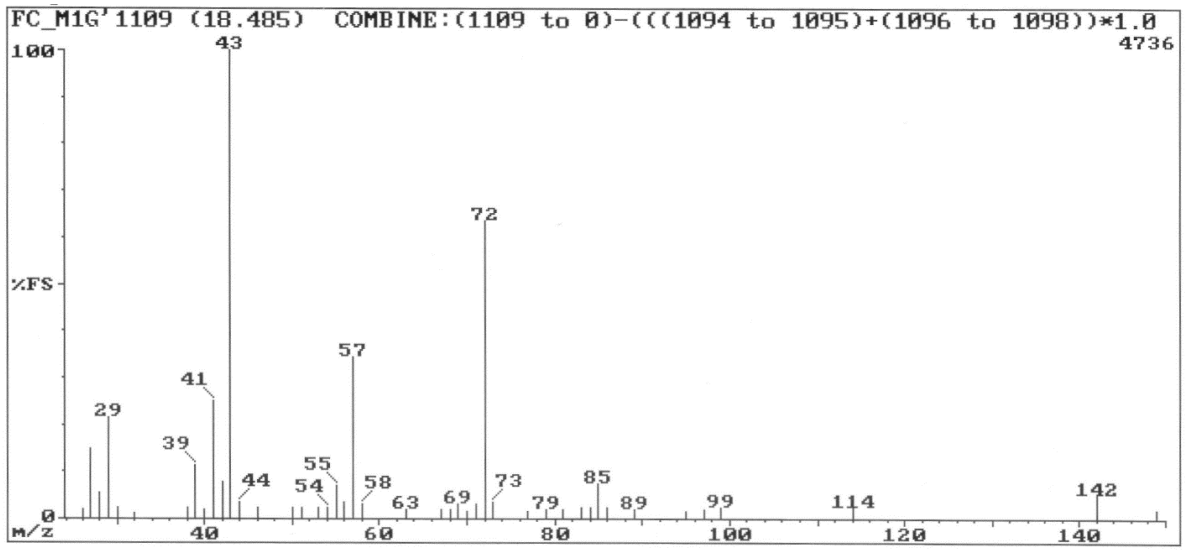


Fig. 3.29: EI-mass spectrum of 3-nonanone (apolar column).

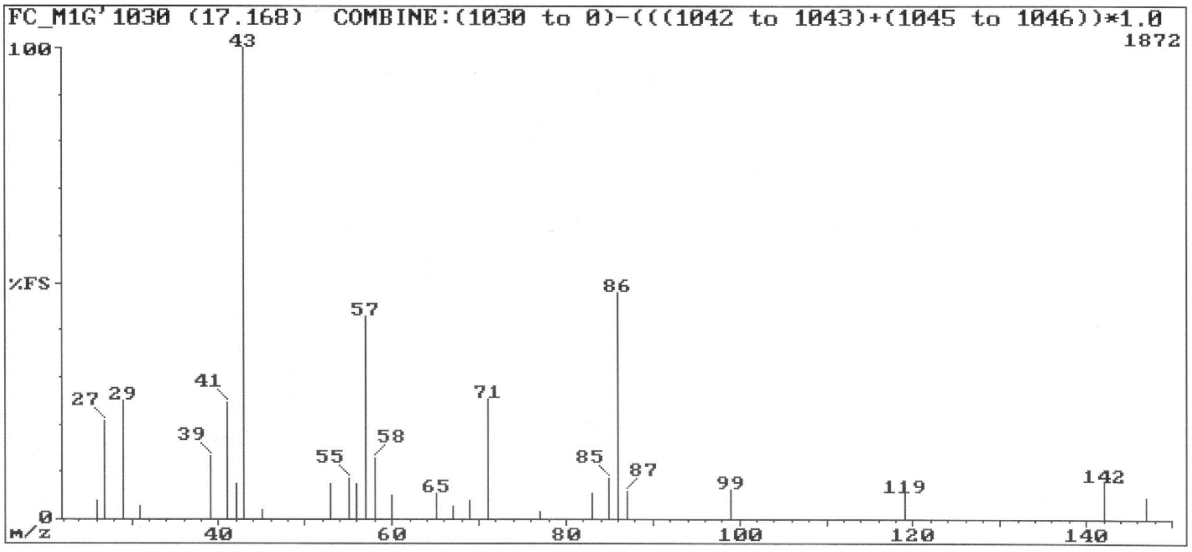


Fig. 3.30: EI-mass spectrum of 4-nonanone (apolar column).

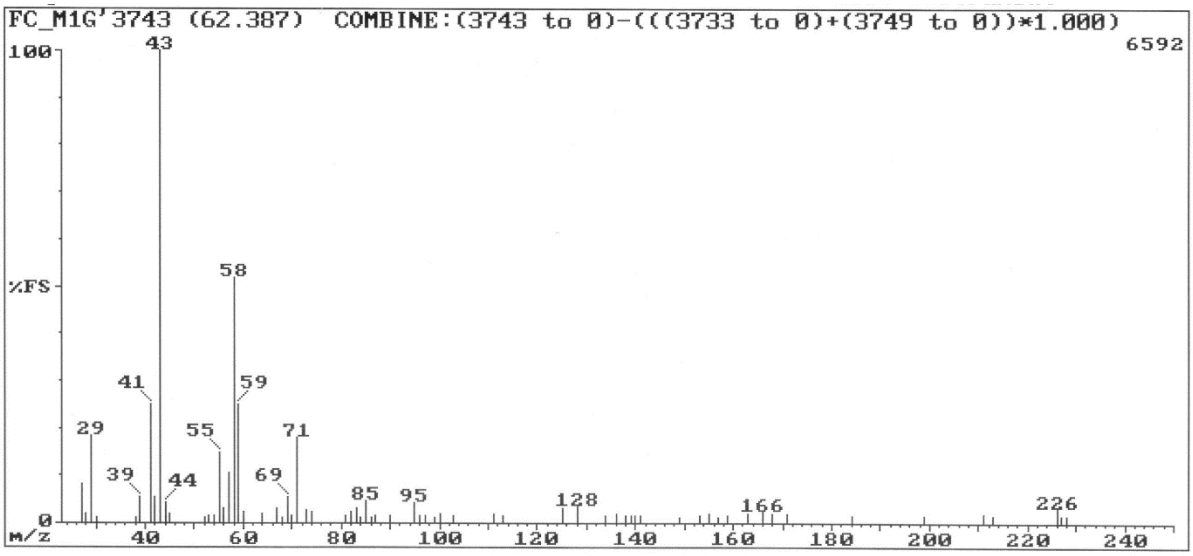


Fig. 3.31: EI-mass spectrum of 2-pentadecanone (apolar column).

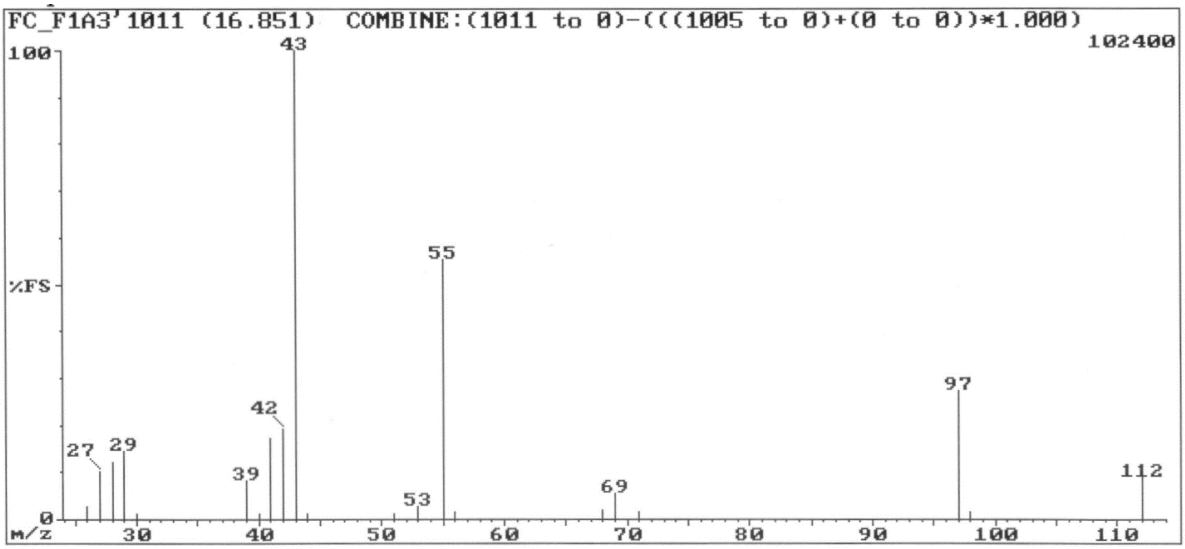


Fig. 3.32: EI-mass spectrum of *trans*-3-hepten-2-one (wax column).

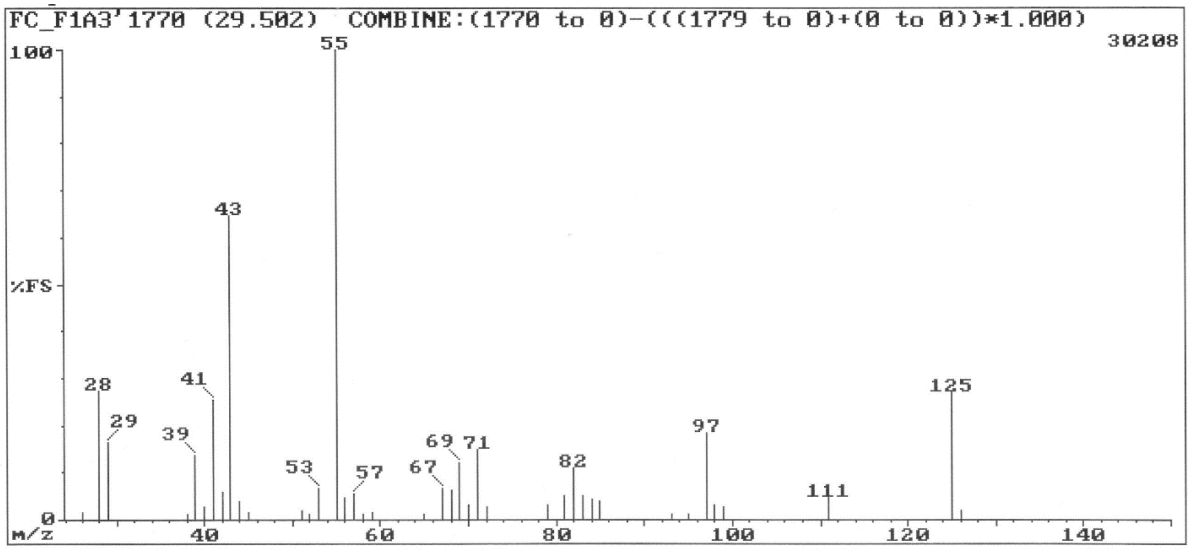


Fig. 3.33: EI-mass spectrum of *trans*-nonen-2-one (wax column).

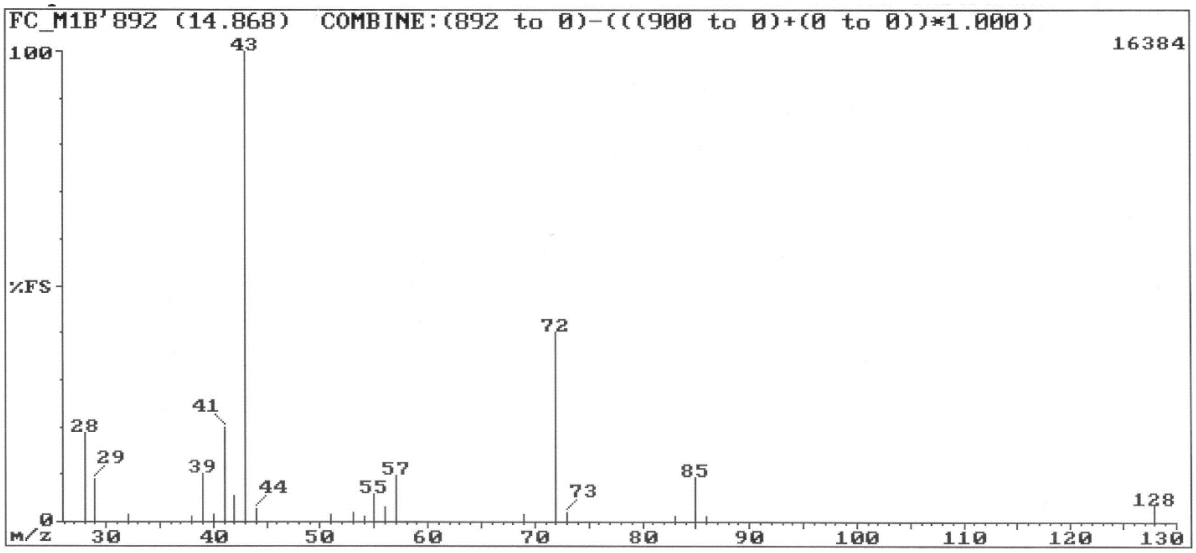


Fig. 3.34: EI-mass spectrum of 3-methyl-2-heptanone (1701column).

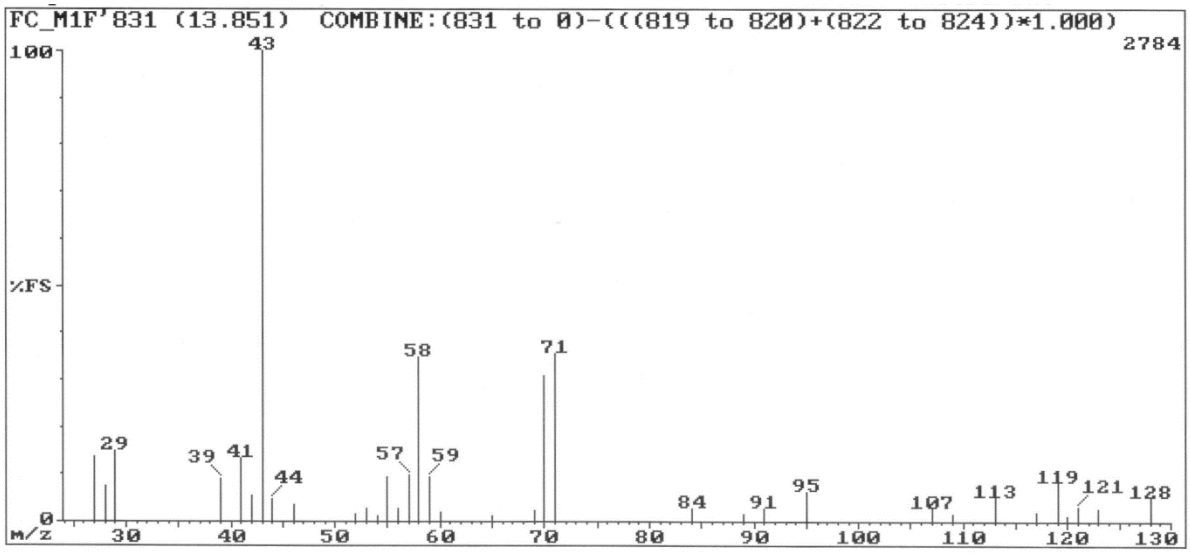


Fig. 3.35: EI-mass spectrum of 5-methyl-2-heptanone (wax column).

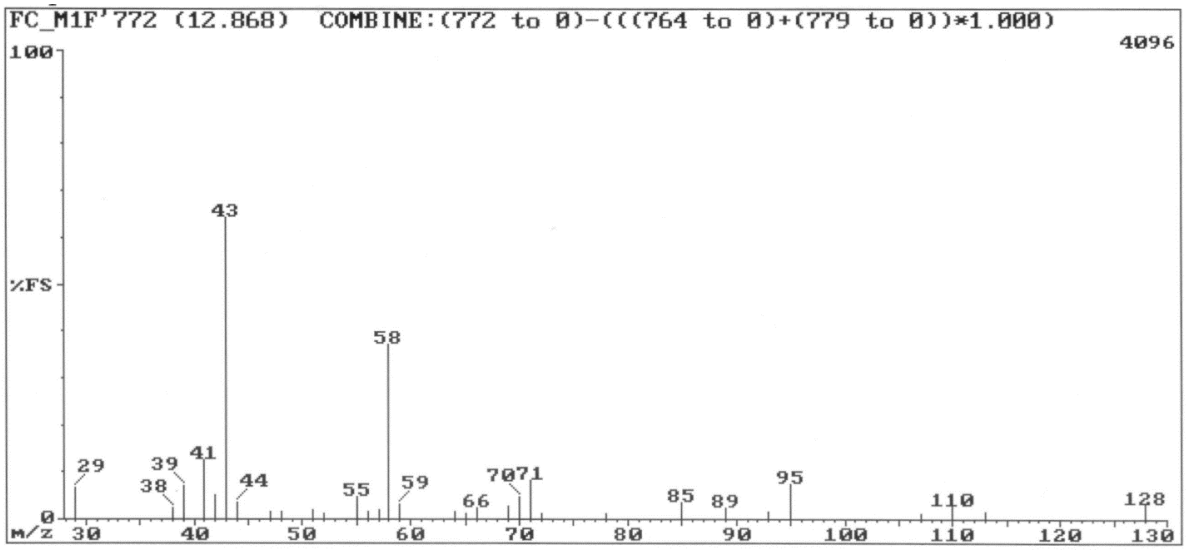


Fig. 3.36: EI-mass spectrum of 6-methyl-2-heptanone (wax column).

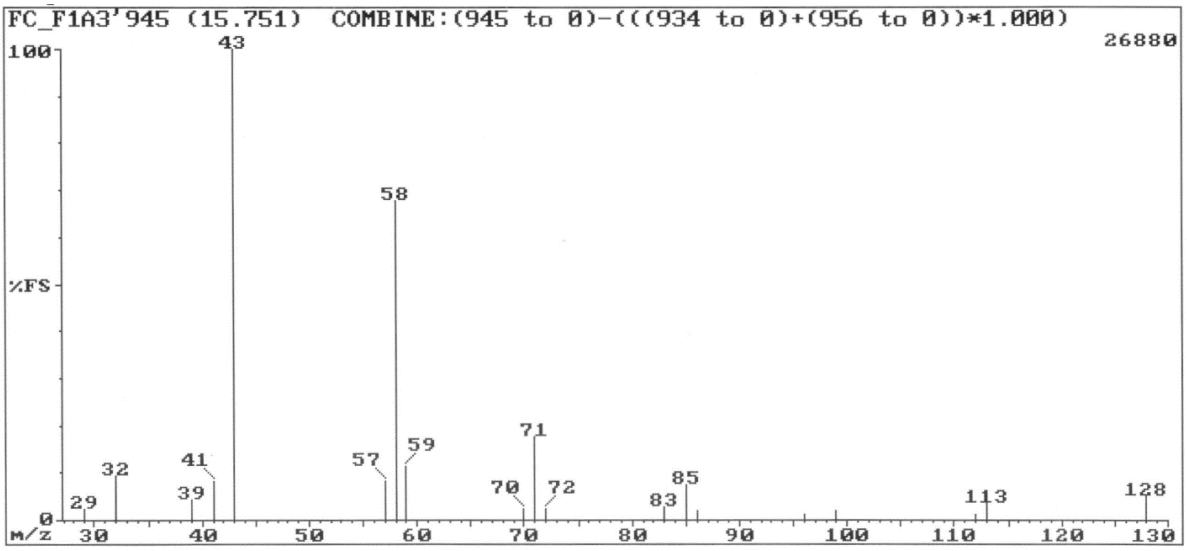


Fig. 3.37: EI-mass spectrum of 2-octanone (wax column).

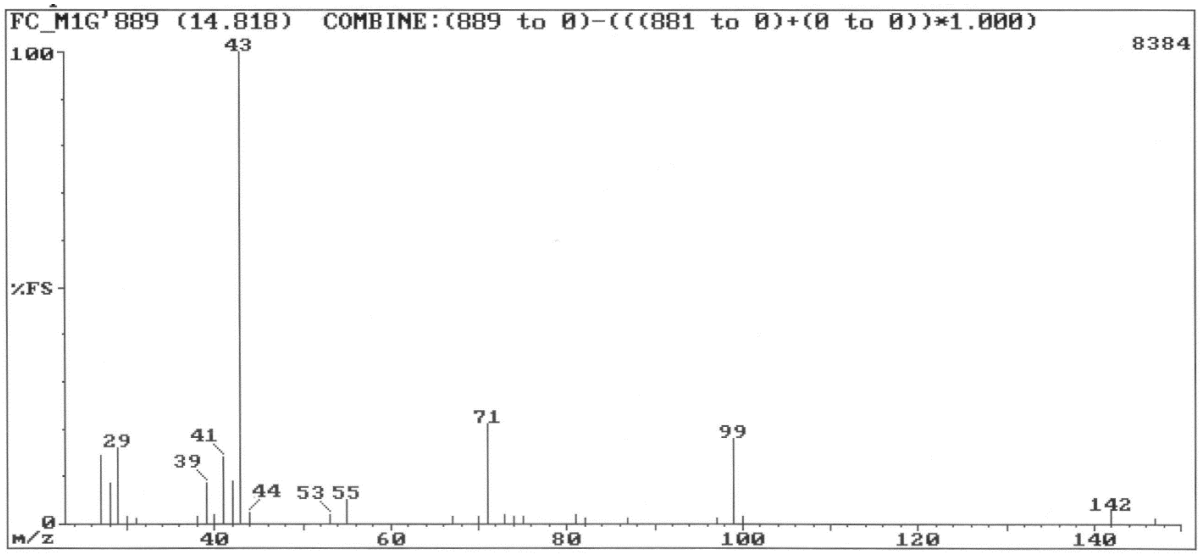


Fig. 3.38: EI-mass spectrum of 2-methyl-3-octanone (apolar column).

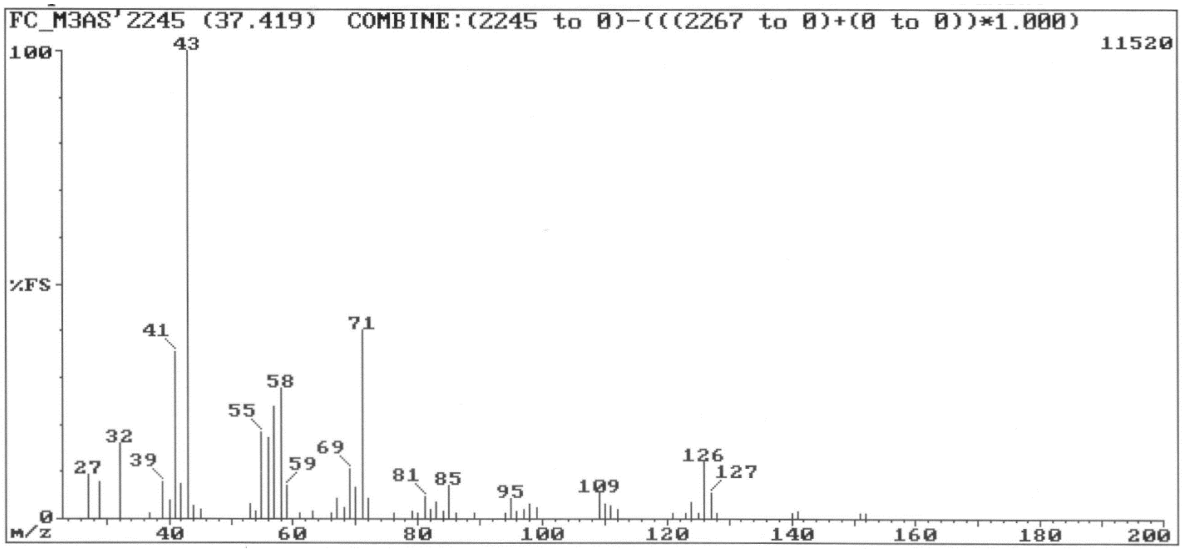


Fig. 3.39: EI-mass spectrum of 5,9-dimethyl-2-decanone (apolar column).

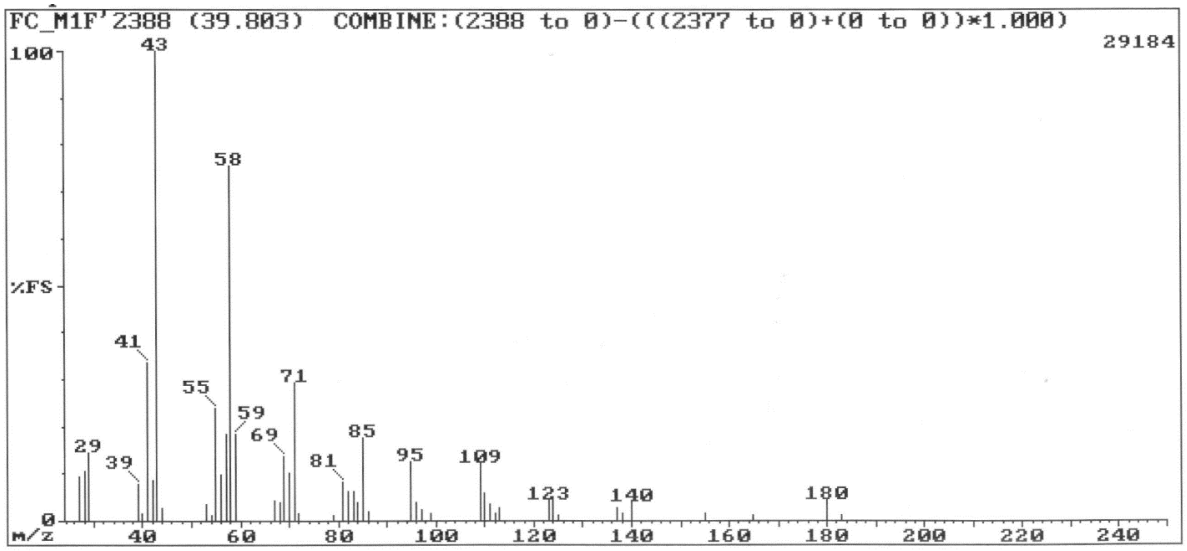


Fig. 3.40: EI-mass spectrum of *trans*-6,10-dimethyl-2-undecanone (wax column).

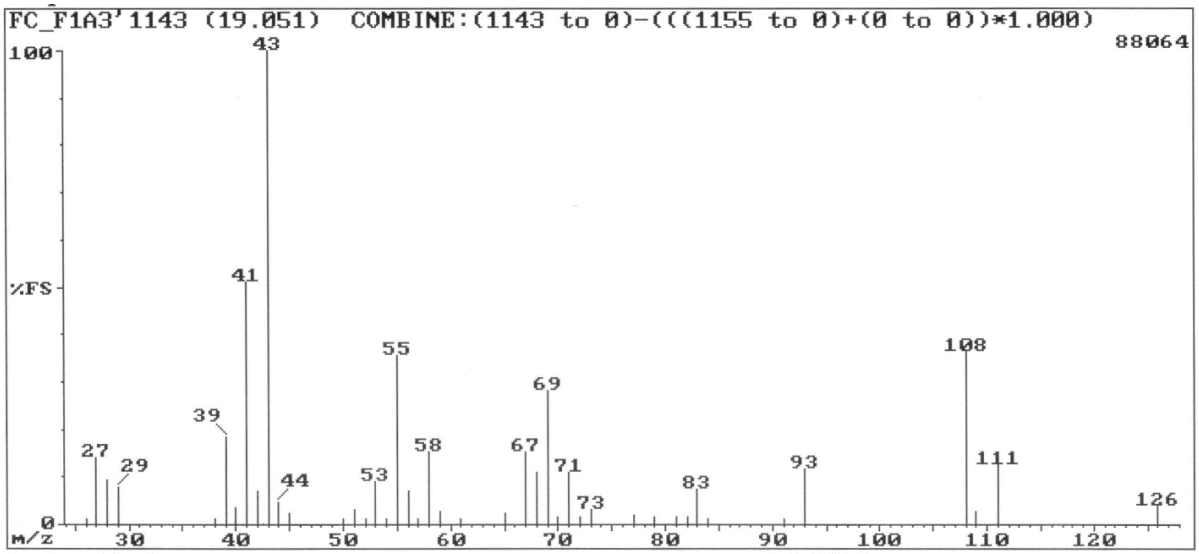


Fig. 3.41: EI-mass spectrum of 6-methyl-5-hepten-2-one (wax column).

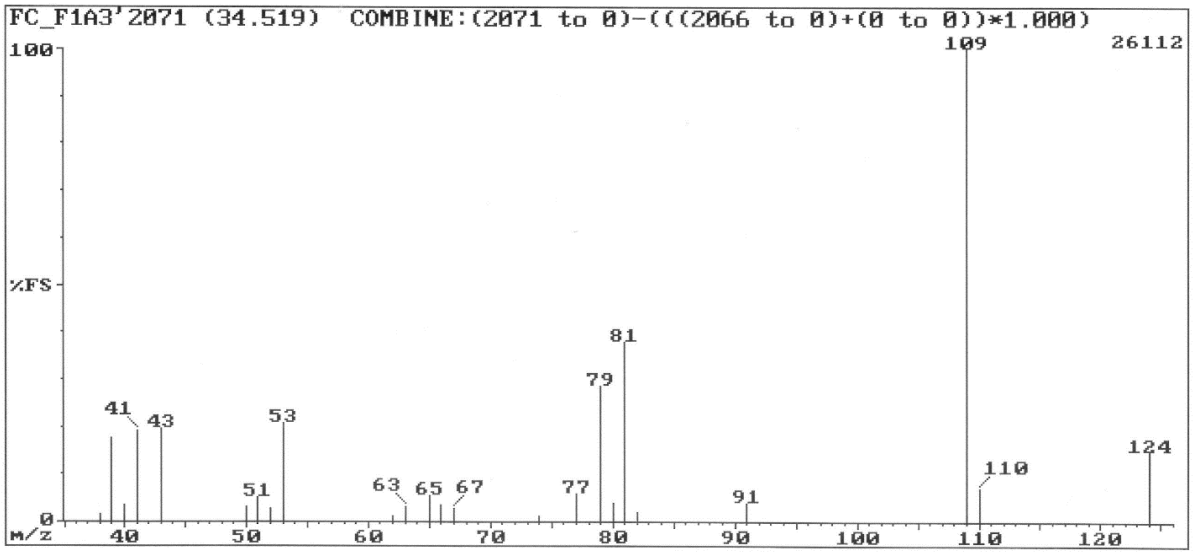


Fig. 3.42: EI-mass spectrum of *trans*-6-methyl-3,5-heptadien-2-one (wax column).

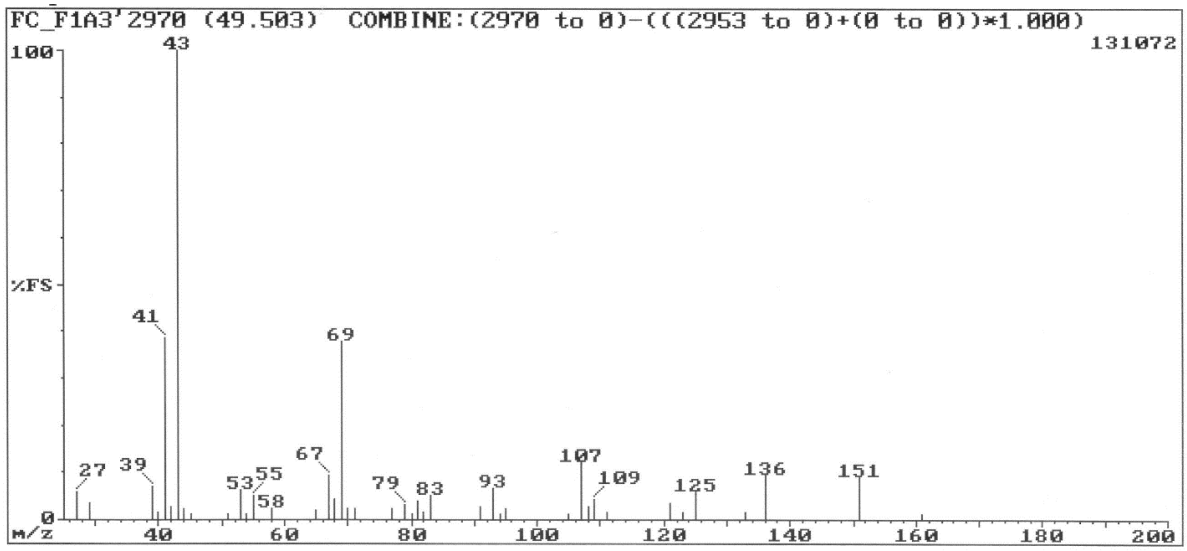


Fig. 3.43: EI-mass spectrum of *trans*-6,10-dimethyl-5,9-undecadien-2-one (wax column).

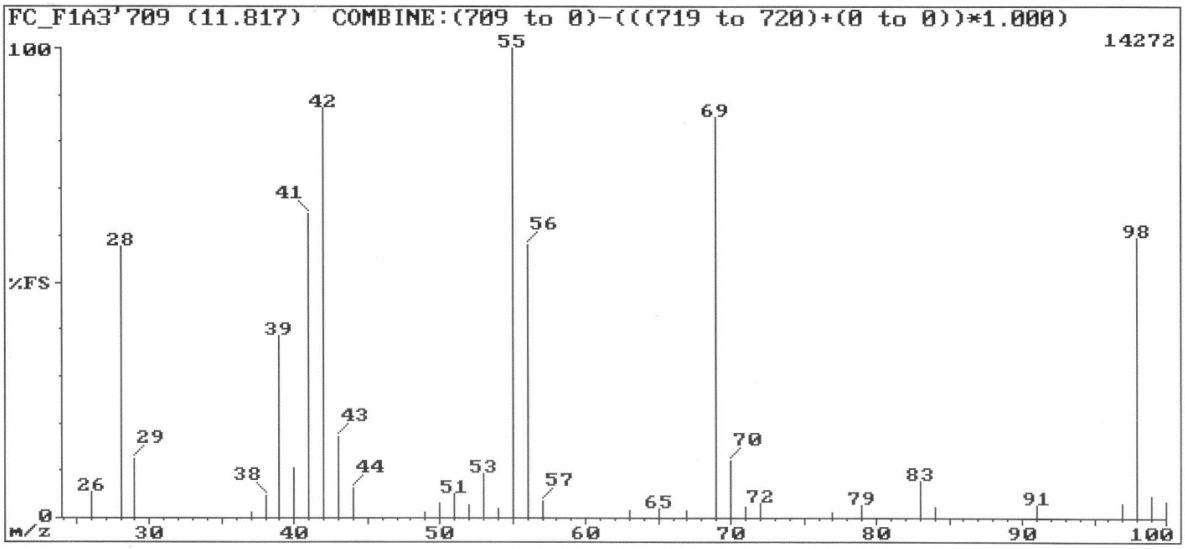


Fig. 3.44: EI-mass spectrum of 3-methylcyclopentanone (wax column).

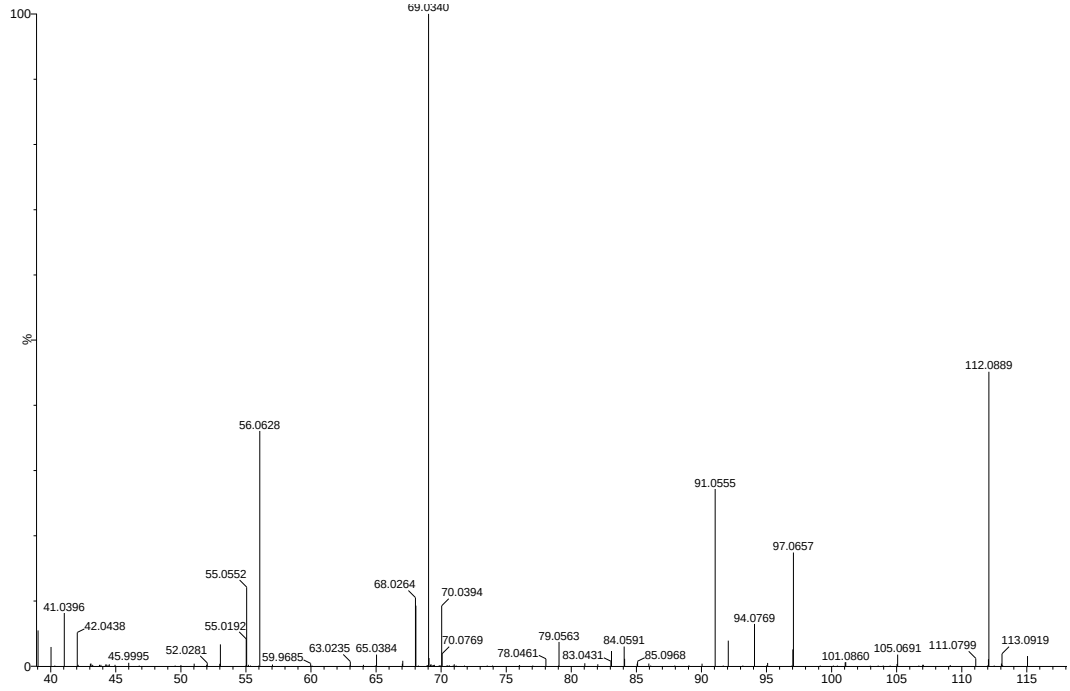


Fig. 3.45: EI-mass spectrum of 3-methylcyclohexanone (apolar column).

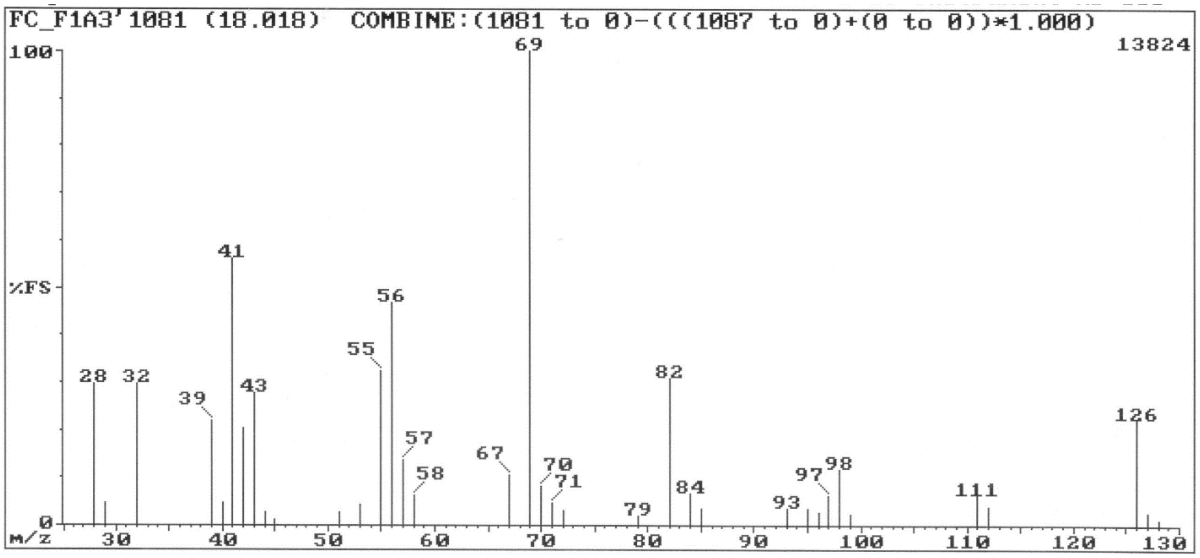


Fig. 3.46: EI-mass spectrum of 3-methylcycloheptanone (wax column).

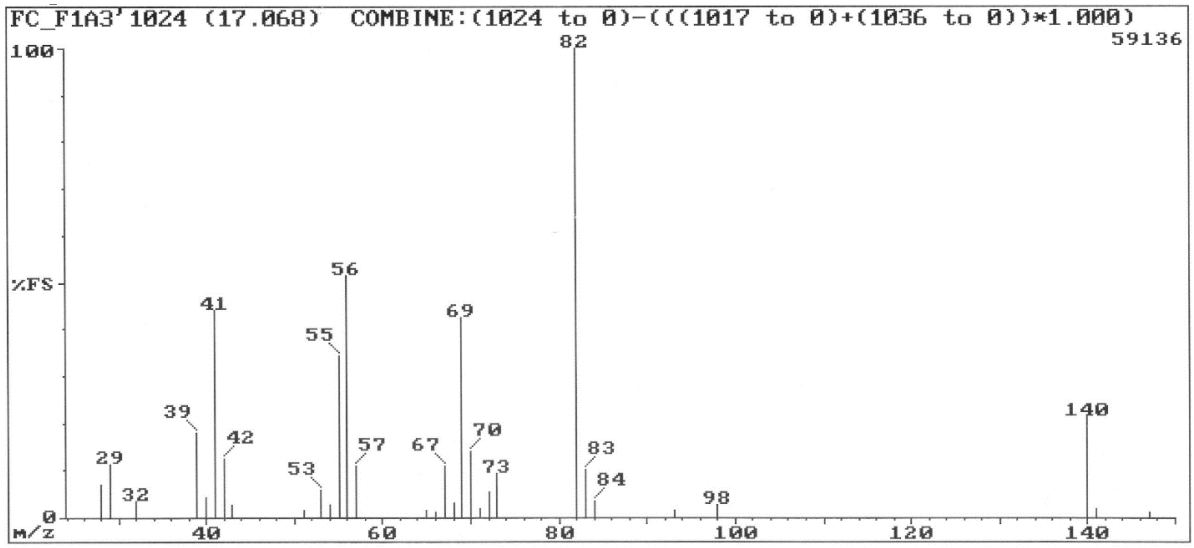


Fig. 3.47: EI-mass spectrum of 2,2,6-trimethylcyclohexanone (wax column).

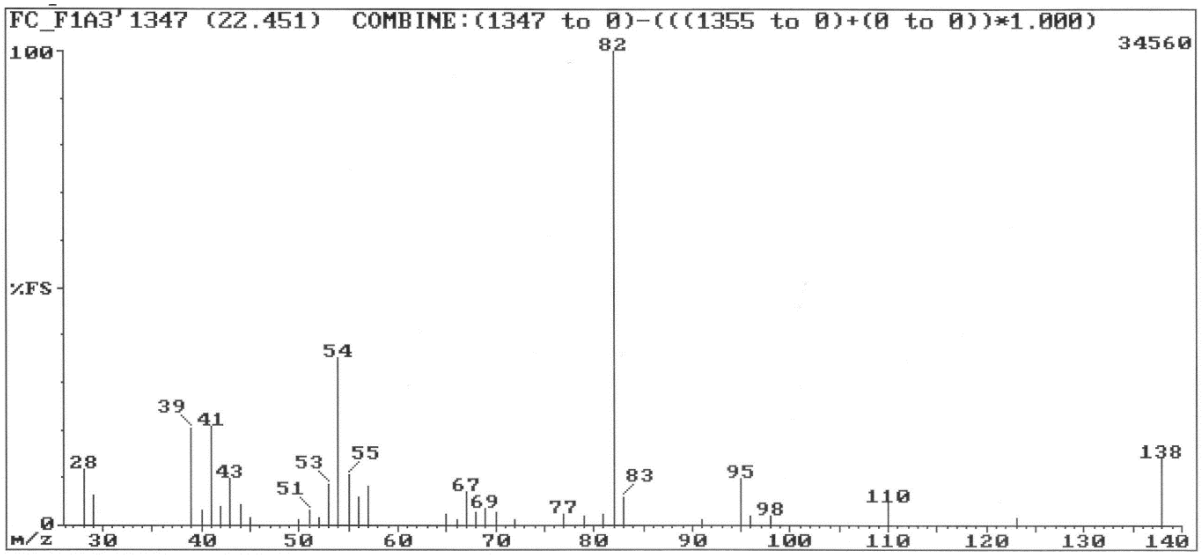


Fig. 3.48: EI-mass spectrum of isophorone (wax column).

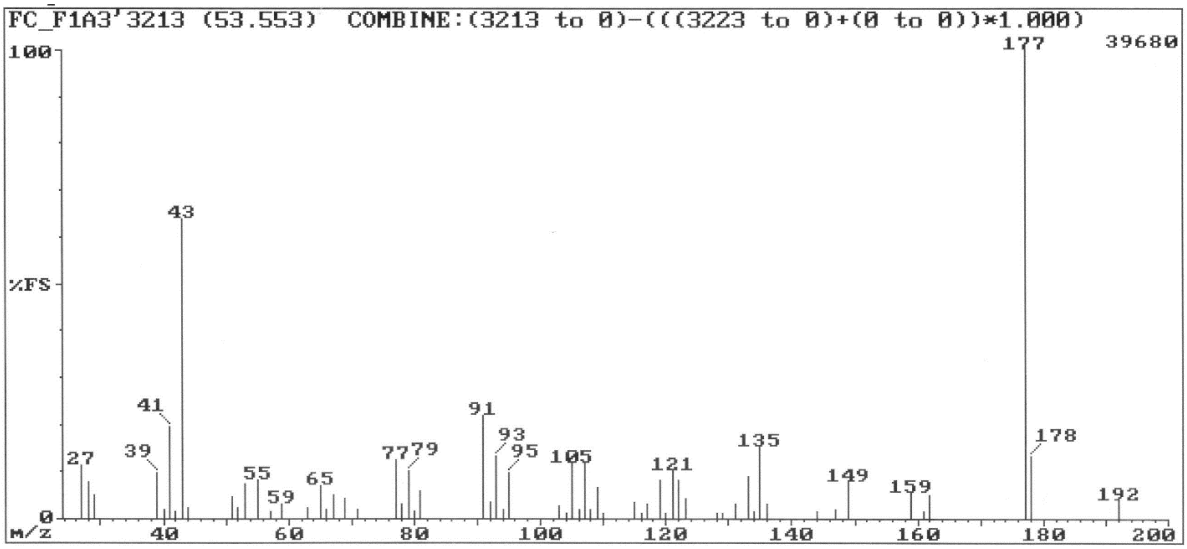


Fig. 3.49: EI-mass spectrum of β-ionone (wax column).

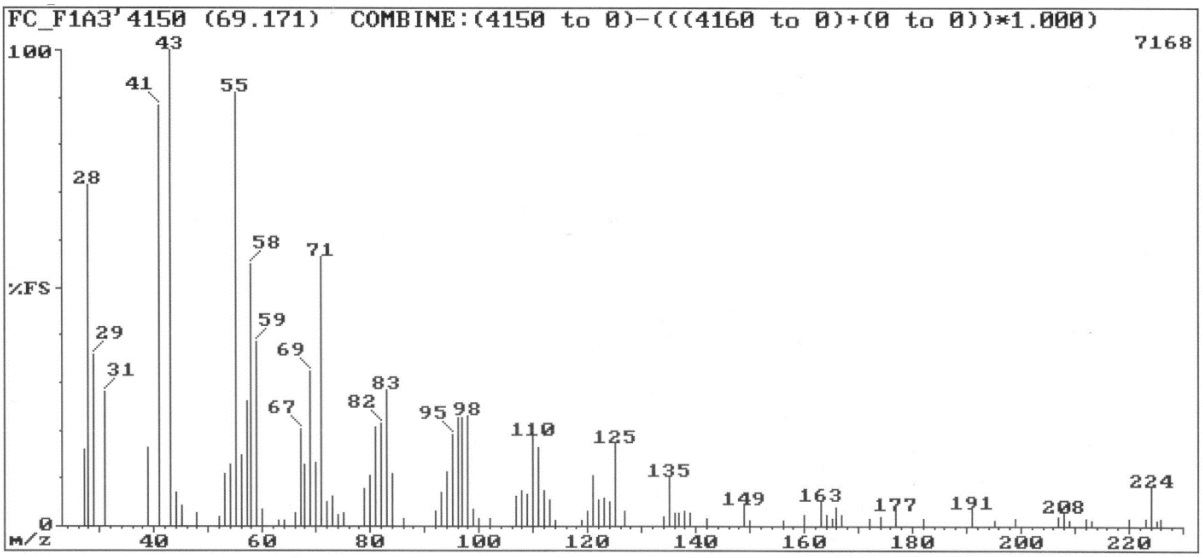


Fig. 3.50: EI-mass spectrum of cyclopentadecanone (wax column).

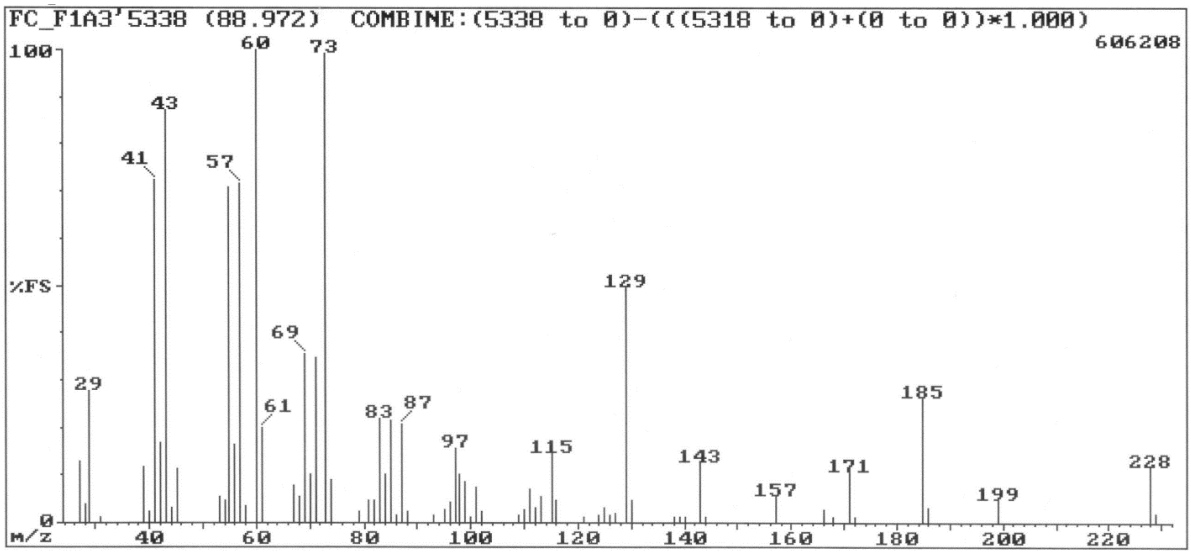


Fig. 3.51: EI-mass spectrum of tetradecanoic acid (wax column).

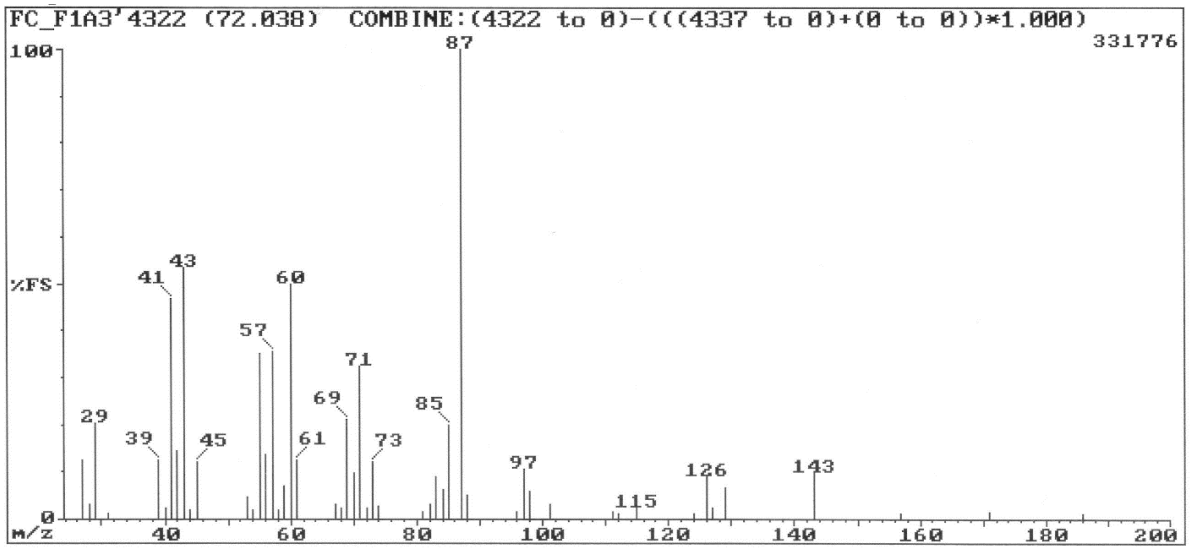


Fig. 3.52: EI-mass spectrum of 3-methyldecanoic acid (wax column).

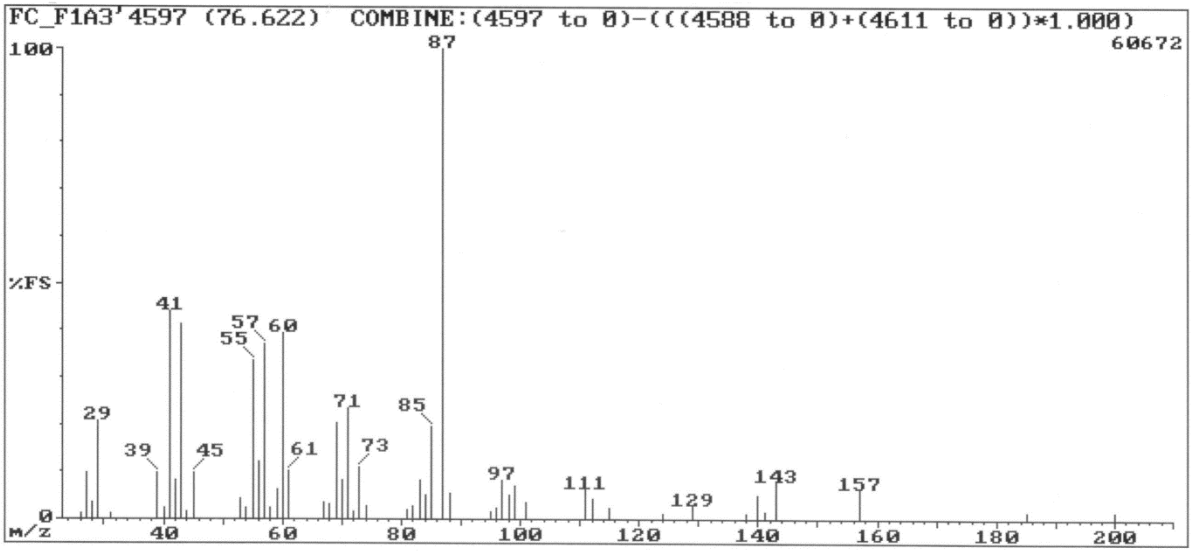


Fig. 3.53: EI-mass spectrum of 2-methylundecanoic acid (wax column).

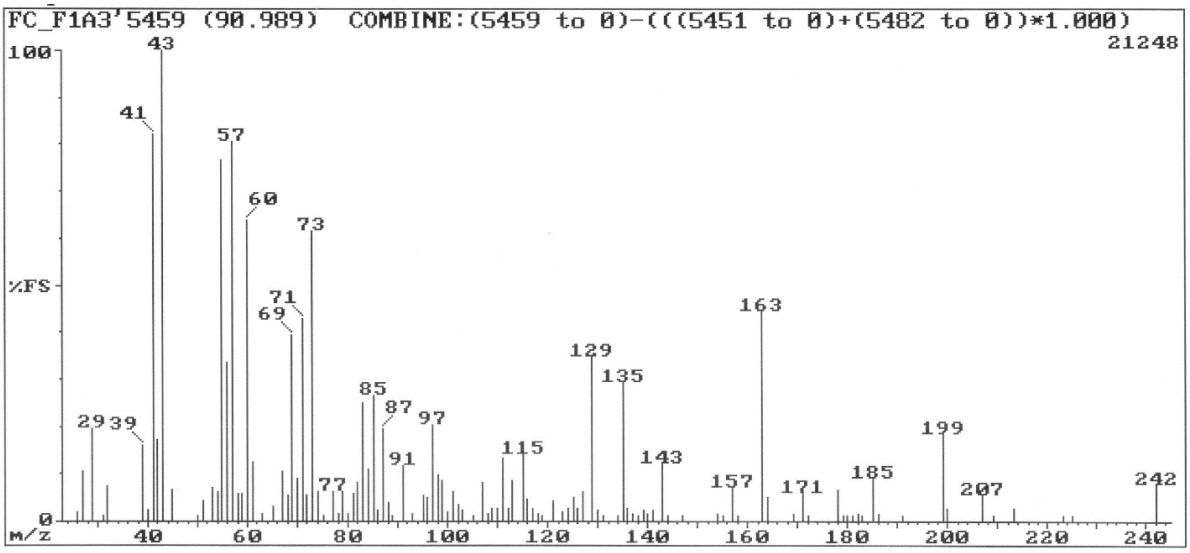


Fig. 3.54: EI-mass spectrum of *iso*-pentadecanoic acid (wax column).

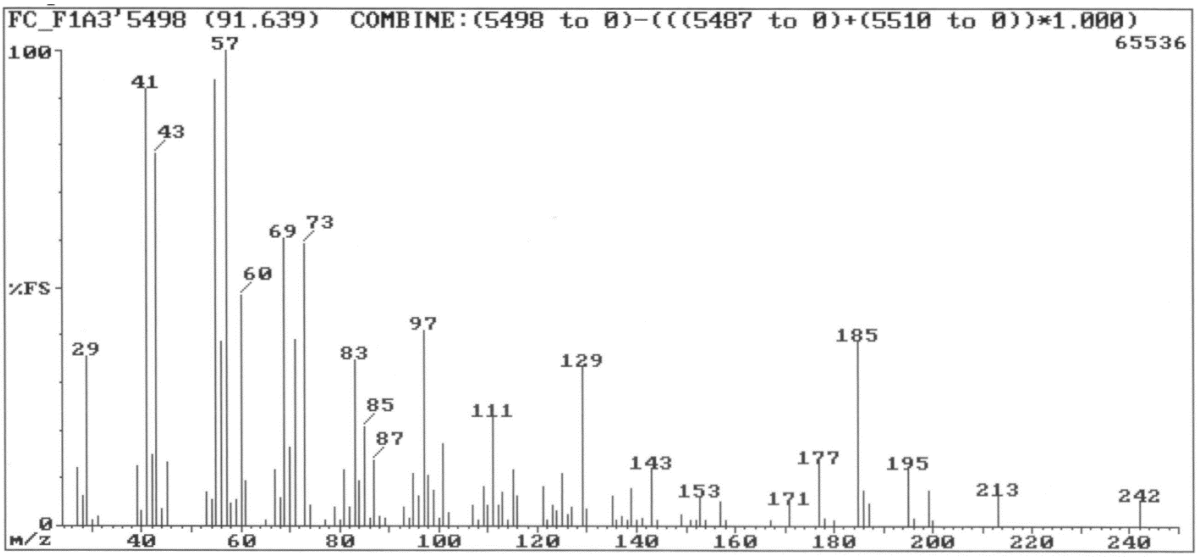


Fig. 3.55: EI-mass spectrum of *anteiso*-pentadecanoic acid (wax column).

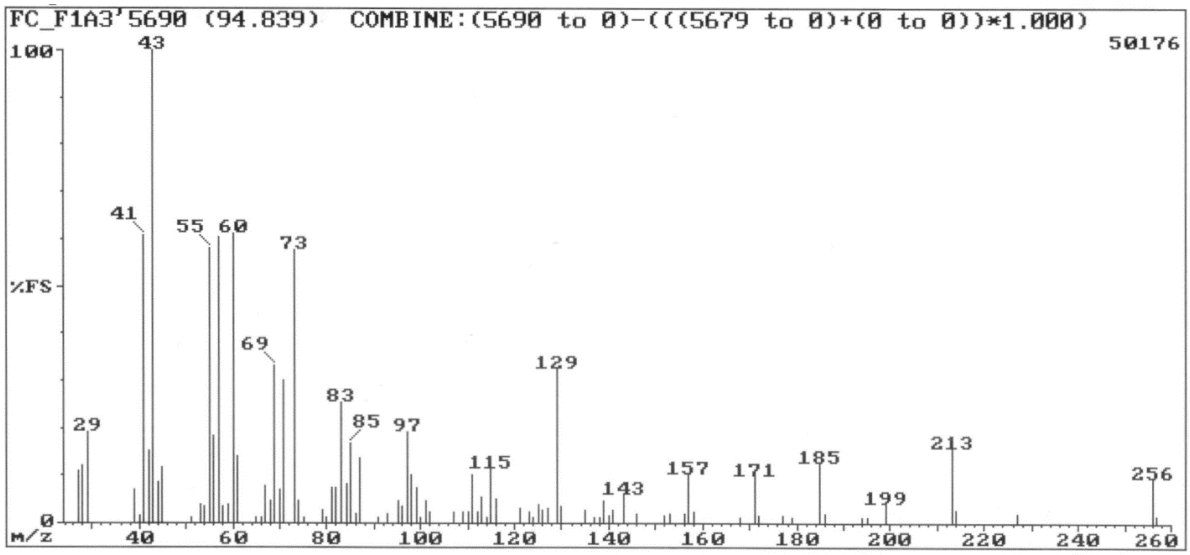


Fig. 3.56: EI-mass spectrum of *anteiso*-hexadecanoic acid (wax column).

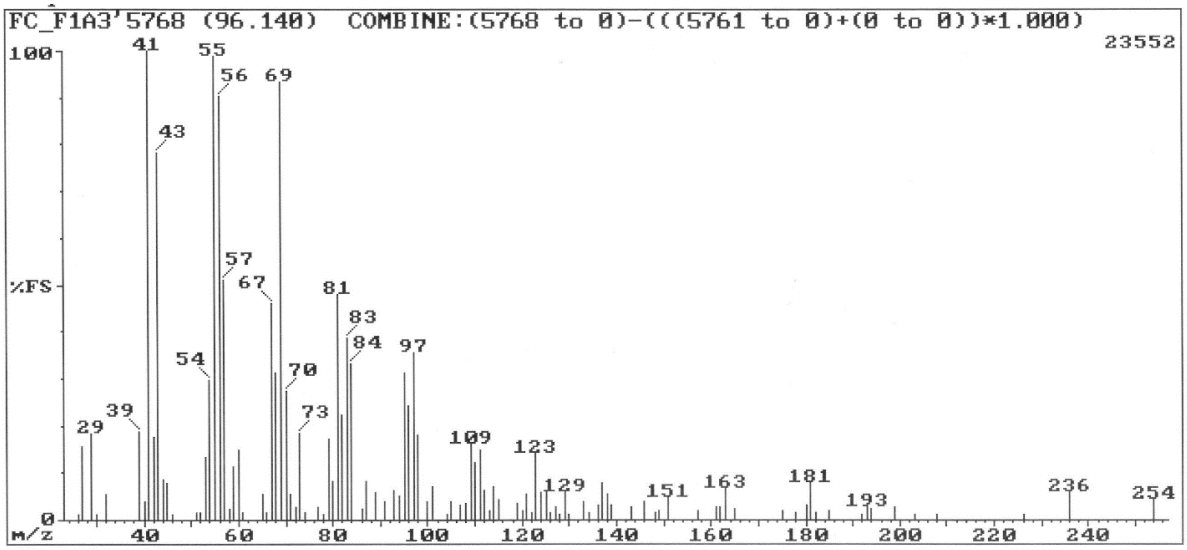


Fig. 3.57: EI-mass spectrum of *cis*-9-hexadecenoic acid (wax column).

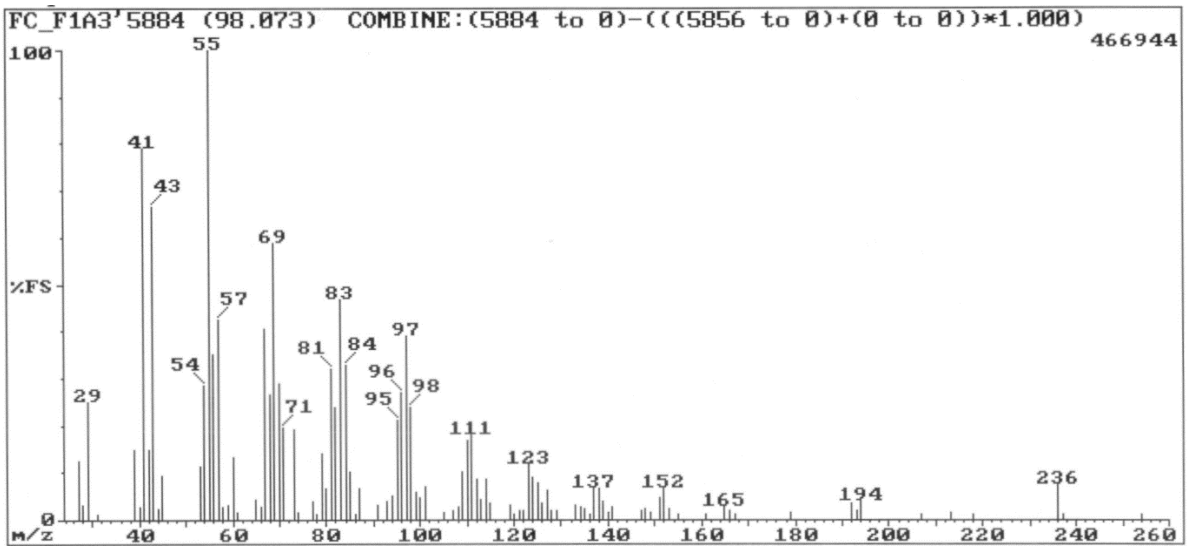


Fig. 3.58: EI-mass spectrum of *trans*-9-hexadecenaoic acid (wax column).

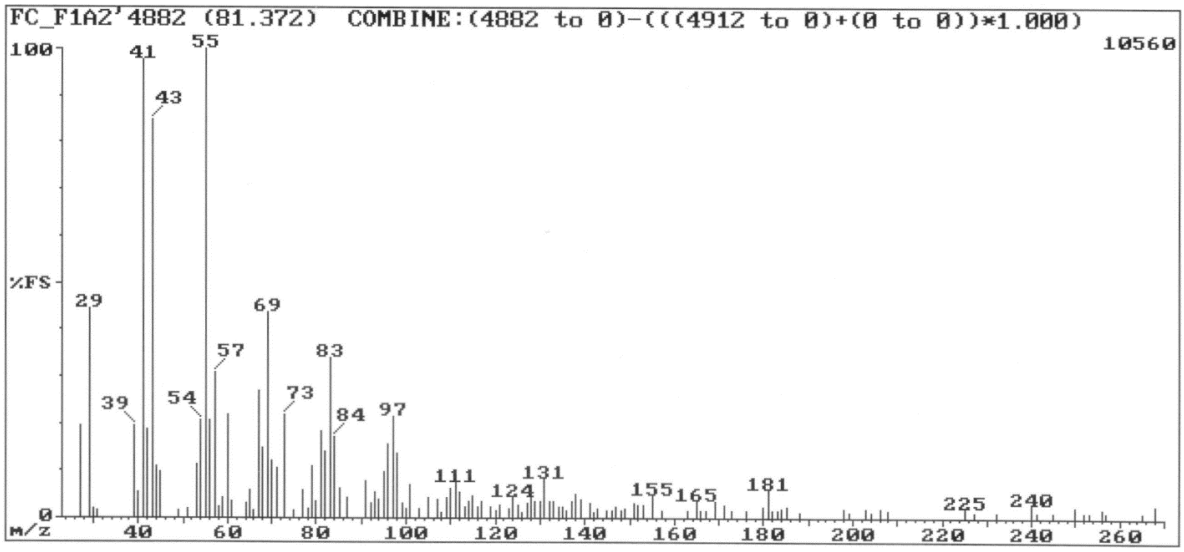


Fig. 3.59: EI-mass spectrum of *cis*-heptadecenoic acid (apolar column).

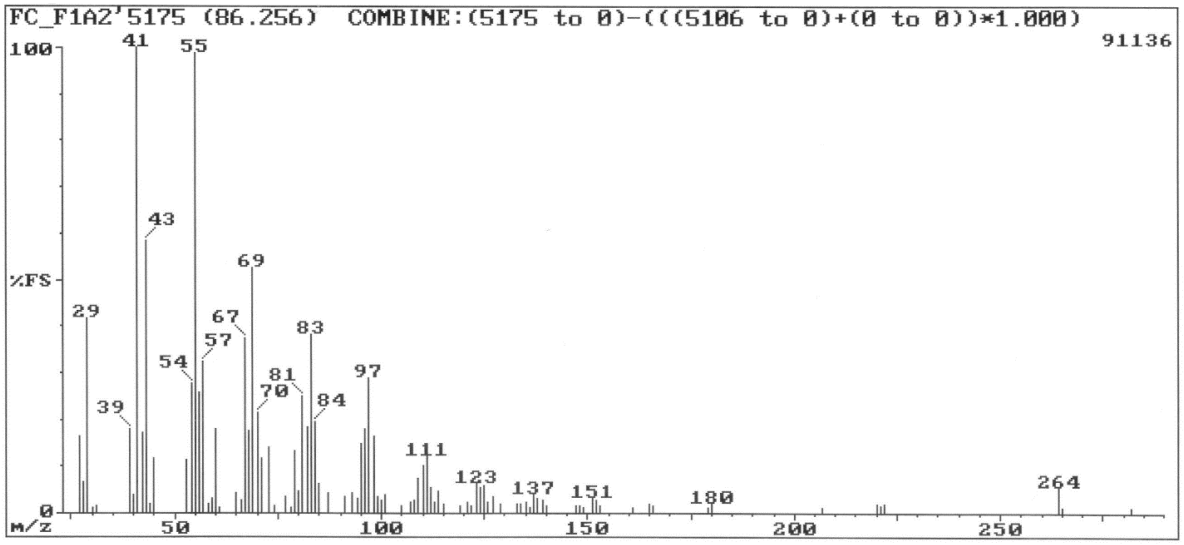


Fig. 3.60: EI-mass spectrum of oleic acid (apolar column).

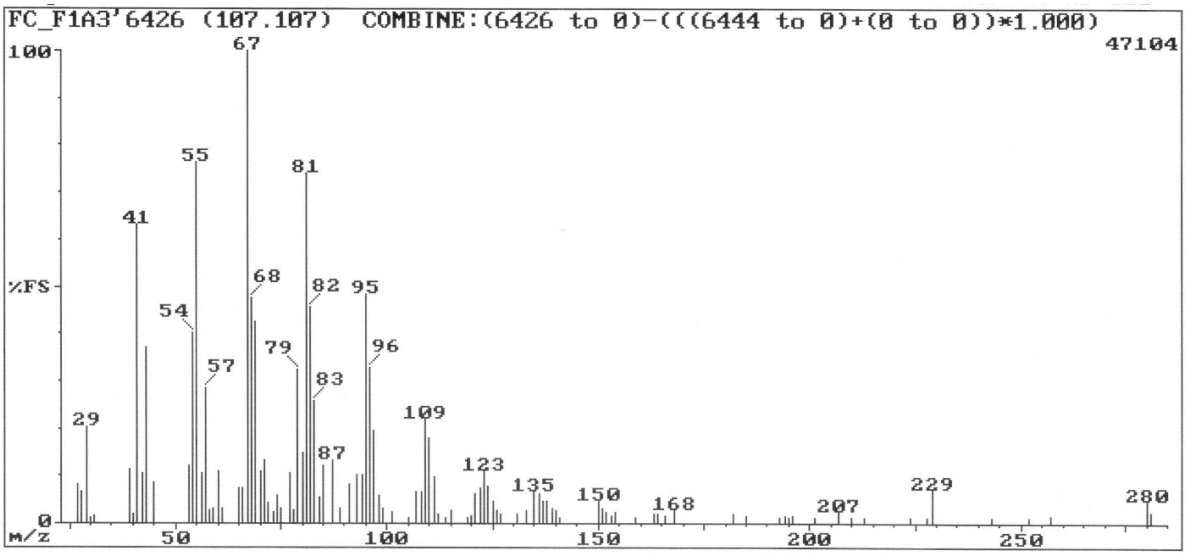


Fig. 3.61: EI-mass spectrum of linoleic acid (wax column).

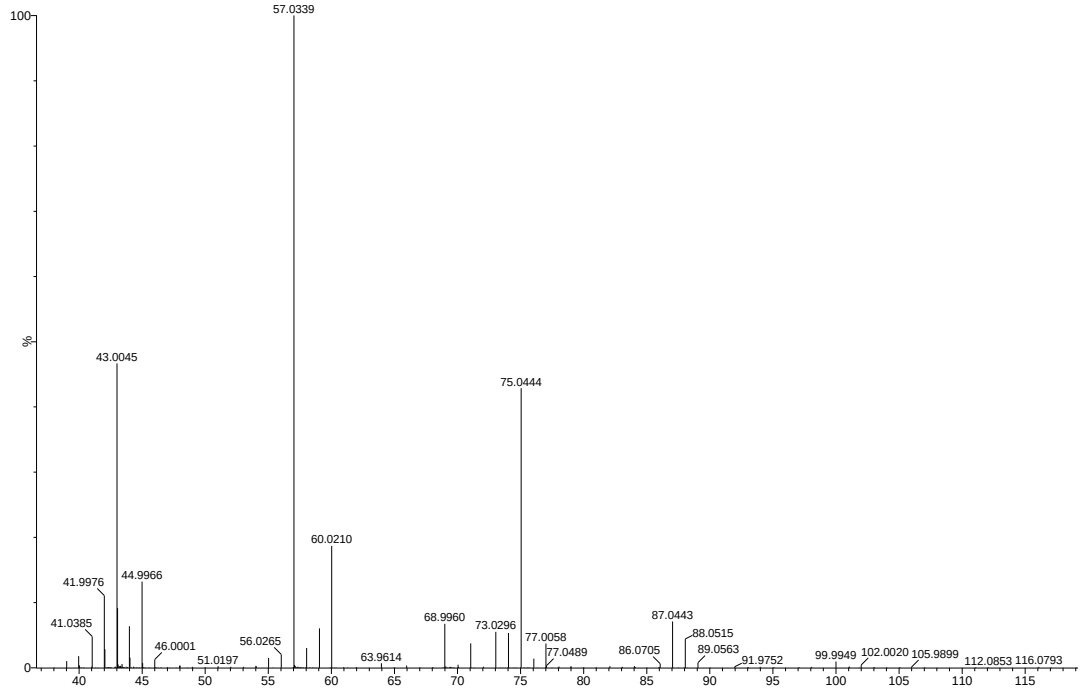


Fig. 3.62: EI-mass spectrum of propyl propanoate (apolar column).

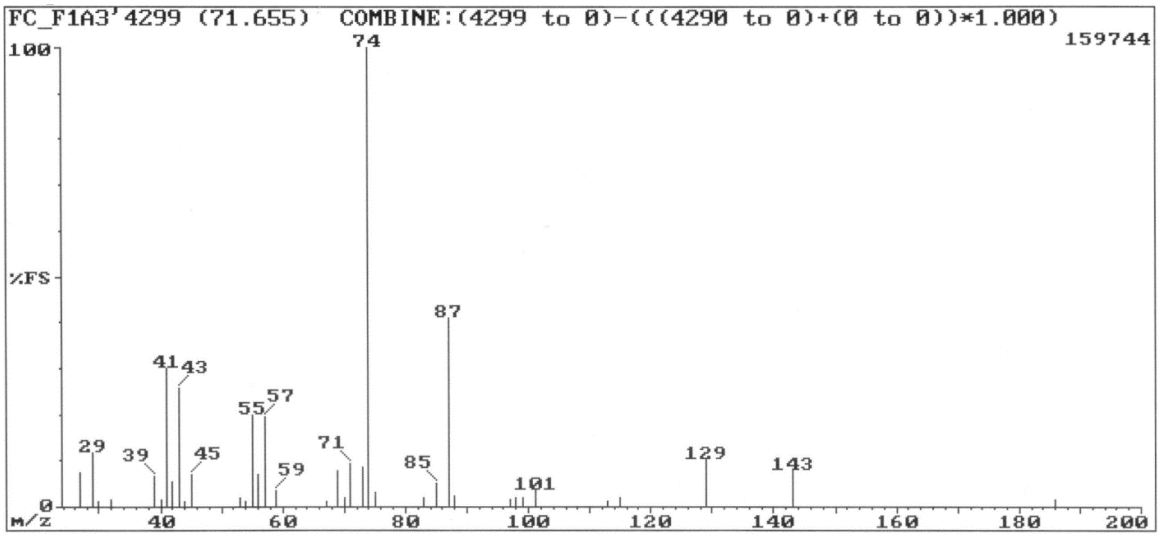


Fig. 3.63: EI-mass spectrum of methyldecanoate (wax column).

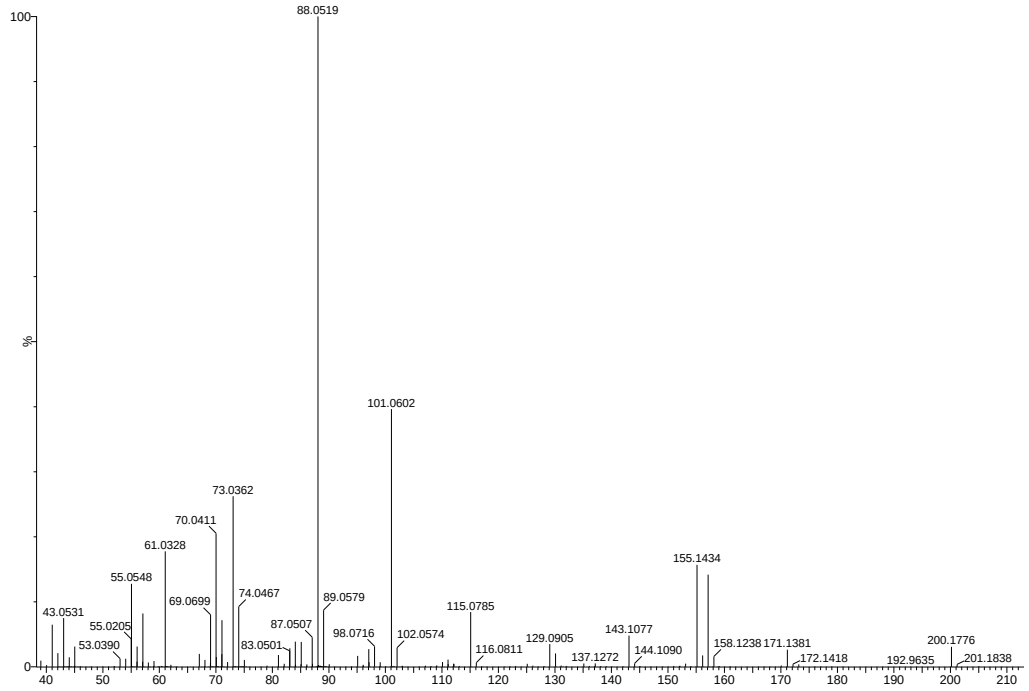


Fig. 3.64: EI-mass spectrum of ethyldecanoate (apolar column).

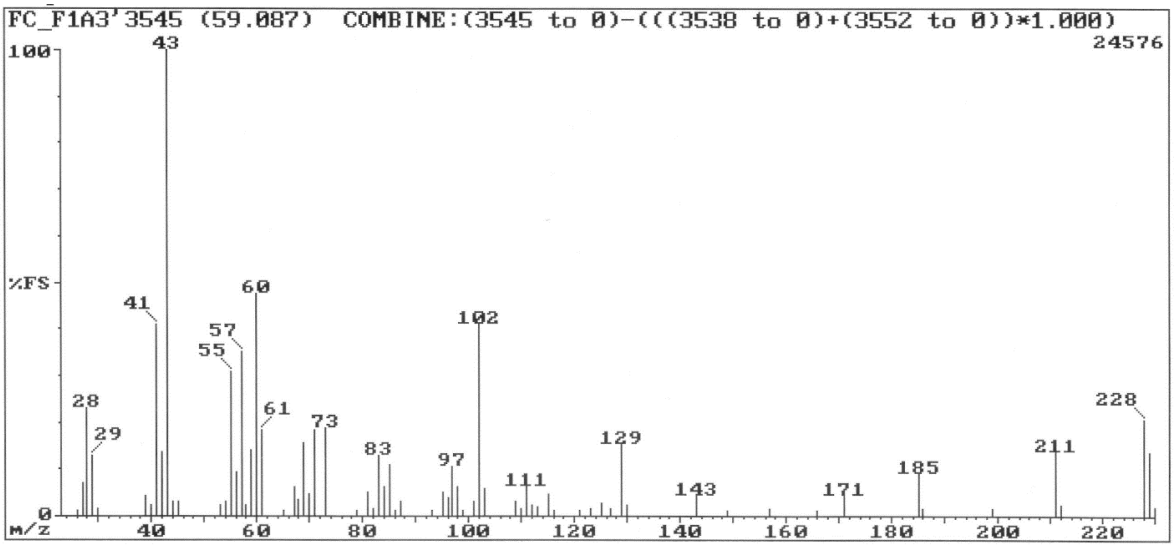


Fig. 3.65: EI-mass spectrum of isopropyltetradecanoate (wax column).

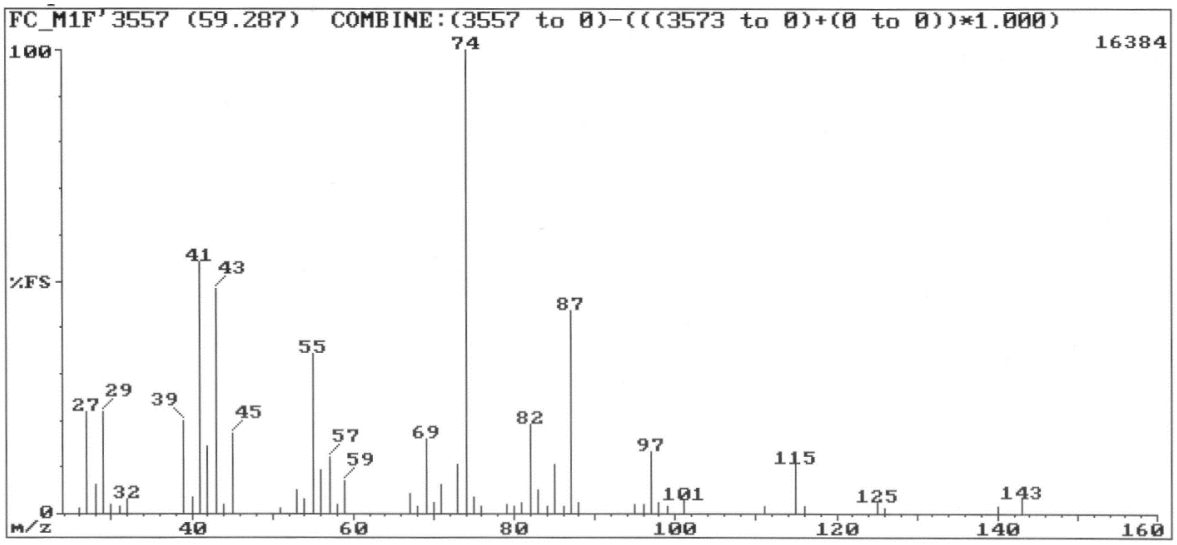


Fig. 3.66: EI-mass spectrum of methyl 6-methylheptanoate (wax column).

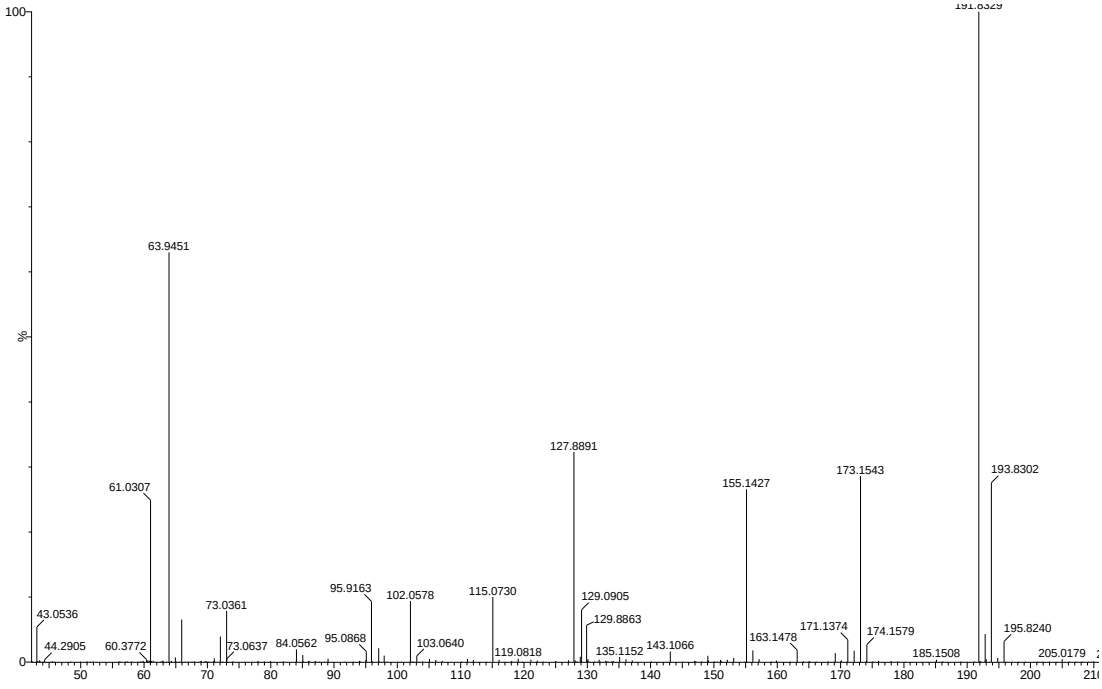


Fig. 3.67: EI-mass spectrum of sulphur (S_6) (apolar column).

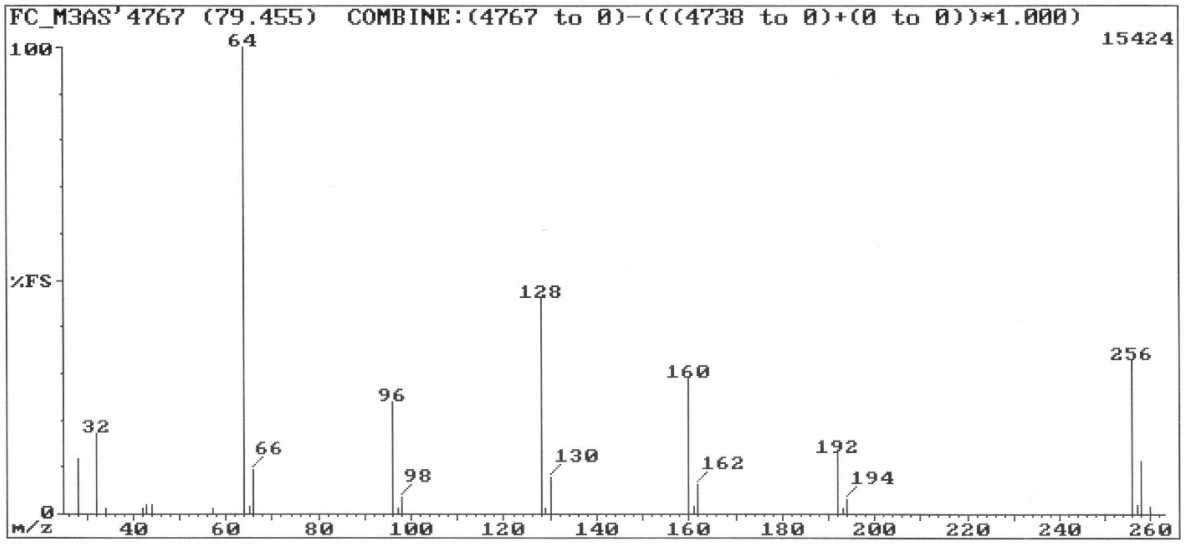


Fig. 3.68: EI-mass spectrum of sulphur (S_8) (apolar column).

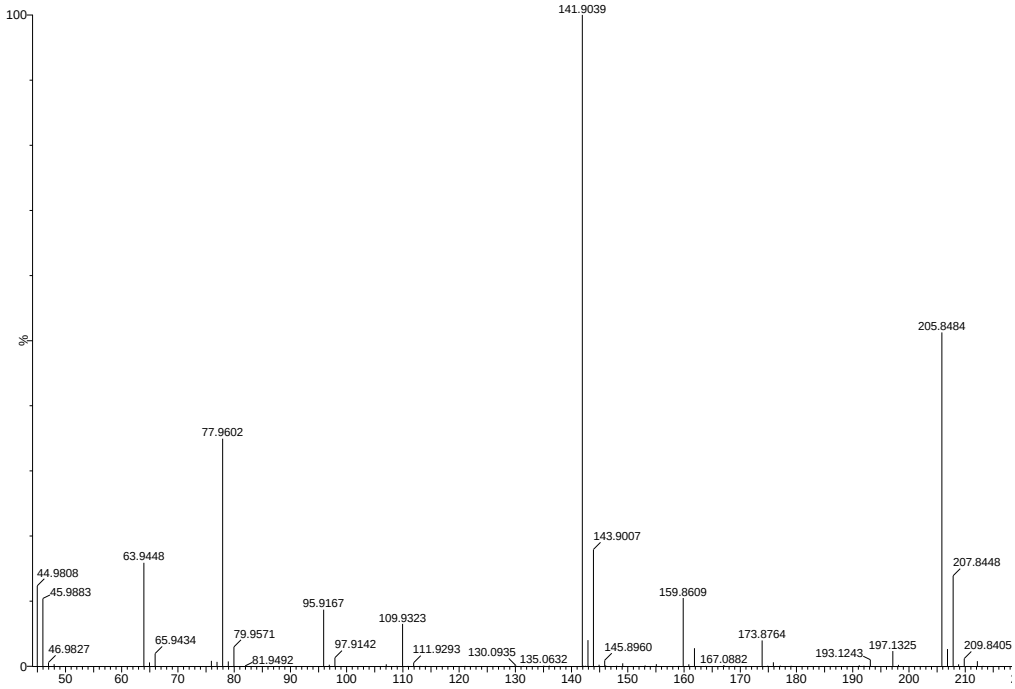


Fig. 3.69: EI-mass spectrum of hexathiepane (CH_2S_6)(apolar column).

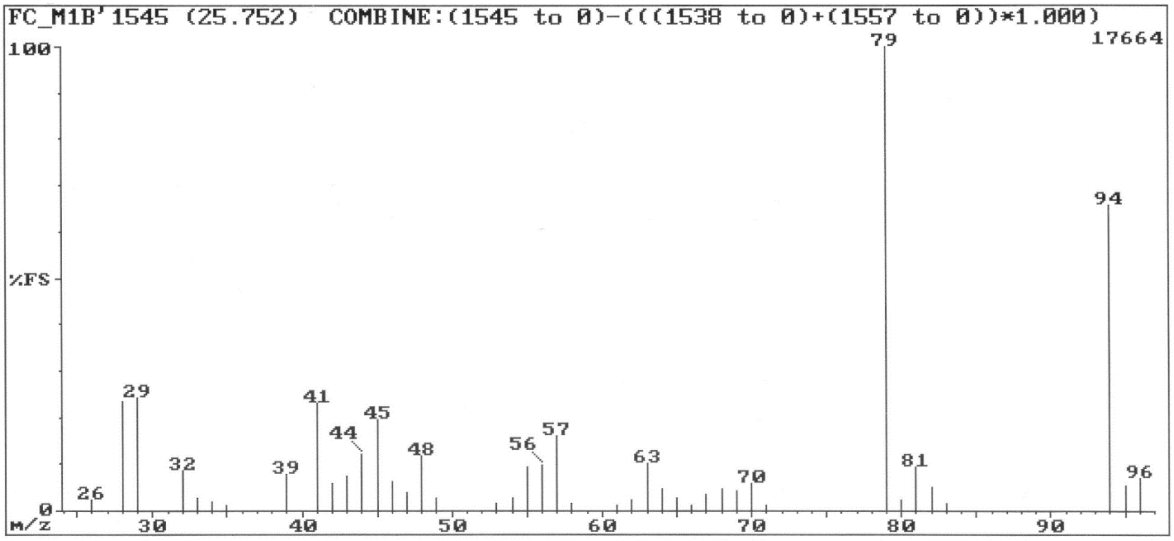


Fig. 3.70: EI-mass spectrum of dimethylsulphone (1701 column).

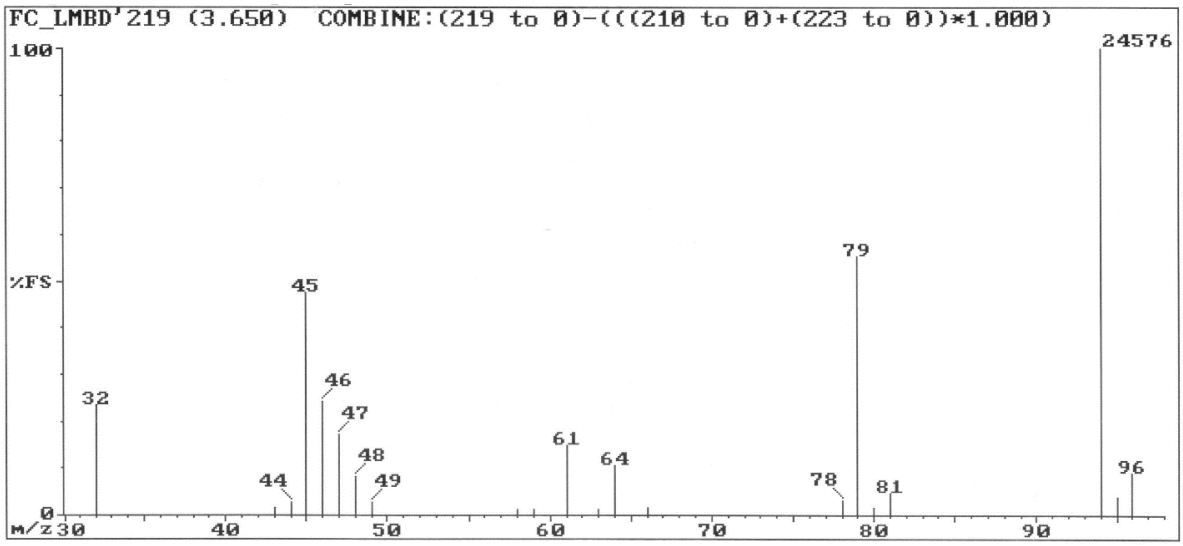


Fig. 3.71: EI-mass spectrum of dimethyldisulphide (apolar column).

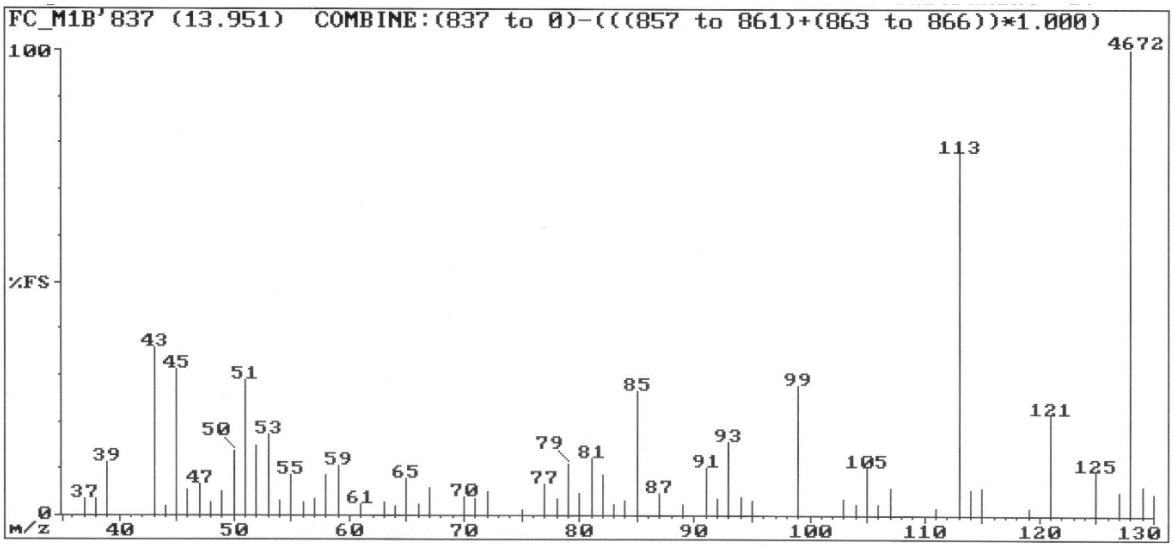


Fig. 3.72: EI-mass spectrum of 2-methyl-5-(methylthio)-furan (1701column).

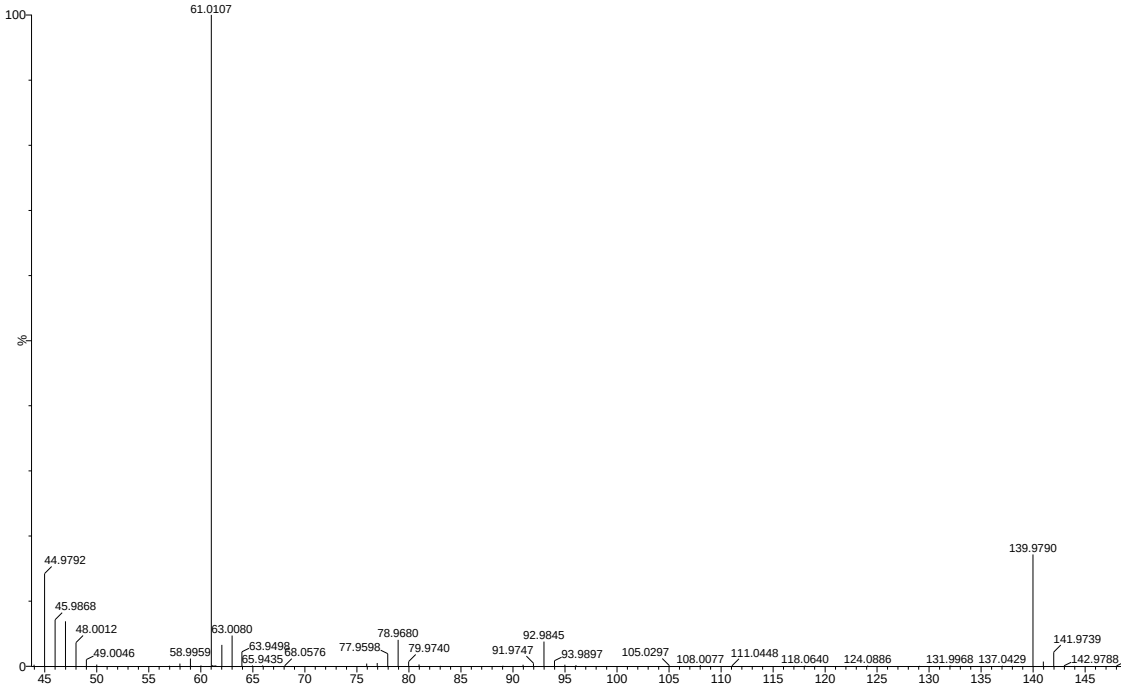


Fig. 3.73: EI-mass spectrum of methyl (methylthio)methyl disulphide (apolar column).

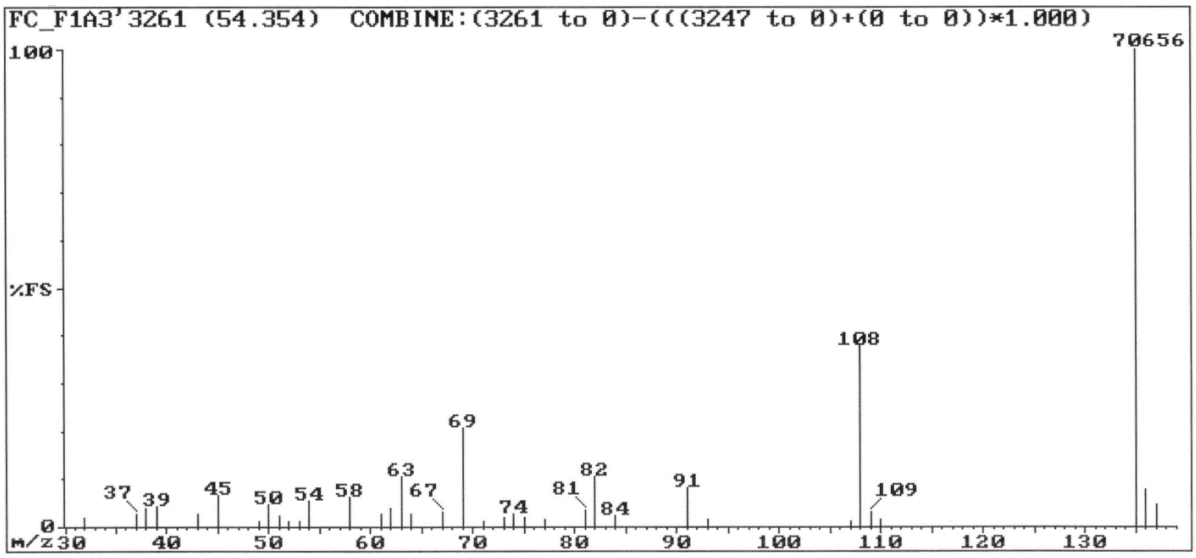


Fig. 3.74: EI-mass spectrum of benzothiazole (wax column).

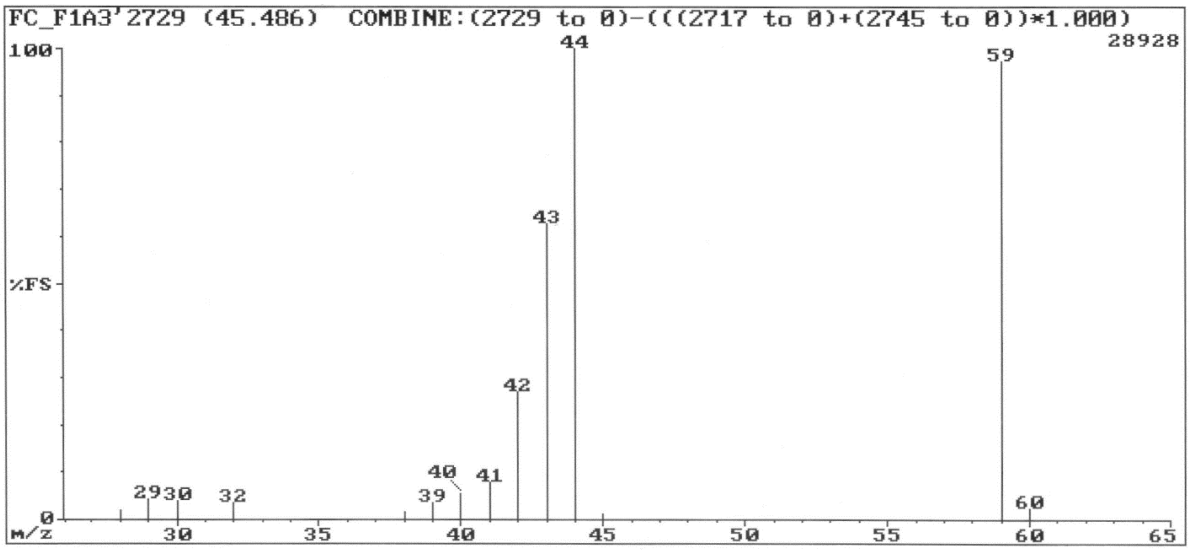


Fig. 3.76: EI-mass spectrum of acetamide (wax column).

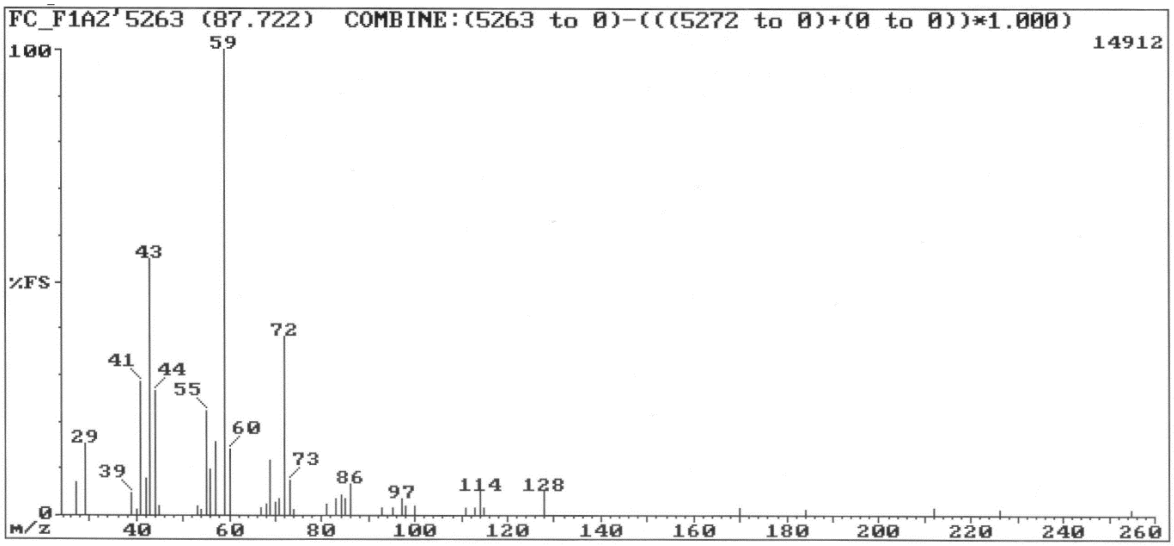


Fig. 3.77: EI-mass spectrum of hexadecanamide (apolar column).

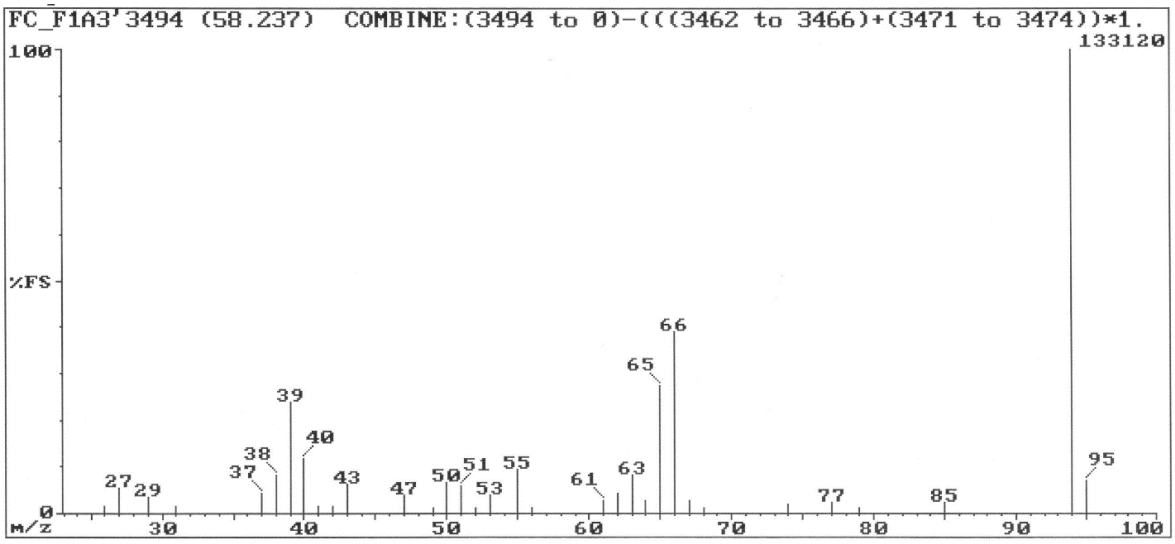


Fig. 3.78: EI-mass spectrum of phenol (wax column).

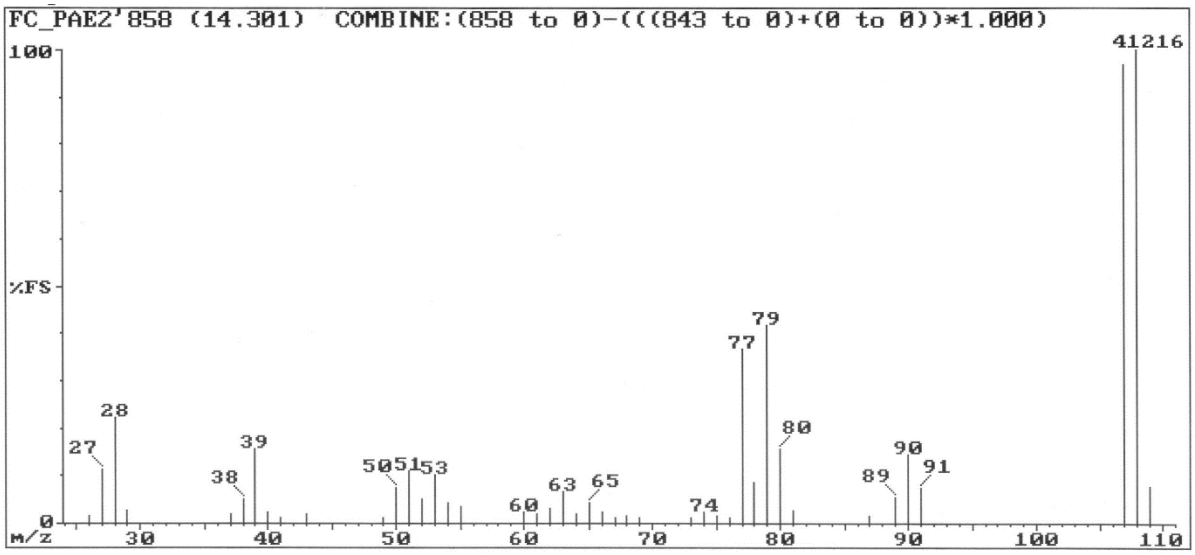


Fig. 3.79: EI-mass spectrum of 3-methylphenol (*m*-cresol) (apolar column).

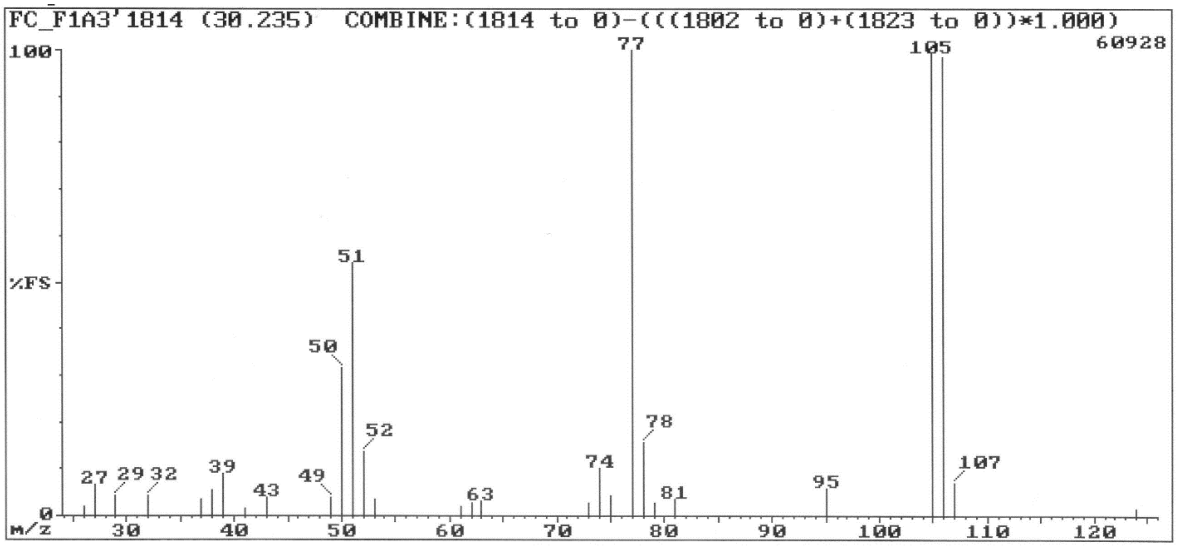


Fig. 3.80: EI-mass spectrum of benzaldehyde (wax column).

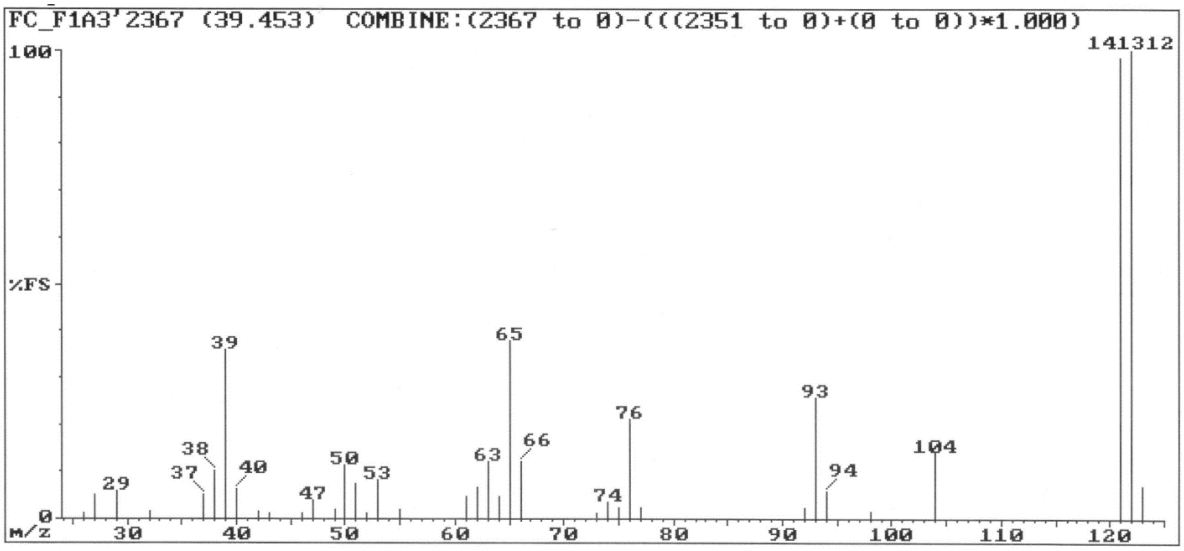


Fig. 3.81: EI-mass spectrum of salicylaldehyde (wax column).

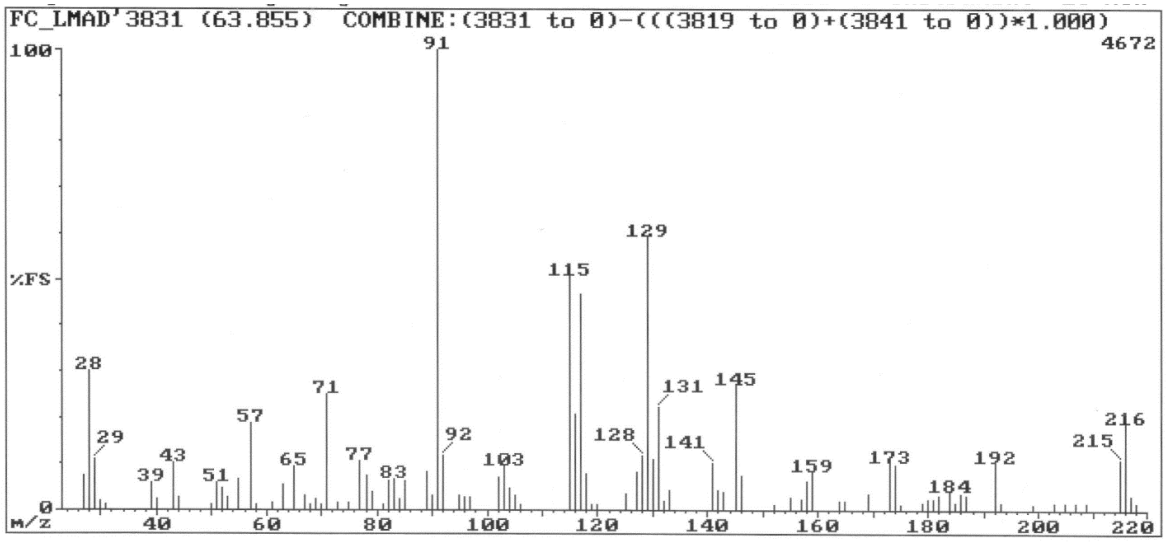


Fig. 3.82: EI-mass spectrum of α-hexylcinnamaldehyde (apolar column).

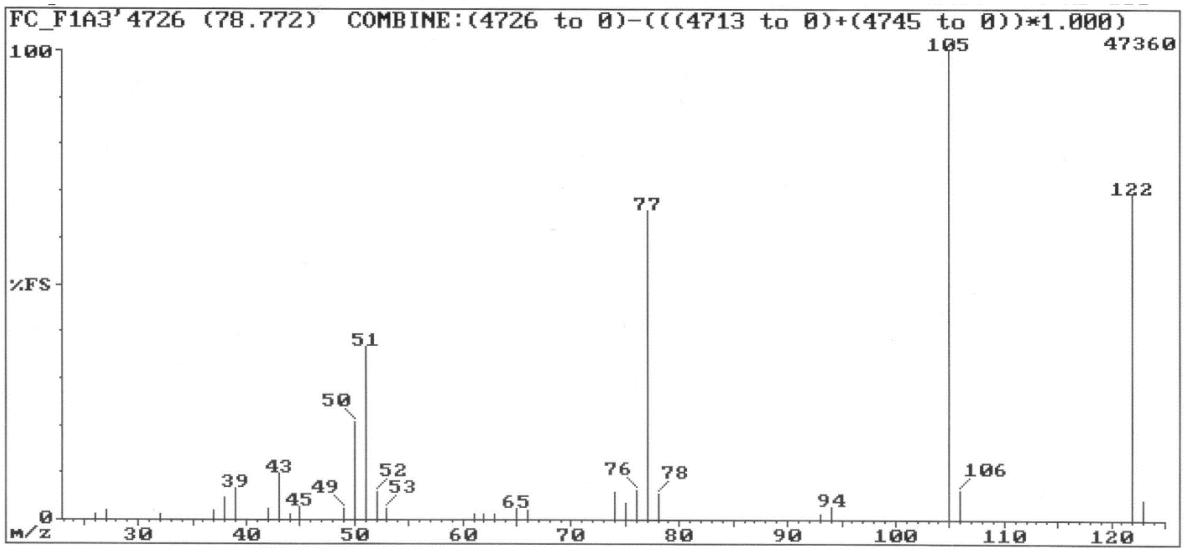


Fig. 3.83: EI-mass spectrum of benzoic acid (wax column).

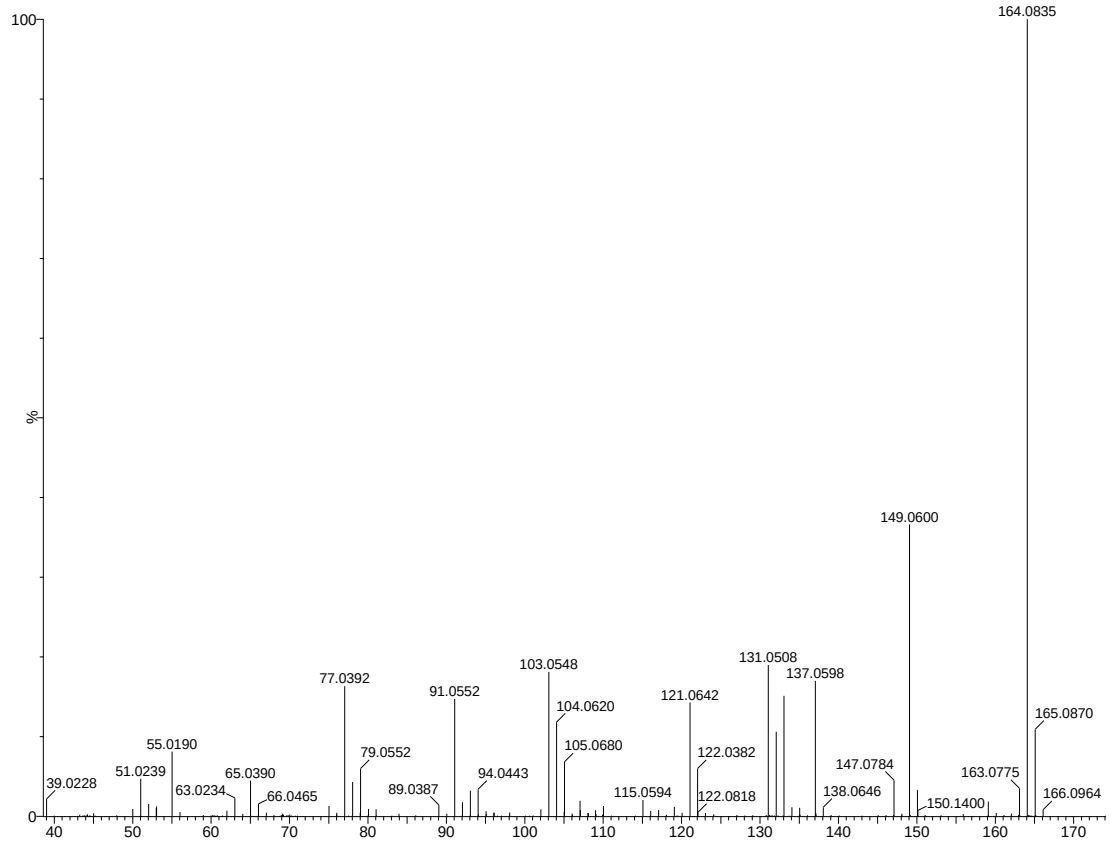


Fig. 3.84: EI-mass spectrum of eugenol (apolar column).

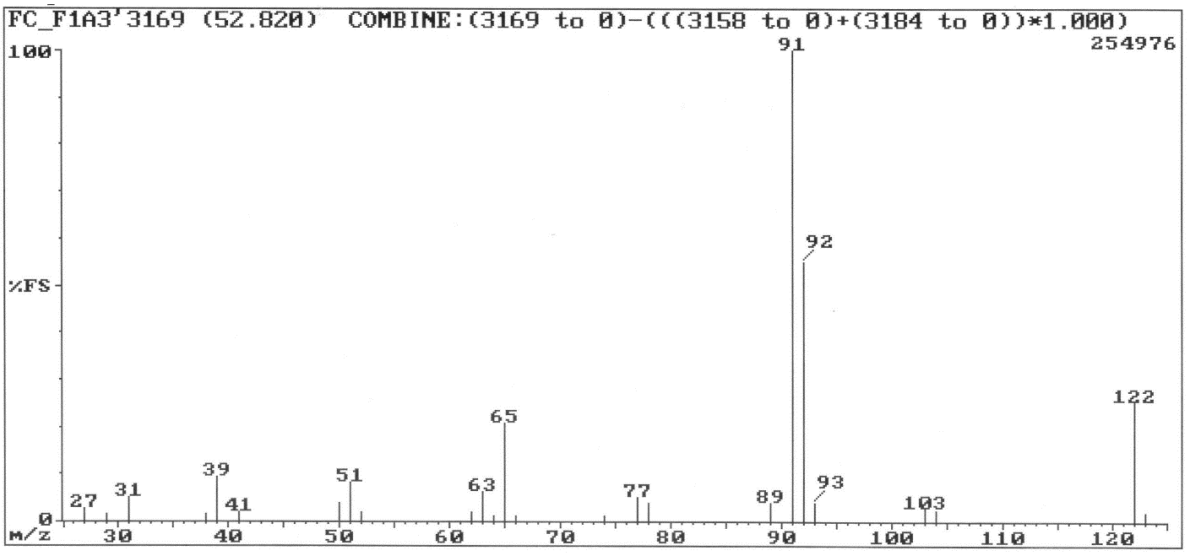


Fig. 3.85: EI-mass spectrum of 2-phenylethanol (wax column).

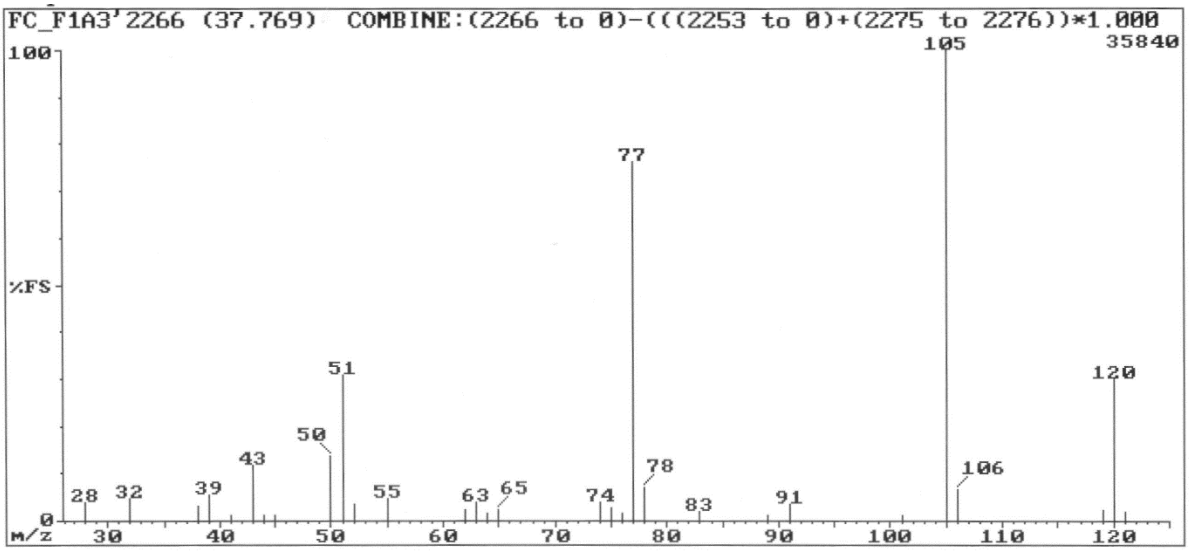


Fig. 3.86: EI-mass spectrum of acetophenone (wax column).

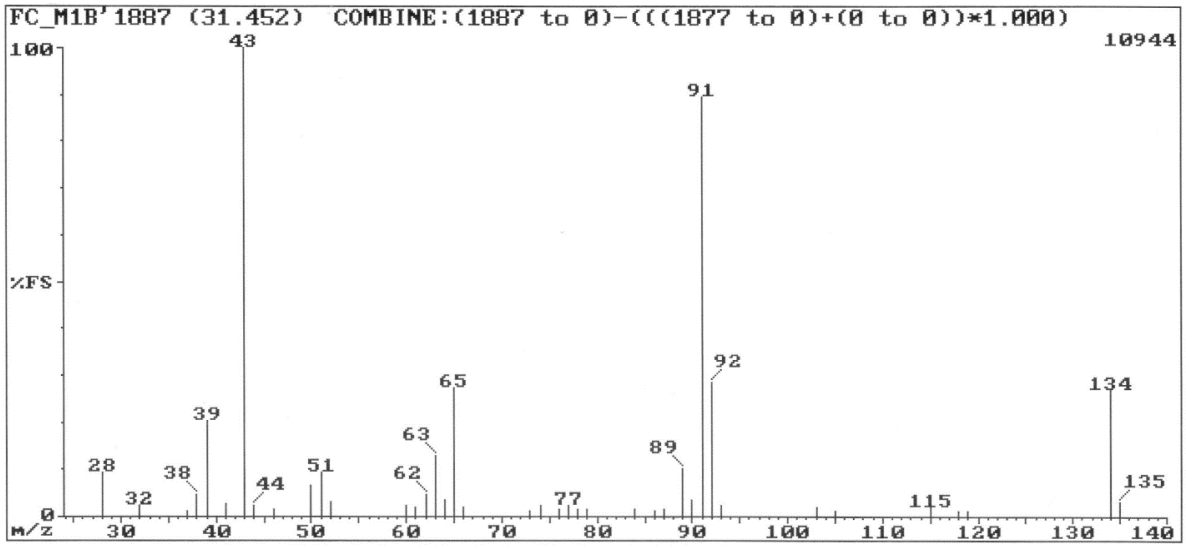


Fig. 3.87: EI-mass spectrum of 1-phenyl-2-propanone (1701 column).

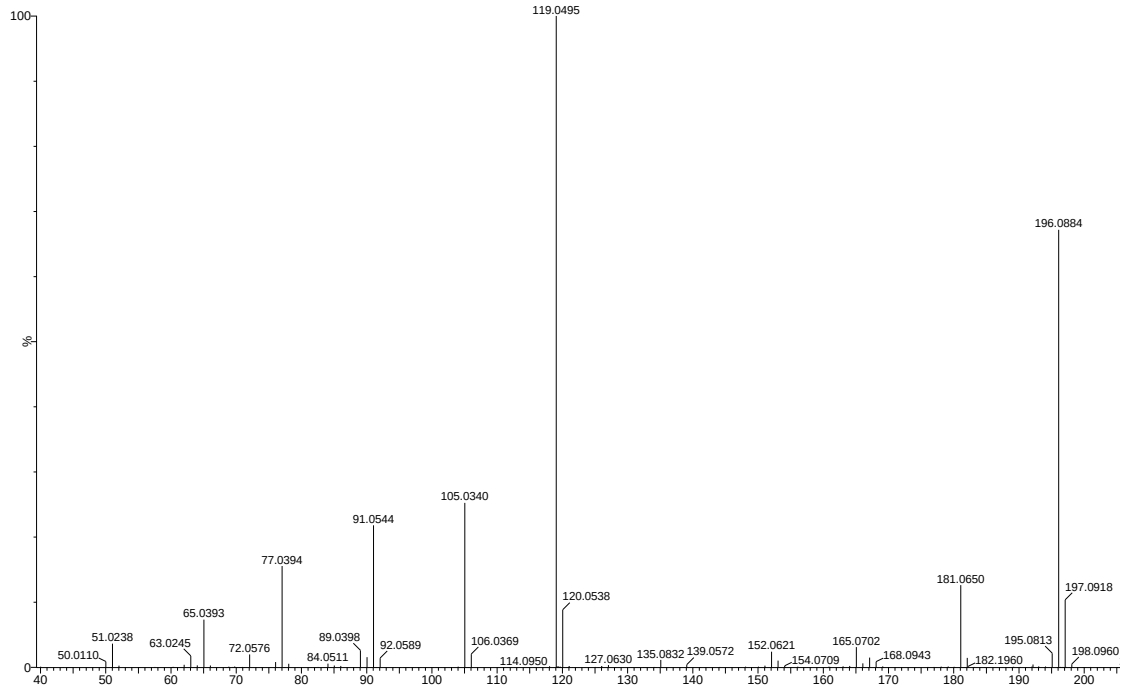


Fig. 3.88: EI-mass spectrum of 4-methylbenzophenone (apolar column).

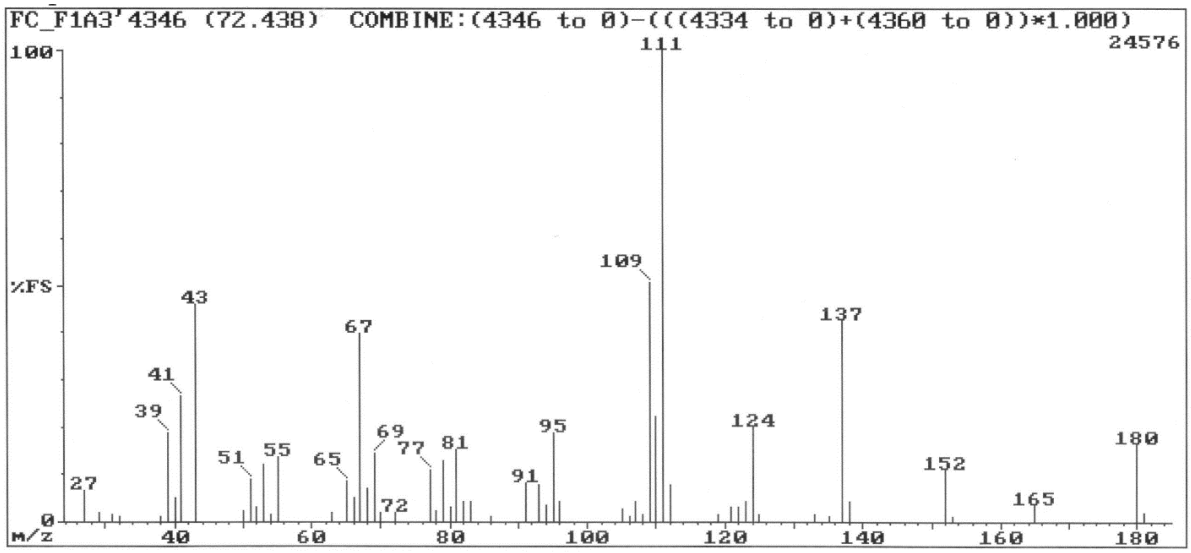


Fig. 3.89: EI-mass spectrum of 4,4,7a-trimethyl-5,6,7,7a-tetrahydro- -2(4H)-benzofuranone (wax column).

Fig. 3.90: EI-mass spectrum trimetylamine (apolar column).

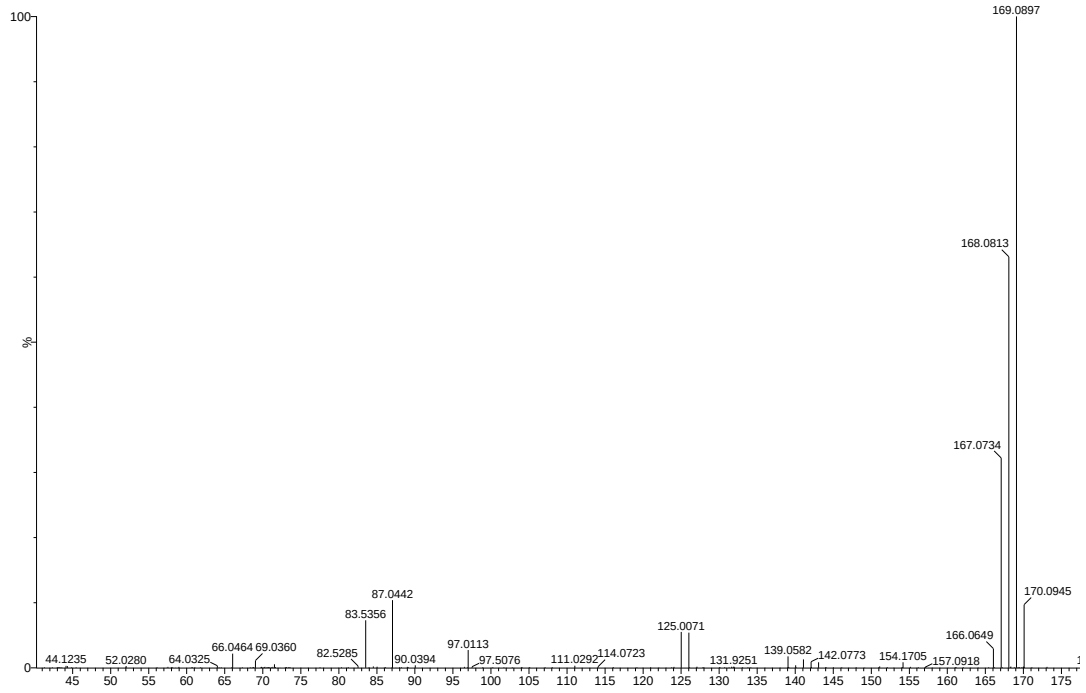


Fig. 3.91: EI-mass spectrum of diphenylamine (apolar column).

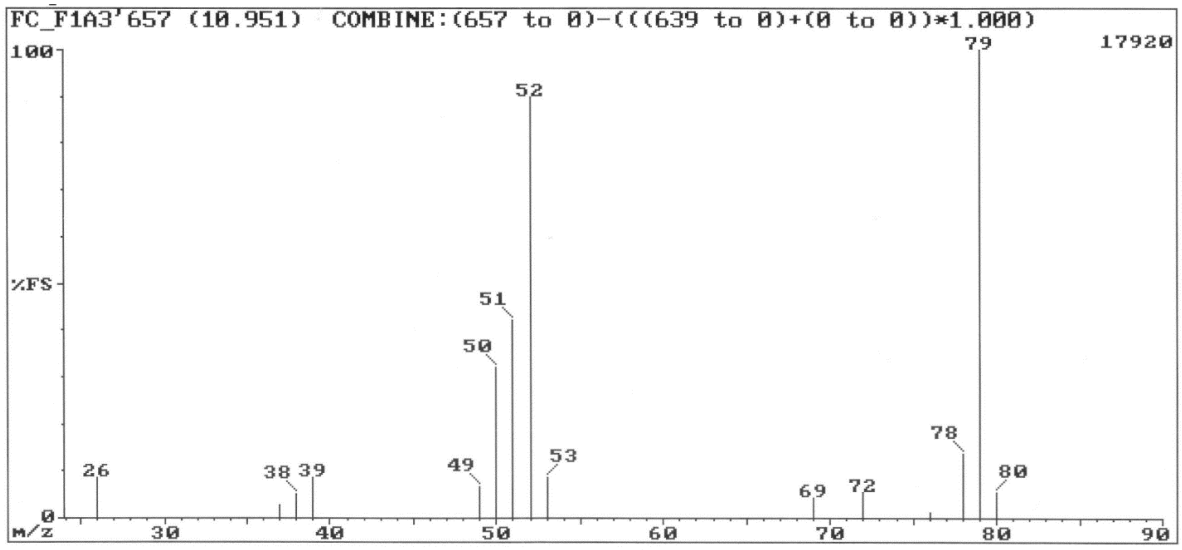


Fig. 3.92: EI-mass spectrum of pyridine (wax column).

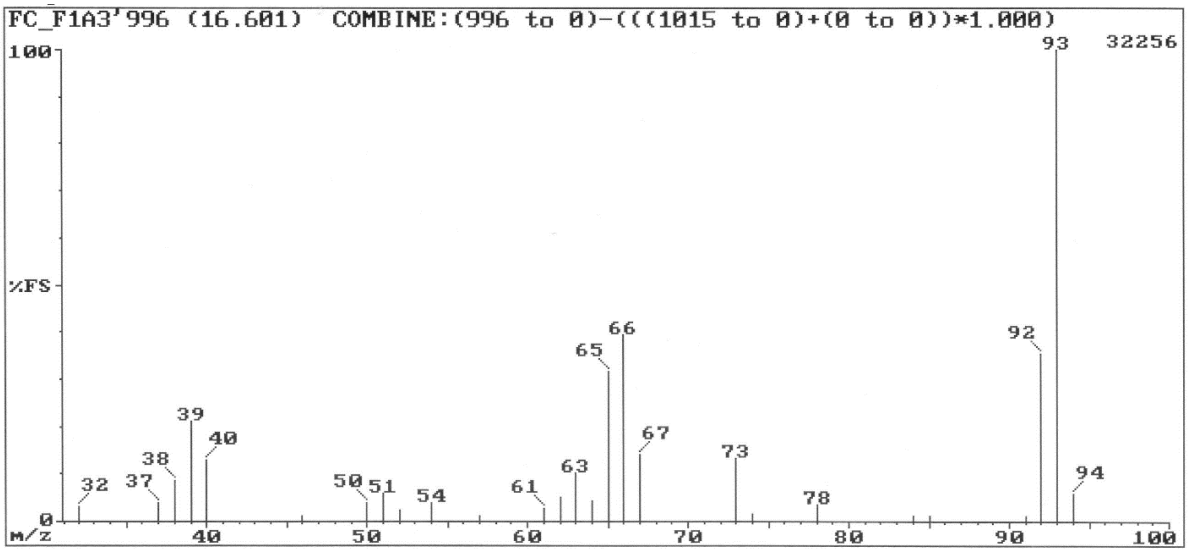


Fig. 3.93: EI-mass spectrum of 3-methylpyridine (wax column).

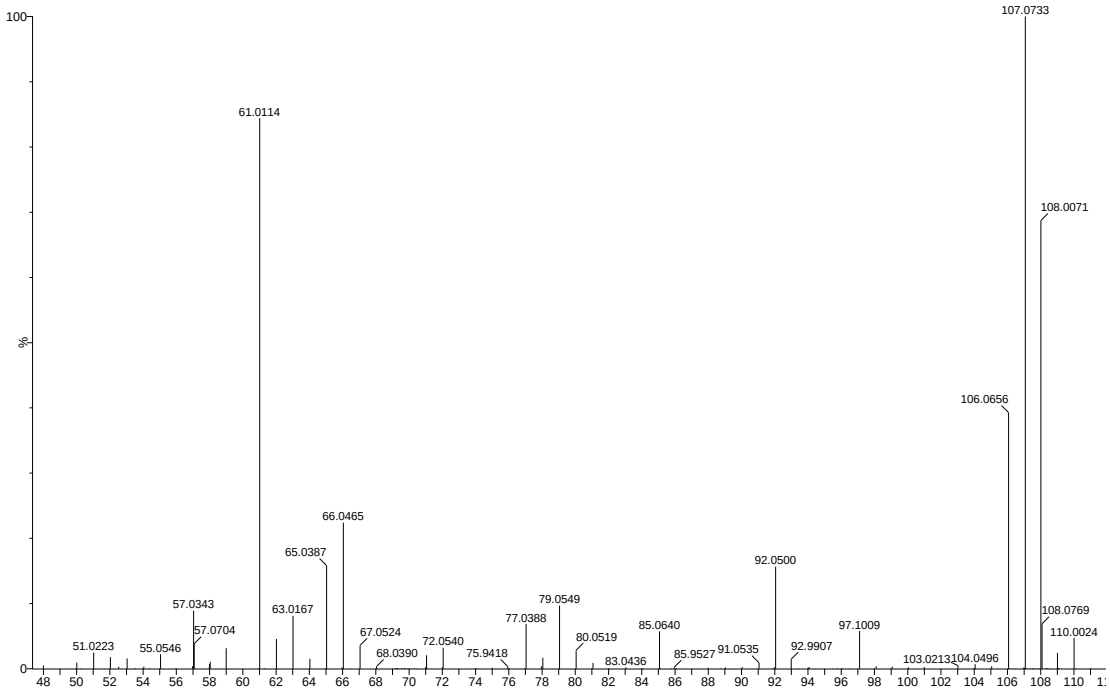


Fig. 3.94: EI-mass spectrum of 2,6-dimethylpyridine (apolar column).

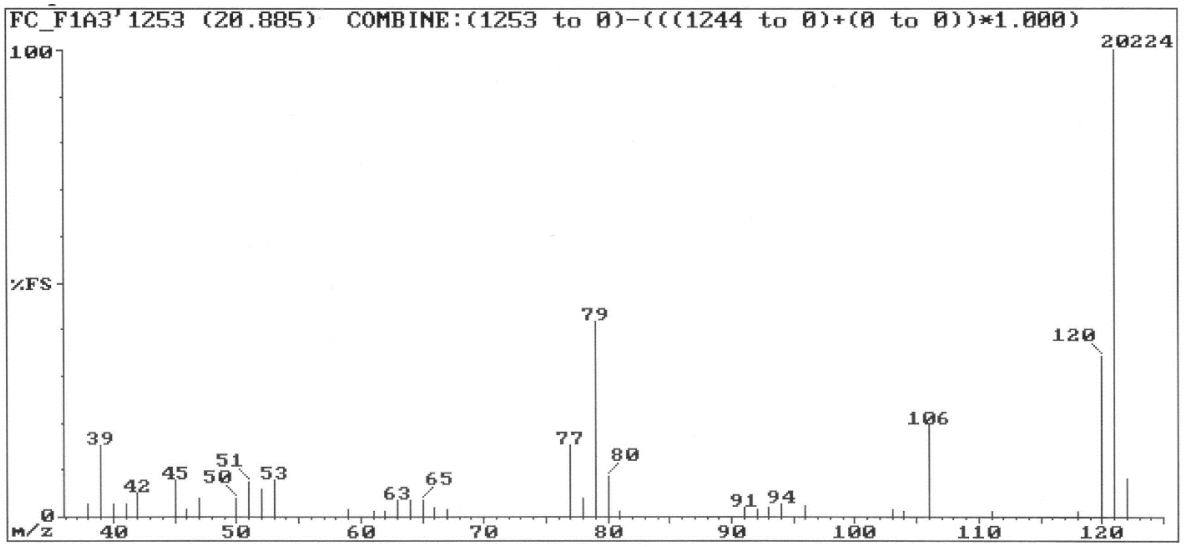


Fig. 3.95: EI-mass spectrum of 2,4,6-trimethylpyridine (wax column).

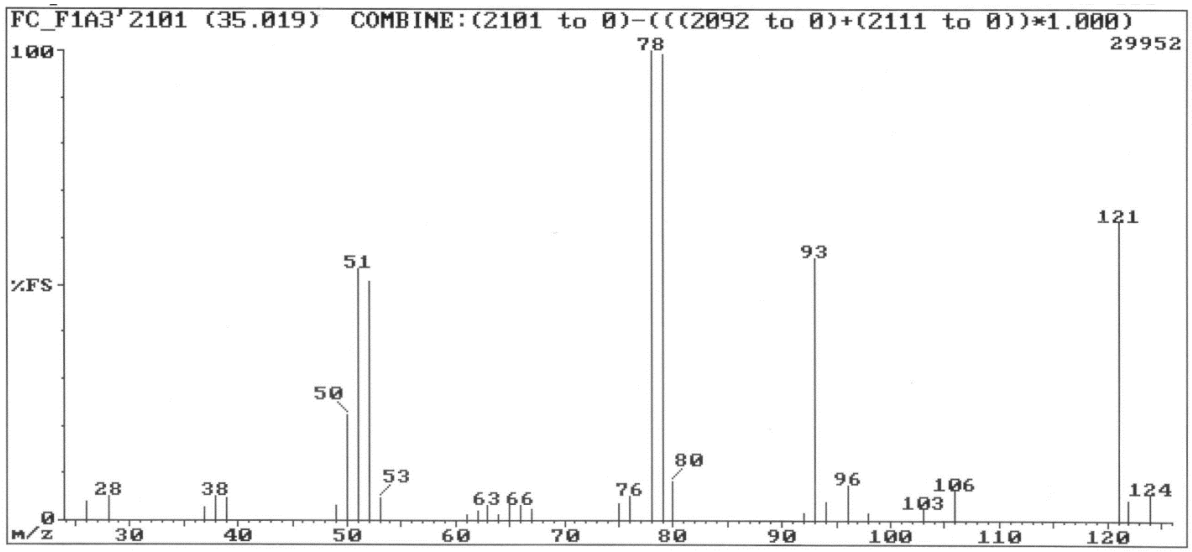


Fig. 3.96: EI-mass spectrum of 2-acetylpyridine (wax column).

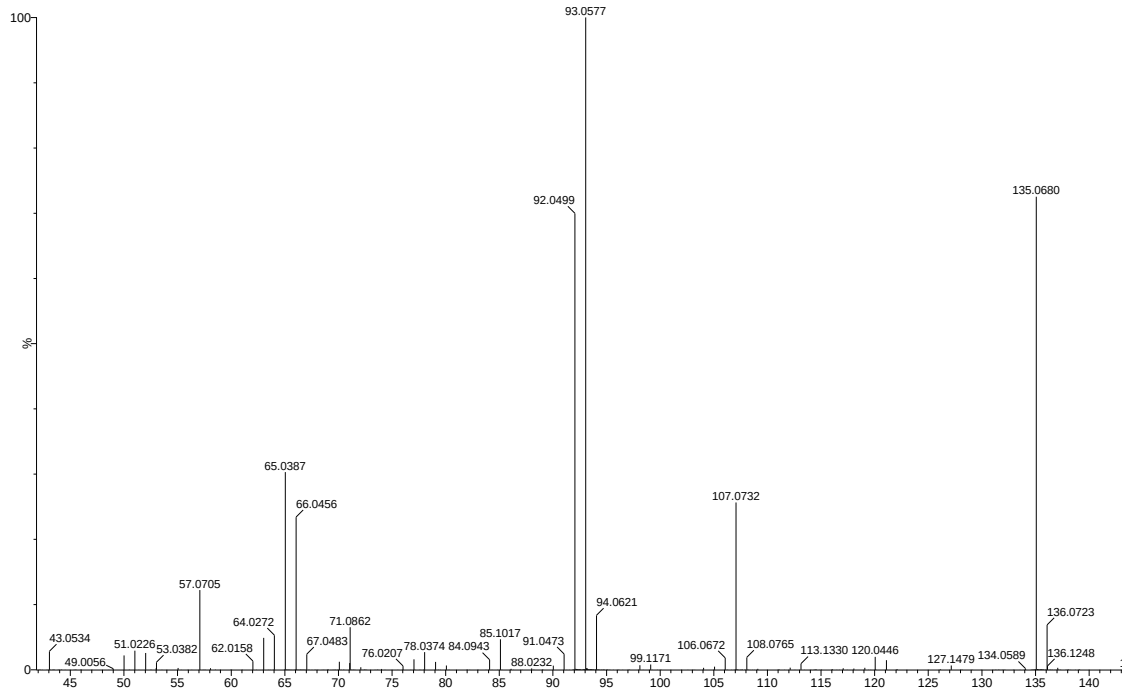


Fig. 3.97: EI-mass spectrum of 2-acetyl-6-methylpyridine (apolar column).

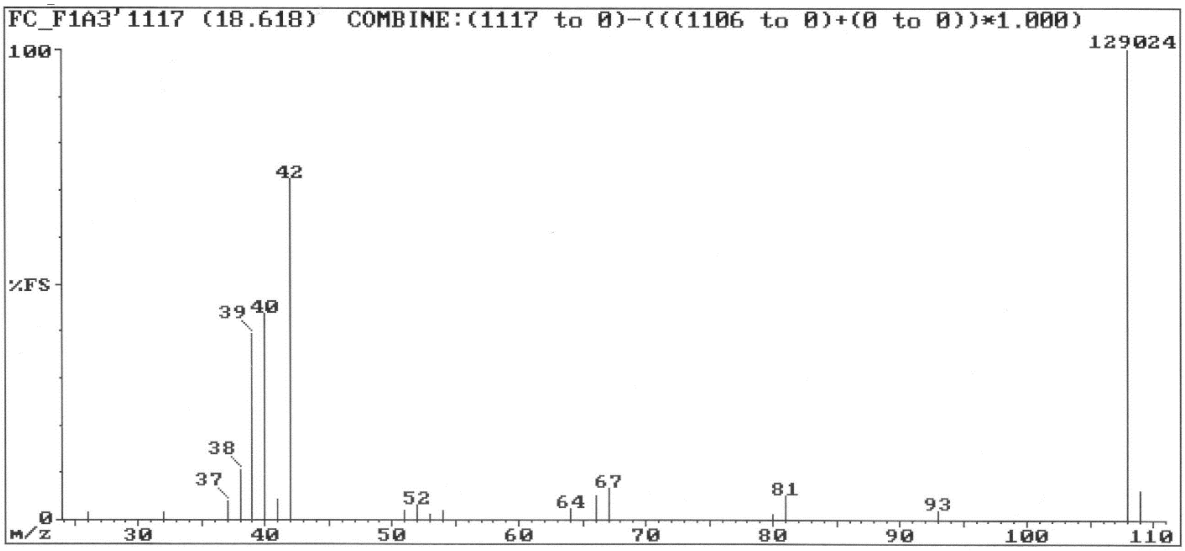


Fig. 3.98: EI-mass spectrum of 2,6-dimethylpyrazine (wax column).

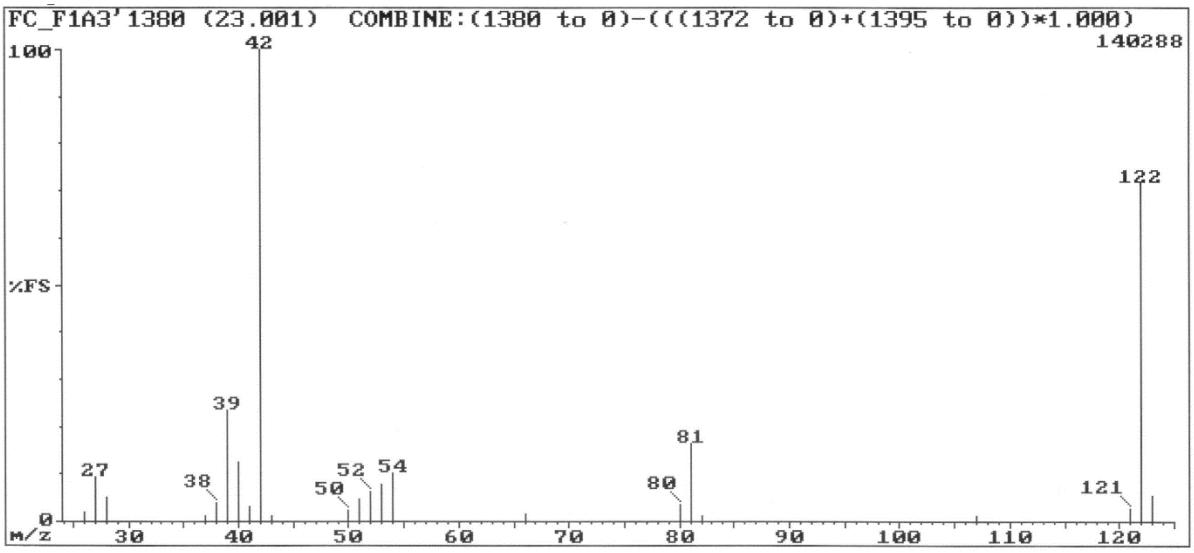


Fig. 3.99: EI-mass spectrum of trimethylpyrazine (wax column).

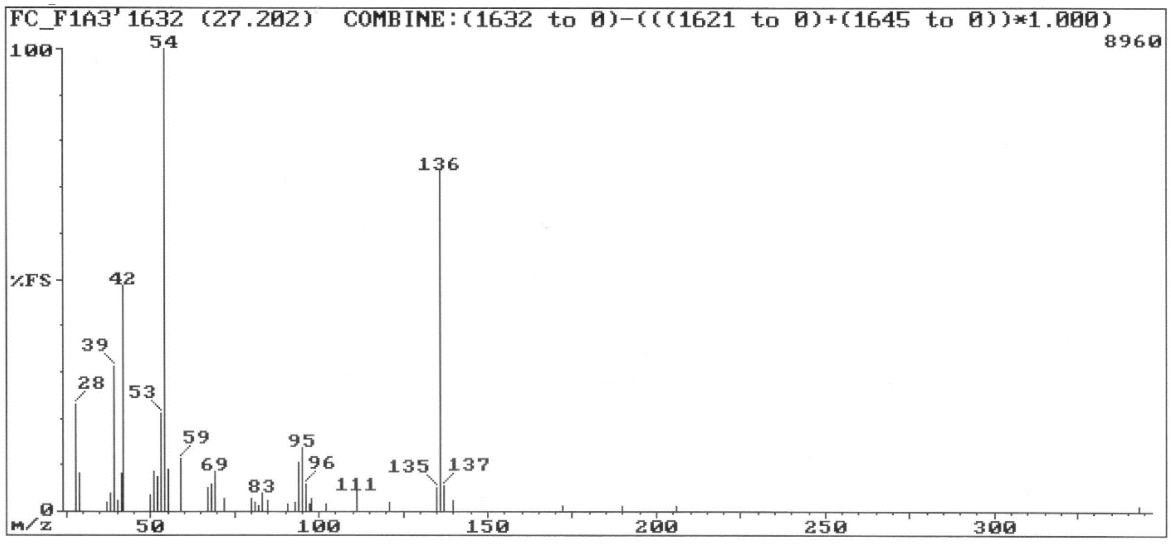


Fig. 3.100: EI-mass spectrum of tetramethylpyrazine (wax column).

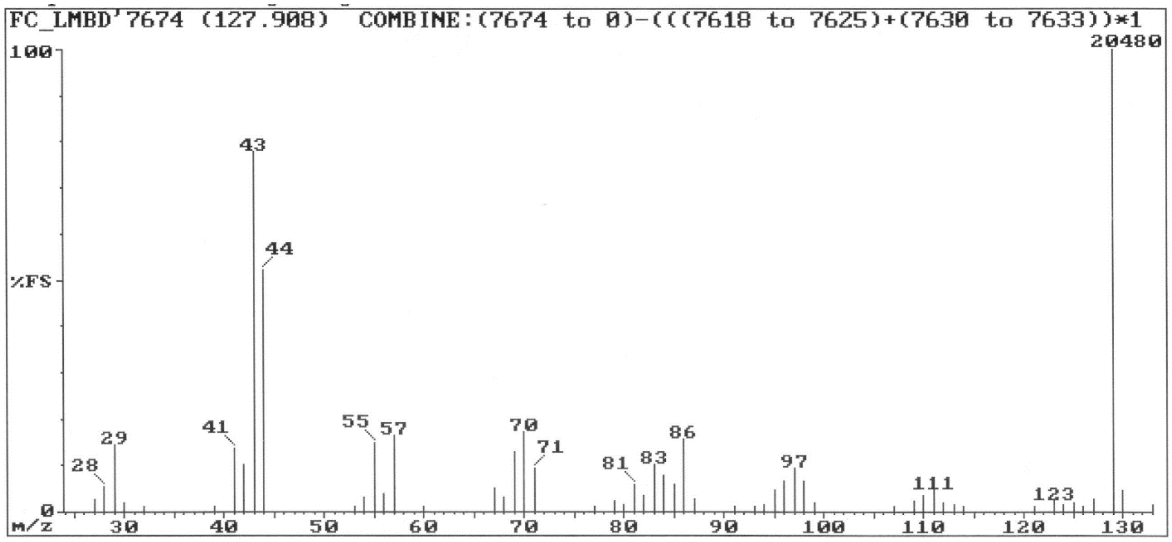


Fig. 3.101: EI-mass spectrum of cyanuric acid (apolar column).

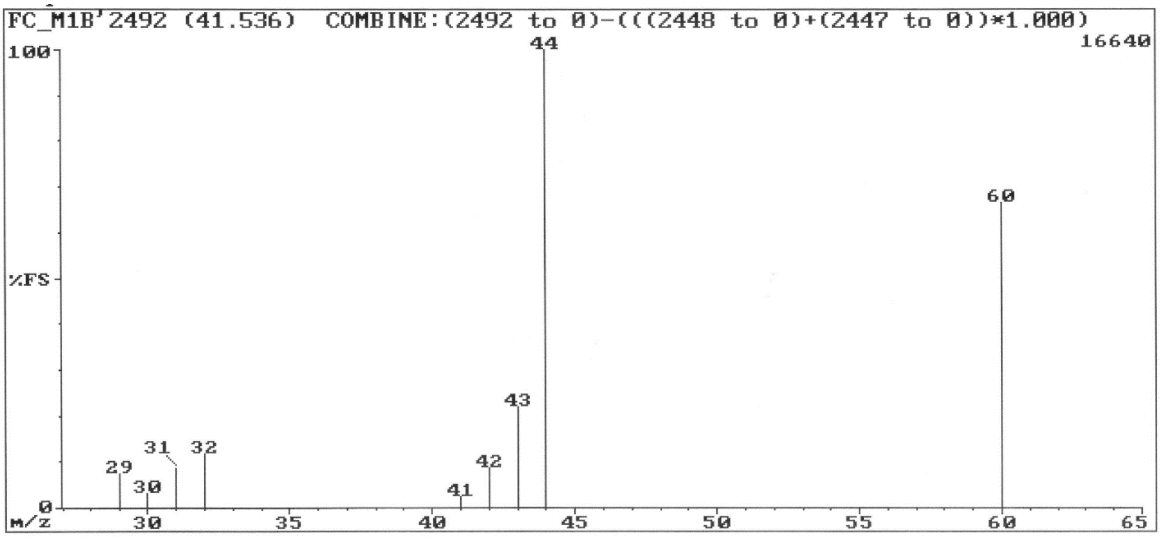


Fig. 3.102: EI-mass spectrum of urea (1701 column).

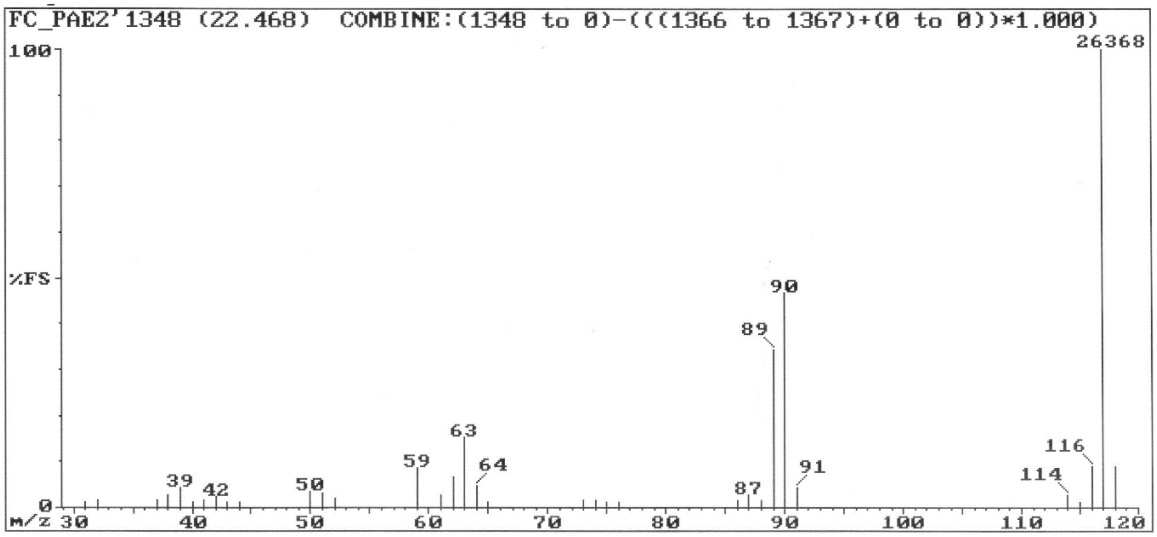


Fig. 3.103: EI-mass spectrum of indole (apolar column).

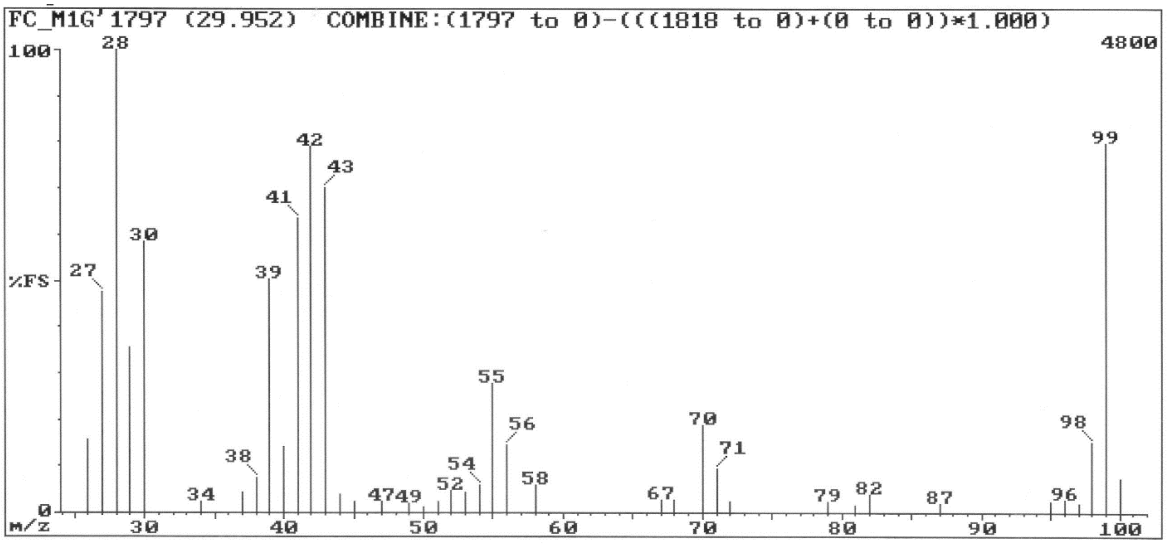


Fig. 3.104: EI-mass spectrum of 2-piperidone (apolar column).

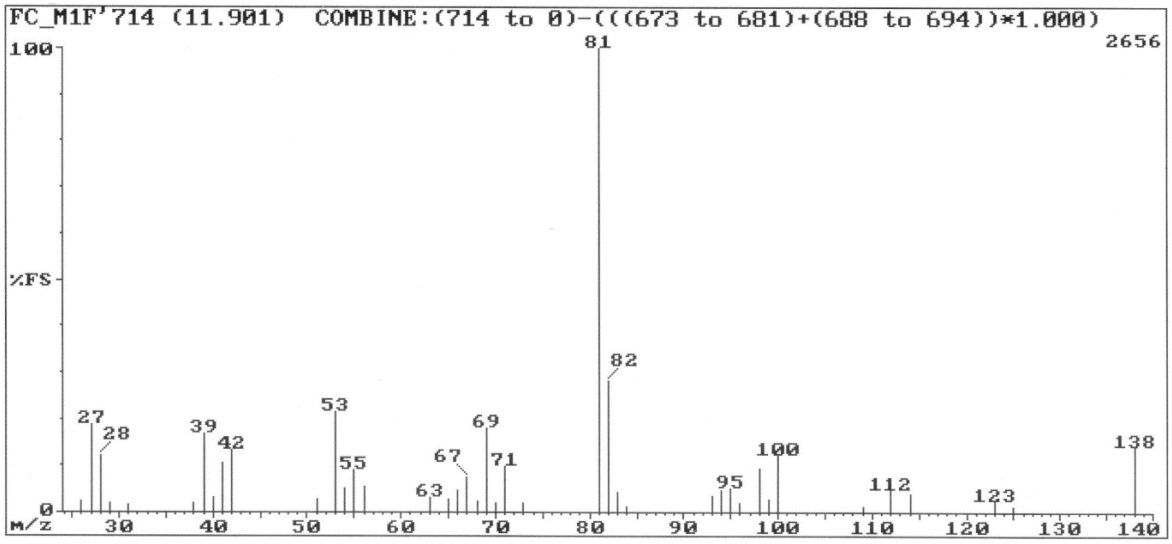


Fig. 3.105: EI-mass spectrum of 2-pentylfuran (wax column).

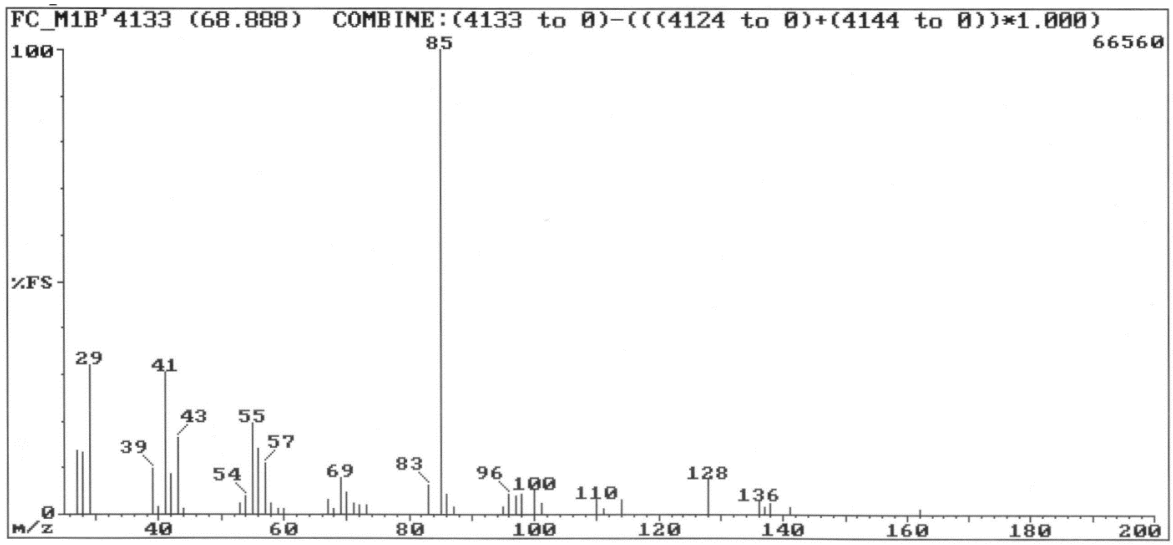


Fig. 3.106: EI-mass spectrum of γ-dodecalactone (1701column).

CHAPTER 4

CHARACTERISATION OF THE URINARY PROTEINS AND THEIR LIGANDS

4.1 Introduction

Several studies on insects and mammals have shown that pheromones bind with proteins. Klusak *et al.* (2003) and Lautenschlager *et al.* (2007) have studied the interaction of pheromones with proteins, specifically, that of bombykol and the pheromone binding proteins in the silkworm moth, *Bombyx mori*. The pheromone and protein interaction for puberty acceleration has also been studied in mammals, mainly in rodents, such as, for example, mice (*Mus domesticus*) (Caretta *et al.*, 1995; Mucignat-Caretta *et al.*, 1999; Novotny, 2003).

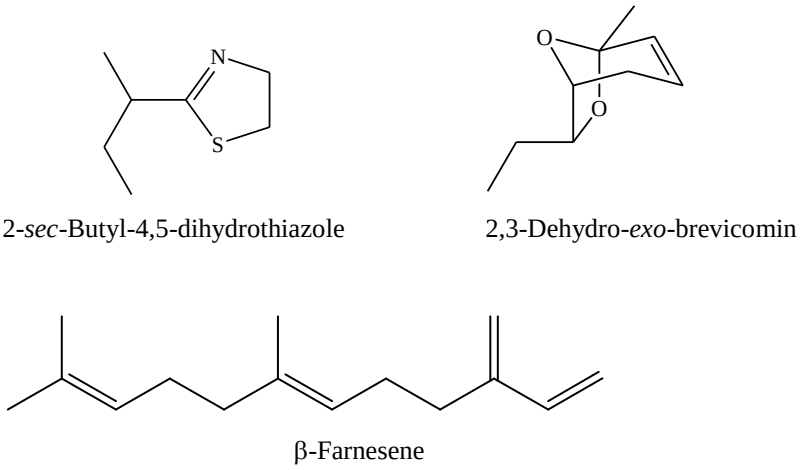
The chemical characterisation of the VOCs and the steroids present in the urine of male and female caracal was discussed in Chapter 3. In an earlier study carried out at LECUS on the territorial marking fluid of the male Bengal tiger, *Panthera tigris* (Burger *et al.*, 2008), it was found that, in addition to urine, the marking fluid of the tiger contained a high percentage of material that appeared to be insoluble in the urine and formed a supernatant layer upon standing. Many observations and studies of the territorial behaviour of tigers in nature and in captivity have revealed that the tiger uses this mixture of urine and lipid material for territorial marking (Poddar-Sarkar *et al.*, 2013).

Except for studies aimed at the determination of the size of the home ranges of male and female caracal (65 and 18.2 square miles, respectively) (Norton and Lawson, 1985), nothing is known about the territorial behaviour of the caracal. Sheep farmers, however, agree that a caracal tends to always follow the same path from lair to hunting range. Experience has shown that more males than females are attracted to traps baited with urine and that male and female urine seem to be equally attractive. Apparently, the caracal is a very inquisitive animal, and farmers using urine to attract caracal often use a piece of glass or a small mirror suspended in such a way, on or near the trap, that it reflects the sunlight to draw the animal's attention. A bunch of feathers moving in the wind is also quite effective. This could indicate that voided caracal urine does not emit a long-range signal. No spray marking has been observed in wild or captive caracal.

Urine samples received from farmers during the course of this investigation were generally clear or only slightly turbid after the frozen urine had thawed and reached room temperature. There was no indication of a layer of lipid material floating on top of the urine. Nevertheless, from the successes achieved by some sheep farmers when using caracal urine as attractant, it was hypothesised that this species probably uses urine marking in some form of intraspecific semiochemical communication. (On the other hand, some farmers claim that the head of a fish works equally well).

The identification of *cis*-4-hydroxydodec-6-enoic acid lactone by Brownlee *et al*. (1969) in the male tarsal scent of the black-tailed deer (*Odocoileus hemionus columbianus*) stimulated interest in the role that mammalian secretions and excretions could play in the behaviour of these animals. The initial euphoria regarding the possibility of using mammalian pheromones to control problem animals has abated. However, a few groups of tenacious researchers have nevertheless achieved remarkable successes in research on the intraspecific semiochemical communication of small rodents, probably because of the availability of experimental animals, in combination with the available experience in handling them in the laboratory. In particular, the influence of intra-species stimuli (pheromones) on the ecology of the mouse has enjoyed increasing interest since the early 1960s. The following putative pheromones were identified in the urine of the male house mouse, *Mus musculus*: 2-(*sec*-butyl)thiazole, 2,3-dehydro-*exo*-brevicomin, and β-farnesene (Schwende *et al.*, 1986; Novotny *et al*., 1990; Caretta *et al.*, 1995). Oestrus synchrony (Whitten, 1958) in grouped adult female mice following their exposure to the urine of sexually mature males (Marsden and Bronson, 1964) and the advancement of puberty in juvenile females are both induced by the same small organic molecules and by their synthetic analogues (Novotny *et al.*, 1999), although larger and less volatile molecules, such as a protein or peptide seemed to be implicated in these phenomena (Vandenberg, 1969; Vandenberg *et al.*, 1975 and 1976). Indeed, Bacchini *et al*. (1992) demonstrated that proteins of the major urinary protein complex of the male mouse selectively bind 2-(*sec*-butyl)thiazole and 2,3-dehydro-*exo*-brevicomin, and concentrate them in the urine. Caretta (1995) attributed puberty acceleration activity to a so-called major urinary protein (MUP), which is a conglomerate of polypeptide isoforms (Finlayson *et al.*, 1965), all having a molecular mass of less than 20 kDa. It is significant that, despite repeated purifications and extensive purging of unfractionated urine for many hours, it retained its odour and remained

biologically active in uterine weight assays (Novotny *et al.*, 1980 and 1999). The ligands identified in the urine of the house mouse that selectively bind to MUP are depicted in Fig. 4.1.



2-*sec*-Butyl-4,5-dihydrothiazole

2,3-Dehydro-*exo*-brevicomin

β-Farnesene

Fig. 4.1: Semiochemicals identified in the urine of the house mouse.

Mice, in common with other rodents, exhibit an obligate proteinuria in the form of MUPs, which constitute a heterogeneous family of 19-kDa proteins. Sequence and structural analysis have assigned MUPs to the lipocalycin superfamily (Cavaggioni *et al*., 1987; Adams and Sawyer, 1990; Bocskei *et al*., 1991; Beynon and Hurst, 2004). This family is characterised by a unique tertiary structure consisting of 8, 9, or 10 β-pleated sheets arranged in a cross-hatched formation, forming a central bowl or calyx, which contains many hydrophobic residues, providing a suitable environment for binding or transport of hydrophobic ligands (North, 1991; Leal *et al*., 2005). Robertson *et al*. (1993) hypothesised that MUPs could serve as a reservoir for pheromonal molecules that could be released gradually from the protein. This hypothesis is in line with the observed persistence of the odour and biological activity observed by Novotny *et al*. (1980 and 1999) and was confirmed by the extraction, characterisation, and binding analysis of 2-(*sec*-butyl)thiazole and 2,3-dehydro-*exo*-brevicomin with MUP of the house mouse, *Mus musculus*.

Felid species, on the other hand, have a protein known as 'cauxin' in their urine. Miyazaki *et al*. (2003) studied the urine of healthy mature domestic cats (*Felis catus*) and found that high concentrations of urinary proteins are excreted. Purification of the urine by gel electrophoresis and trypsin digestion followed by mass spectrometric determination of the peptides of the urine resulted in the identification of a protein with a mass of 70 kDa, which is similar to proteins of the

serine hydrolase family, such as mammalian carboxylesterase. This protein was designated as cauxin (carboxylesterase-like urinary excreted protein) (McLean *et al*., 2009).

Bearing in mind that caracal have been successfully trapped using fish as bait, it was thought possible that malodourous compounds present in the animal's urine could be involved in some way in the attraction of the animals to traps baited with caracal urine. For this reason, comprehensive analyses were first carried out on the urine of as many individuals as possible. This was followed by an investigation of the putative role of proteins in this animal's intraspecific semiochemical communication.

4.2. Proteomics

4.2.1 Exploratory analysis of putative proteins in caracal urine

UPLC-ESI-MS/MS was employed to profile the protein content of caracal urine, upon which the later selection of appropriate analytical techniques for the characterisation of the urinary proteins were to be based. Male urine samples that were reliably collected and transported to LECUS from Aurora and Fraserburg (see map in Chapter 5) were chosen for these analyses (see also § 2.4.5). Analyses were carried out on the ACQUITY UPLC instrument (specified in Chapter 2), using a C18 column (100 × 2.1 mm, 1.7 µm) at a column temperature of 50 °C. As mobile phases A and B, 2% formic acid (FA) and 98% acetonitrile were used, respectively. The analytes were eluted at a flow rate of 0.350 ml/min. A linear gradient from 5% B to 44% B over 20 min was used, followed by the use of 100% B for 2 min. The total run time was 25 min. The UPLC was coupled to a Synapt G2 quadrupole/TOF mass spectrometer (Waters, Milford, USA). Positive electrospray with a capillary voltage of 3 kV and a cone voltage of 15 V was employed. Leucine-enkephalin was used as lock mass reference for accurate mass determination.

The chromatograms (Figs 4.2 and 4.4) resulting from the analyses of the Aurora and Fraserburg samples both have a broad peak at a retention time of *ca.* 14.5 min. The characteristic peak patterns of the mass spectra depicted in Figs 4.3 and 4.5 are typical for multiply charged proteins (Loo, 2000).

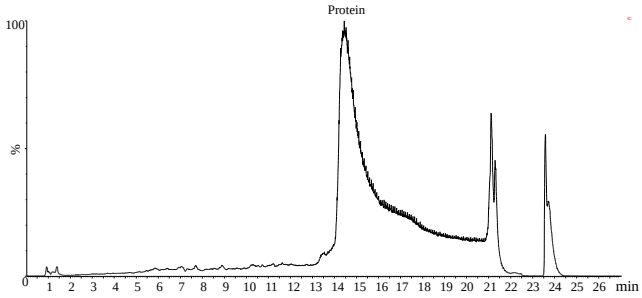


Fig. 4.2: The TIC of male caracal urine (Aurora). ESI-UPLC; (Waters HSS C18, 2.1 mm × 150 mm, 1.7 μm) and Synapt G2 quadrupole TOF mass spectrometer. The two sharp peaks resulted from solvent polarity changes.

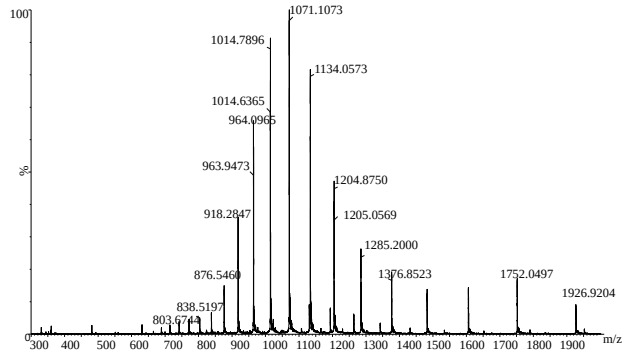


Fig. 4.3: Mass spectrum taken at retention time 14.5 min in the chromatogram depicted in Fig. 4.2. The *m/z* values of the respective peaks are given at their apexes.

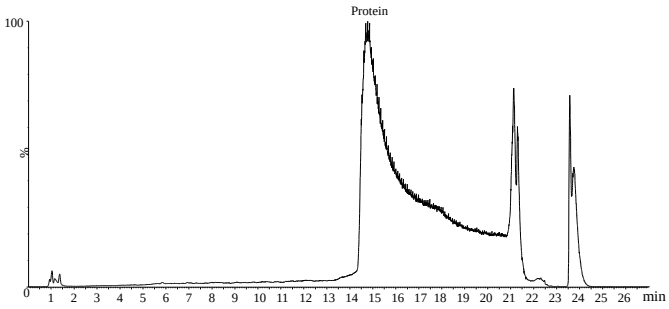


Fig. 4.4: The TIC of male caracal urine (Fraserburg). ESI-UPLC; (Analytical conditions as in Fig. 4.4.)

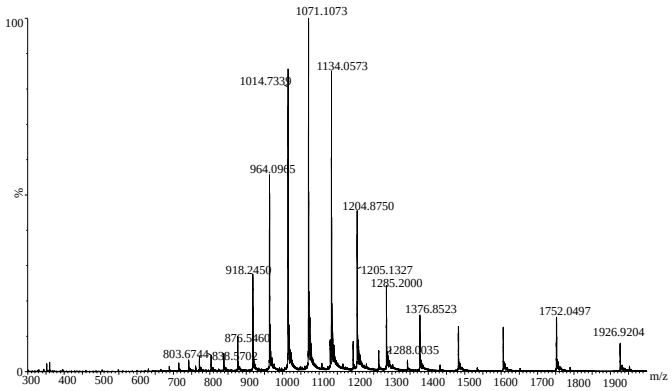


Fig. 4.5: Mass spectrum taken at retention time 14.5 min in the chromatogram depicted in Fig. 4.4.

Such a typical mass spectrum of a protein provides information regarding the charge of each peak and the mass of the protein (Griffiths *et al*., 2001). Each peak in the mass spectrum of the protein represents a protein with one charge less than its immediately preceding neighbour of lower *m/z*. Using equation 1, in which peaks 1 and 2 are adjacent peaks, and H as the mass of a proton (for protonated or deprotonated species), the charge on the respective ions can be calculated.

$$Z = \frac{peak2 - H}{peak1 - peak2} \qquad (1)$$

The charges on the ions *m/z* 1752.097 and 1926.9204, for example, are 11^+ and 10^+, respectively. The mass, M, of the protein can be calculated by using the *m/z* values and charge of the ions for *m/z* 1752.097 and 1926.9204 and the charges, 11^+ and 10^+, in equation 2 (Griffiths *et al*., 2001).

$$M = Z\left(\frac{m}{z} - H\right) \qquad (2)$$

The calculated masses are 19262.067 and 19259.204, respectively. The average of the results of similar calculations for the other ions in the spectrum yields the molecular mass of the protein, in this case, 19.262 kDa.

4.2.2 Quantitation of the urinary protein

Using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, USA), quantitative protein determinations were carried out according to Smith (1985) on urine samples collected from two different geological areas. Fresh samples (0.1 ml) were diluted with distilled water (0.9 ml) to bring the protein concentration to within the protein standard curve range of the method used. Bovine serum albumin (BSA) standards ranging from 200 to 2000 ppm (0.2 to 2 mg/ml) were prepared and used for protein concentration determination of the urine samples. The concentration standards and the samples were pipetted in triplicate into the wells of a 96-well microtiter plate. To each well, bicinchoninic acid assay (BCA assay) working reagent was added using a multichannel pipette. The peptide bonds in the protein reduced Cu^{2+} in the BCA assay to Cu^{1+} and the colour of the urine samples changed to purple on addition of the BCA reagent, confirming the formation of a complex. The plates were then incubated at 37 °C for 30 min. After allowing the plates to cool to room temperature, the absorbance of the blank (distilled water), the protein standards, and the urine samples were spectrophotometrically compared at 540 nm using a Multiskan EX microplate spectrophotometer or microplate reader (Thermo Scientific) and Software version 2.6.

Total protein content determinations were carried out on several of the available urine samples. To obtain an idea of protein losses incurred during dialysis, determination of the protein content of one male and one female sample was also carried out after the urine samples had been dialysed. Results of these determinations are tabulated in Table 4.1.

Table 4.1: The total protein content of urine samples collected from geologically different areas.

Geographical area (Sample number)	Male (mg/ml)		Female (mg/ml)	
	Before dialysis	After dialysis	Before dialysis	After dialysis
Laingsburg	11.83	9.21	7.92	2.23
Prince Albert (1)	5.72			
Prince Albert (2)	9.05			
Prince Albert (3)	8.51		10.76	
Prince Albert (4)	5.69			
Porterville			7.02	

The differences in the protein concentrations before and after dialysis could probably be ascribed to the removal of smaller proteins and urea by dialysis. Urea was present in significant concentrations in many urine samples.

4.2.3 Protein dialysis

As expected, caracal urine contains VOCs and proteins. As a sample preparation step, semipermeable membrane separation (dialysis) was used to remove small molecules from samples of urine intended for protein analysis.

Fresh urine samples (15 ml) were dialysed for 72 h against a 20% aqueous solution of polyethylene glycol (PEG) (35 kDa; Sigma-Aldrich, Schnelldorf, Germany) using the custom-built apparatus depicted in Fig. 4.6. This apparatus had been specifically designed for dialysis and simultaneous concentration of protein-containing samples (Swart, 1981: 192). For this project, the apparatus consisted of a 15-cm length of seamless semipermeable cellulose dialysis tube (3.5 kDa, 20.4 mm i.d.) containing the caracal urine, suspended in a glass tube (50 mm i.d.), which served as housing of the apparatus. The 20% aqueous PEG solution was circulated to and from a PEG reservoir and through the glass tube with a peristaltic pump (AC-2110; ATTA, Japan) at a flow rate of 1 ml/min. The PEG solution in the reservoir was replaced every 4 h with fresh PEG solution. The urine was concentrated to a final volume of *ca.* 3 ml (about one-fifth of the volume of the original urine samples). Samples of the resulting male and female protein concentrates were used for protein quantitation, gel electrophoresis, the detection of putative protein ligands, and for protein characterisation.

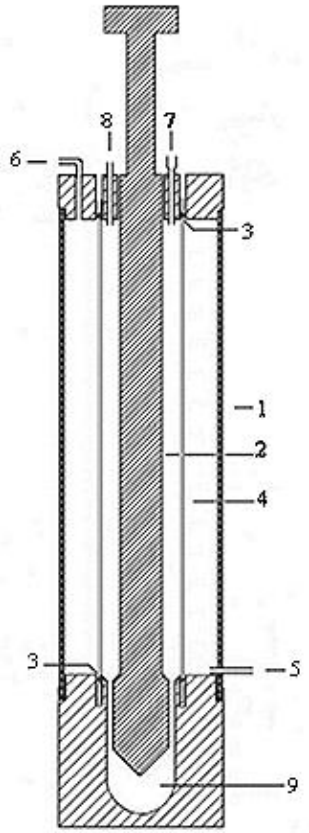


Fig. 4.6: Schematic diagram of the protein dialysis apparatus used for the concentration of urine samples: 1. housing (thick-walled glass tube), 2. central spacing rod and membrane support, 3. rubber O-rings, 4. dialysis tube, 5. PEG solution inlet, 6. PEG solution outlet, 7. urine introduction port, 8. vent, 9. sample collection chamber.

4.2.4 Gel electrophoretic separation of the urinary proteins

Gel electrophoretic separation of proteins is based on differences in the size and charge of proteins. Thus, proteins with smaller molecular mass and/or a higher charge travel faster and longer distances than proteins with a higher mass and/or a lower charge. The polyacrylamide gel on which the electrophoresis is carried out is formed by cross-linking polymerisation of two organic monomers, acrylamide and the cross-linking agent *N,N'*-methylene-*bis*-acrylamide. *N,N,N',N'*-tetramethylethylene-diamine (TEMED) is added to the mixture to initiate polymerisation (Raymond and Weintraub, 1959). The solubilising and denaturing agent sodium dodecyl sulphate (SDS) is widely used in the gel electrophoretic separation of proteins (Laemmli, 1970). SDS has a high affinity for proteins. The negatively charged detergent molecules coat the proteins and mask their native charge. The proteins separated by SDS-PAGE thus migrate according to their molecular weight regardless of their charge. The SDS causes protein denaturation and unfolding

of proteins into linear structures. As the resolution of SDS-PAGE is insufficient for some applications, a more efficient two-dimensional technique, 2D SDS-PAGE, was developed, in which different separation mechanisms are applied in the two dimensions (Washburn *et al*., 2001). Using this more advanced gel electrophoresis technique, the molecular mass as well as the isoelectric point (P*I*) values of proteins can be determined.

In this research project, 1D SDS-PAGE and non-denaturing (native) PAGE were used. Samples of male and female urine (15 ml) were dialysed and concentrated as described in § 4.2.3. SDS-PAGE gels were run as described by Laemmli (1970). The set-up consisted of a resolving gel in which the proteins are separated and a stacking gel that is used to concentrate the proteins prior to their entering the resolving gel. The difference in the composition of the two gels and the electrophoresis buffer results in resolution of the proteins according to their molecular weight. The gels used for the separation of the urinary proteins in this project had a total length of 74 mm and a thickness of 1 mm. The gels were made up as follows:

8% Running gel: 0.375 M Tris(hydroxymethyl)aminomethane (Tris-HCl) pH 8.8; 0.1% SDS (v/v); 8% (v/v) 30% acryl/bis-acrylamide stock solution (Sigma); 0.75% ammonium persulphate (v/v); and 0.075% TEMED (v/v).

4% Stacking gel: 0.125 M Tris-HCl pH 6.8; 0.1% SDS (v/v); 4% (v/v) 30% acryl/bis-acrylamide stock solution (Sigma); 0.75% ammonium persulphate (v/v); and 0.075% TEMED (v/v).

A vertical slab gel was cast at room temperature by first preparing the running gel and allowing it to polymerise before overlaying it with the stacking gel. This was done on a Bio-Rad Mini-Protean II cell (Bio-Rad, Hercules, USA). Prior to PAGE analyses, the protein-containing samples were incubated at 37 °C for 10 min. Kaleidoscope mass markers (combination of proteins with different masses) and the Bio-Rad Mini-Protean II assay (cat# 161-0375) were used to determine the approximate molecular mass of proteins.

A 30-µg protein sample was treated in a 1:1 ratio with 2× treatment buffer (120 mM Tris-HCl pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol, and 0.02% (w/v) bromophenol blue). Each sample was first heated to 90 °C for 10 min and then allowed to cool. The samples were briefly centrifuged before being loaded onto the 12% polyacrylamide running gel. The SDS-PAGE was run at 4 °C and 200 V until the indicator front was 1 cm from the bottom of the gel. Proteins were stained with Coomassie Brilliant Blue R-250 (0.25% (w/v) in 45% methanol/10%

glacial acetic acid) according to the procedure of Sambrook and Russell (2001). Destaining was carried out in five changes of destaining solution (45% methanol, 10% glacial acetic acid). A typical separation of the urinary proteins on a SDS-PAGE gel is depicted in Fig. 4.19.

4.3 Ligand identification

To determine whether the caracal's urinary proteins could possibly contain ligands, 15-ml samples of male and female urine were dialysed for three days against a 20% aqueous solution of PEG. The headspace VOCs of the dialysed and concentrated material (*ca.* 1.9 ml) were sampled by SEP50 enrichment for 24 h at 22 °C and analysed by GC-MS analysis as described in § 2.3.1. The samples were then heated at 95 °C for 10 min in tightly closed SEP sampling bottles to denature the proteins. After the bottles and their contents had cooled to room temperature, the VOCs were again analysed by SEP50-GC-MS to determine whether denaturing the urinary proteins resulted in an increase in the concentration of the putative protein ligands.

In an experiment with urine from Fraserburg, not even cyclopentadecanone (see § 4.3.4) could be detected in dialysed material before denaturation. In other experiments only cyclopentadecanone could be detected before denaturation and in others only low concentrations of some of the ketones. The TICs of one set of experiments carried out with male and female dialysed material from the same area are depicted in Figs 4.7–4.10. An increase in the concentrations of these ketones produced after protein denaturation was particularly evident the case of female material. These experiments produced clear evidence that these ketones are ligands bound with the non-denatured urinary protein(s) of the caracal.

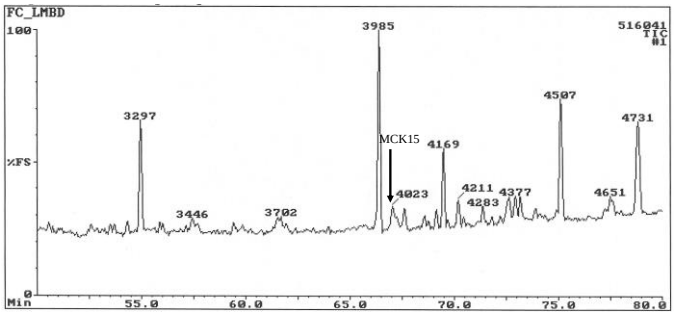


Fig. 4.7: SEP60-GC-MS analysis of dialysed male caracal urine (Laingsburg sample LM 2). Apolar column, 40–280 °C at 2 °C/min. MCK15, cyclopentadecanone.

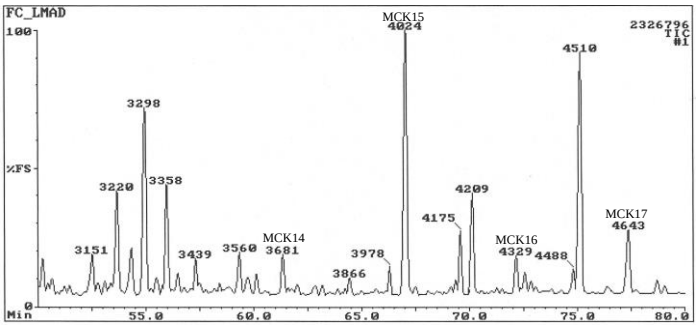


Fig. 4.8: SEP60-GC-MS analysis of male caracal urine (Laingsburg sample LM 2) after heating the urine sample at 95 °C for 10 min (protein denaturation). Apolar column, 40–280 °C at 2 °C/min. MCK14, cyclotetradecanone; MCK15, cyclopentadecan-one; MCK16, cyclohexadecanone; MCK17, cycloheptadecanone.

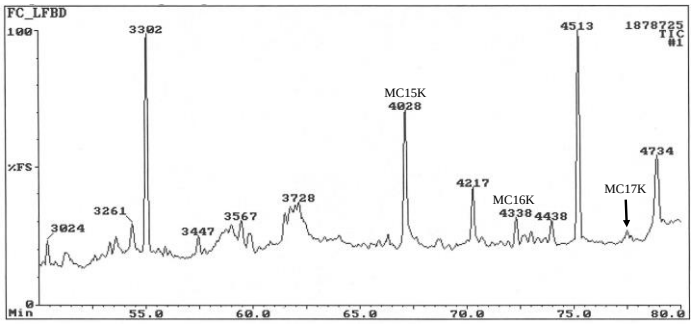


Fig. 4.9: SEP60-GC-MS analysis of dialysed female caracal urine (Laingsburg sample LF4). Apolar column, 40–280 °C at 2 °C/min.MCK15, cyclopentadecanone; MCK16, cyclohexadecanone; MCK17, cycloheptadecanone.

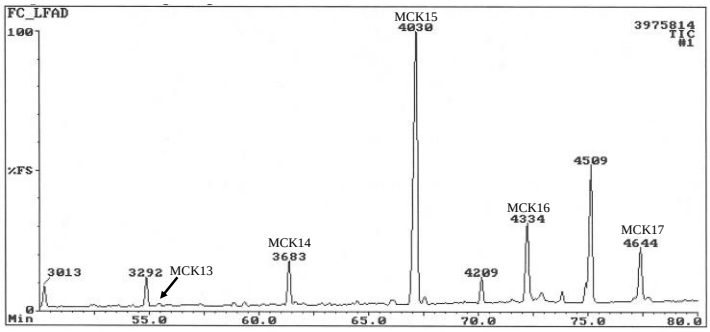


Fig. 4.10: SEP60-GC-MS analysis of urinary protein dialysed and concentrated from female urine sample LF4 (Laingsburg) after proteindenaturation at 95 °C for 10 min. Apolar column, 40–280 °C at 2 °C/min. MCK13, cyclotridecanone; MCK14, cyclotetradecanone; MCK15, cyclopentadecanone; MCK16, cyclohexadecanone; MCK17, cycloheptadecanone.

4.3.1 Ultrafiltration of urinary proteins

Results of the experiments discussed above indicated that the ligands of the urinary proteins of the caracal are not lost during dialysis. It was then considered necessary to attempt to denature individual proteins of protein fractions to determine which protein carries a specific ligand. SEP-GC-MS analysis of protein bands excised from SDS-PAGE or native PAGE gels was chosen as a possible method to achieve this goal. The recovery of proteins from gels by electrophoretic elution (Strålfors and Belfrage, 1983; Shoji *et al*., 1995) and passive diffusion (e.g., Kurien and Scofield, 2012) was considered problematic, mainly due to the lack of the necessary equipment in our laboratory. Recovery by maceration extraction (Scheer and Ryan, 2001) was repeatedly carried out without any success. Recovering the proteins from gels was thus abandoned in favour of protein fractionation by stepwise ultrafiltration (UF), a technique that is based on differences in the molecular size of the proteins.

A series of Vivaspin VS6 concentrators (Sartorius, Göttingen, Germany) with molecular weight cut-off points (MWCO) of 100, 50, 30, 10, and 5 kDa was used to separate protein fractions for subsequent detection of any putative protein ligands in the individual fractions. A sample of male caracal urine from Laingsburg (6 ml) was introduced into a 100 kDa concentrator and centrifuged at 3000 × g for 180 min on a Multifuge 3 S-R (Heraeus, Hamburg, Germany), after which the filtrate was centrifuged in a 50 kDa concentrator at 5000 × g for 90 min. This procedure was repeated with the 30, 10 and 5 kDa concentrators at 5000 × g. It was assumed that the proteins would be retained on the different membranes. All fractions were washed (centrifuged) twice with cold deionised water. The procedure was repeated with female urine from Laingsburg.

The two 100 kDa concentrators on which the proteinaceous material from the male and female urine were retained were removed from their housings and samples were taken for SDS-PAGE. Comparison of the resulting gel, depicted in Fig. 4.11, with previous gels indicated that separation of the proteins was not achieved by gel filtration, presumably because the proteins formed clusters that were retained on the 100 kDa concentrator.

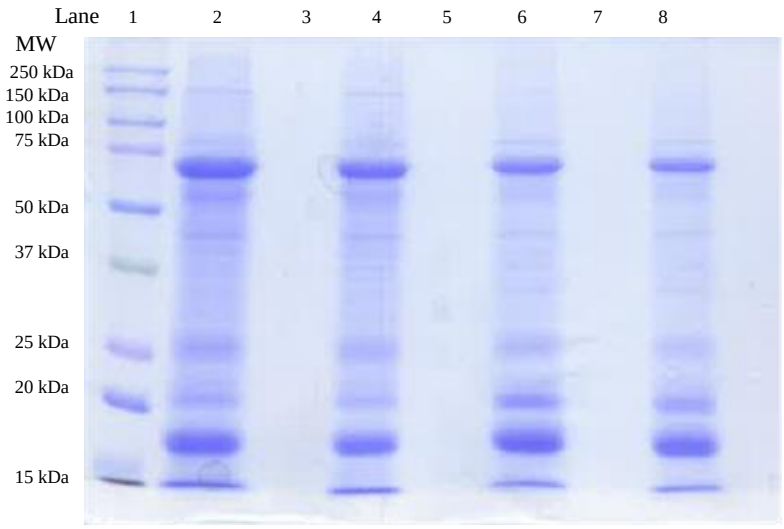


Fig. 4.11: SDS-PAGE of the protein retained on the 100-kDa concentrator in an attempted separation of the urinary proteins by UF of male and female caracal urine. Lane 1, mass markers; lanes 2 and 4, male proteins; lanes 6 and 8, female proteins. Lanes 3, 5 and 7 were left empty to prevent possible carryover.

Despite this disappointing result, the experiment nonetheless gave worthwhile information. The 100 kDa concentrator used for the attempted separation of the male proteins, with its contents, was placed in a 50 ml SEP sampling bottle and was tightly closed with a cap fitted with a Teflon-faced septum. The sampling bottle with its contents was heated from 65 °C to 95 °C at 2 °C/min and the final temperature was held for 10 min. After the bottle had cooled to room temperature, a SEP30 sampling device was installed in the bottle and any VOCs present in the headspace gas of the denatured proteins were sampled at 23 °C for 24 h. SEP-GC-MS analysis of the enriched material was carried out in the usual manner. The 50, 30, 10, and 5 kDa concentrators were treated likewise. These procedures were repeated with the female urinary protein.

By constructing a selected ion chromatogram for the molecular ion of cyclopentadecanone at *m/z* 224 and for the ions at *m/z* 96 and 98, which are typically present in the mass spectra of unbranched macrocyclic ketones, this ketone was unambiguously detected in the male and female protein retained on the 100 kDa concentrators. The ketone was not detected in the headspace gas of the other concentrators that had been subjected to these procedures.

4.3.2 Strong anion-exchange chromatographic separation of proteins

In a previous project carried out in LECUS, strong anion-exchange chromatography was used successfully for the isolation of protein fractions from the urine of the Bengal tiger (Burger *et al.*, 2008). Fresh samples (15 ml) of male and female urine from Laingsburg were dialysed for 72 h against a 20% aqueous solution of PEG (35 kDa) as described in § 4.2.3. Low pressure LC on a 5 ml HiTrap Q strong anion-exchange column (GE Healthcare, Uppsala, Sweden) was employed for the separation of proteins present in these samples, following a procedure described by Armstrong *et al.* (2005). The column was equilibrated with several column volumes of binding buffer, after which a linear salt gradient (0–1 M NaCl in binding buffer) was used to elute the bound protein from the column. The resulting chromatograms of the male and female proteins are depicted in Figs 4.12 and 4.13, respectively.

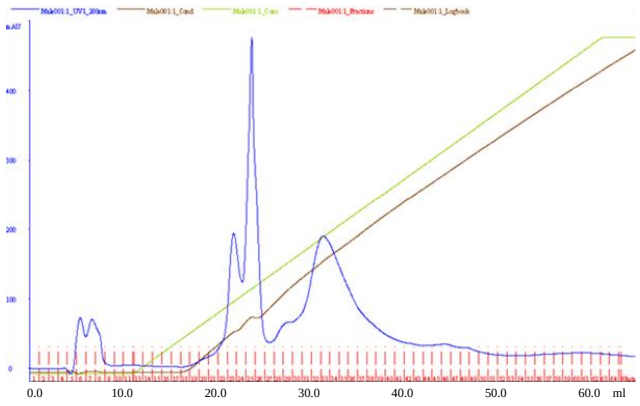


Fig. 4.12: Strong anion-exchange chromatogram of dialysed male protein.

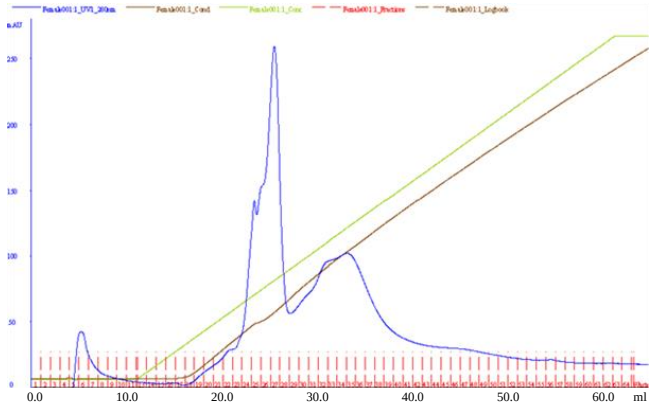


Fig. 4.13: Strong anion-exchange chromatogram of dialysed female protein.

The collected fractions (1 ml), numbered from 1 to 60 (indicated on the x-axes in Figs 4.12 and 4.13), were sampled on PAGE gels that were used for Orbitrap mass spectrometric identification of the putative ligand-carrying protein(s). Fractions 6, 8, 13 and 33 obtained in the separation of the male protein were also subjected to thermal denaturing, followed by SEP enrichment of VOCs and GC-MS analysis. However, no indication could be found of the presence of ligand-carrying proteins in these fractions.

4.3.3 Gel electrophoretic separation of the urinary proteins

The SDS-PAGE and native PAGE gels of the material sampled from the chromatographic fractions isolated in the previous experiment are depicted in Figs 4.14–4.17.

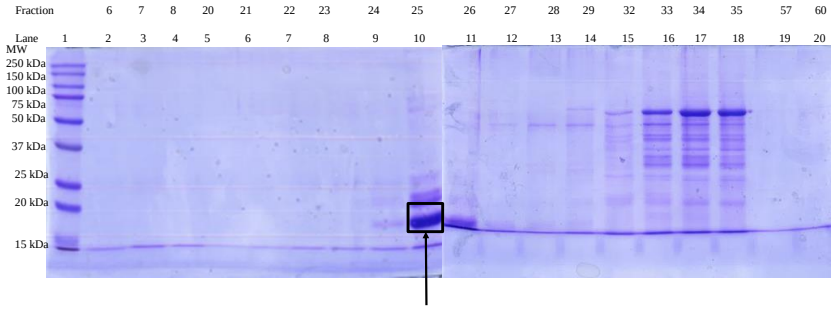


Fig. 4.14: SDS-PAGE gel of male urinary proteins (Laingsburg sample LM4): Lane 1, mass markers; lanes 2–4, fractions 6–8; lanes 5–14, fractions 20–29; lanes 15–18, fractions 32–35; lane 19, fraction 57; lane 20, fraction 60. The arrow indicates the protein band excised for mass spectrometric protein analysis.

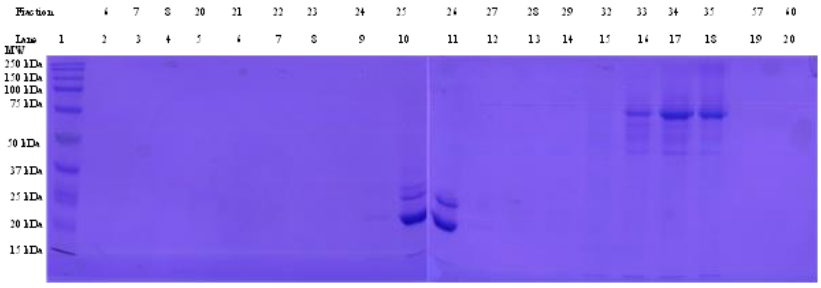


Fig. 4.15: Native PAGE gel of male urinary proteins (Laingsburg sample LM4): Lane 1, mass markers; lanes 2–4, fractions 6–8; lanes 5–14, fractions 20–29; lanes 15–18, fractions 32–35; lane 19; fraction 57; lane 20, fraction 60.

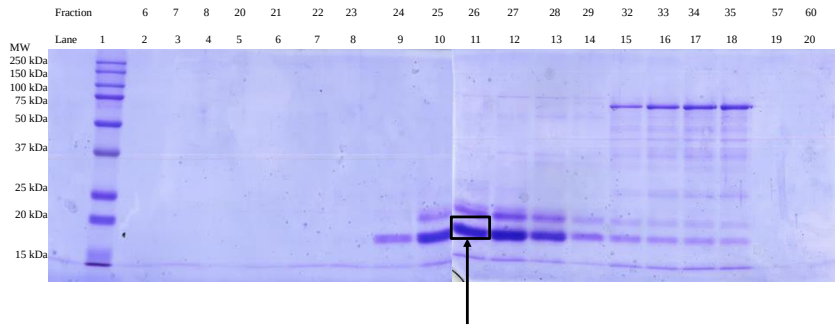


Fig. 4.16: SDS-PAGE gel of female urinary proteins (Laingsburg sample LF4): Lane 1, mass markers; lanes 2–4, fractions 6–8; lanes 5–14, fractions 20–29; lanes 15–18, fractions 32–35; lane 19, fraction 57; lane 20, fraction 60. The arrow indicates the protein band excised for mass spectrometric protein analysis.

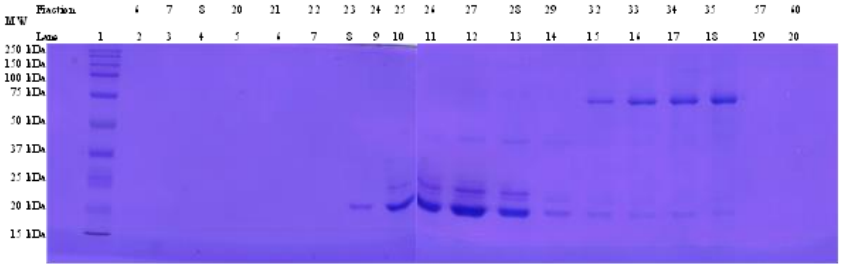


Fig. 4.17: Native PAGE gel of female urinary proteins (Laingsburg sample LF4): Lane 1, mass markers; lanes 2–4, fractions 6–8; lanes 5–14, fractions 20–29; lanes 15–18, fractions 32–35; lane 19, fraction 57; lane 20, fraction 60.

Fractions 25 and 26 were collected over the apex of the major peak in each of the chromatograms shown in Figs 4.11 and 4.12, and were sampled in lanes 10 and 11 of the gels depicted in Figs 4.14–4.17.

4.3.4 Ligand detection in chromatographic fractions

The chromatographic fractions collected from the anion-exchange column that contained the highest concentrations of protein were subjected to SEP30-GC-MS analysis for the detection of any putative protein ligands. Cyclopentadecanone was detected in very low concentrations in fractions 25 and 26 collected from the anion-exchange separation of both the male, and female urinary proteins. These fractions were then heated at 95 °C for 10 min. After these samples had cooled to room temperature, SEP30-GC-MS analyses were carried out to determine whether the thermal denaturation of the proteins resulted in an increase of the putative ligands in the headspace gas of the denatured proteins. Denaturation of the proteins resulted in large increases in the headspace concentration of the macrocyclic ketones, especially in the case of the female material.

4.4 Orbitrap mass spectrometric characterisation of the urinary protein(s)

The main steps that are commonly taken in proteome analyses are summarised in Fig. 4.18 (Peng and Gygi, 2001).

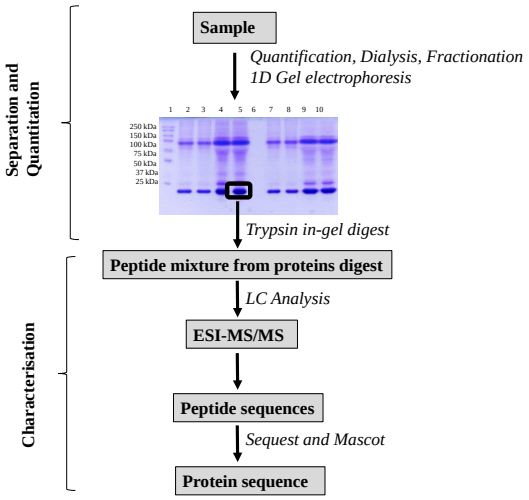


Fig. 4.18: Techniques employed in proteome analysis.

4.4.1 SDS-PAGE separation

SDS-PAGE gel separation of dialysed and undialysed male and female caracal protein was carried out. The resulting gel is depicted in Fig. 4.19.

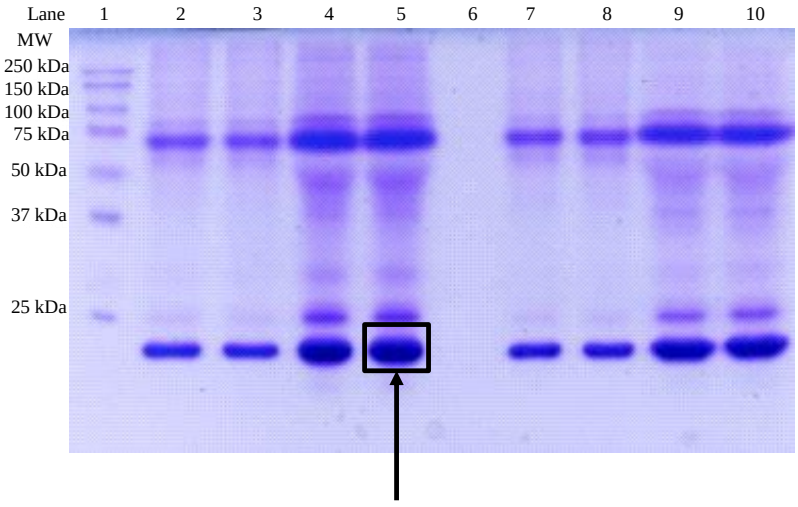


Figure 4.19: SDS-PAGE of caracal protein (Laingsburg samples LM4 and LF4). Lane 1, mass markers; lanes 2 and 3, undialysed male caracal protein; lanes 4 and 5, dialysed male protein; lane 6, open; lanes 7 and 8, undialysed female caracal protein; lanes 9 and 10, dialysed female caracal protein. The arrow indicates the protein band that was excised for Orbitrap mass spectral analysis.

4.4.2 In-gel tryptic digestion

A protein band (male material) was excised from lane 5 (Fig. 4.19) and digested in-gel, using trypsin to break down the proteins into peptides. Trypsin cleaves peptide bonds at the carboxyl side of lysine and arginine residues. In-gel digestion is commonly used for this, although it requires a higher concentration of enzyme compared to in-solution digestion.

The peptides were reduced and modified prior to digestion according to Shevchenko *et al*. (1996). Excised gel bands were destained in Eppendorf 1.5 ml tubes with 200 mM NH_4HCO_3/acetonitrile (1:1) (Sigma-Aldrich) until clear. Samples were dehydrated and desiccated before reduction with 2 mM triscarboxyethyl phosphine (TCEP) (Fluka) in 25 mM NH_4HCO_3 for 15 min at room temperature with agitation. Excess TCEP was removed and the gel pieces again dehydrated. Cysteine residues were thiomethylated with 20 mM *S*-methyl methanethiosulphonate (Sigma-Aldrich, St. Louis, USA) in 25 mM NH_4HCO_3 for 30 min at room temperature. After thiomethylation, the gel pieces were dehydrated and washed with 25 mM NH_4HCO_3, followed by another dehydration step. Proteins were then digested by rehydrating the gel slices in trypsin solution (Pierce, Rockford, USA) (20 ng/μl) and incubation at 37 °C overnight. Peptides were extracted from the gel pieces once with 50 μl water and once with 50% acetonitrile. The samples were dried and re-suspended in 30 μl 2% acetonitrile/water, 0.1% FA. Residual digest reagents were removed using an in-house manufactured C18 stage tip (Empore octadecyl C18 extraction discs) (Supelco, Bellefonte, USA). The samples were loaded onto the stage tip after activating the C18 membrane with 30 μl methanol and equilibration with 30 μL 2% acetonitrile/water; 0.05% trifluoroacetic acid (TFA). The bound sample was washed with 30 μl 2% acetonitrile/water, 0.1% TFA before elution with 30 μl 50% acetonitrile/water, 0.05% TFA. The eluate was evaporated to dryness. The dried peptides were dissolved in 2% acetonitrile/ water, 0.1% FA for LC-MS analysis.

4.4.3 Liquid chromatography

The peptide solution was subjected to nano rapid separation liquid chromatography (nano-RSLC) on a Thermo Scientific Ultimate 3000 RSLC instrument equipped with a 2 cm × 100 μm C18 trap column and a 35cm × 75 μm in-house manufactured C18 column (Luna C18, 5 μm) (Phenomenex, Torrance, USA) analytical column. The following solvent system was employed as mobile phase: solvent A, 2% acetonitrile/water containing 0.1% FA; solvent B, 100% acetonitrile. The samples

were loaded onto the trap column using loading solvent at a flow rate of 15 µl/min from a temperature controlled auto sampler set at 7 °C. Loading was performed for 5 min before the sample was eluted onto the analytical column. The flow rate was set to 500 nl/min and the gradient was generated as follows: 2.0%–10% B over 5 min; 5%–25% B from 5–50 min; 25%–45% from 50–65 min, using Chromeleon nonlinear gradient 6. Chromatography was performed at 50 °C. The effluent was delivered to the mass spectrometer through a stainless steel nanobore emitter.

4.4.4 Mass spectrometry

The successful characterisation of proteins depends, largely, on the availability of advanced mass spectrometric techniques (Aebersold and Mann, 2003; Domon and Aebersold, 2006; Scigelova and Makarov, 2006). The development of the Orbitrap mass analyser has significantly contributed to the progress that has been made in this field during recent years.

The mass spectrometric analyses were carried out on a Thermo Scientific Fusion spectrometer equipped with a nanospray flex ionisation source. The sample was introduced through a stainless steel emitter. Data were collected in the positive mode with spray voltage set to 2 kV and ion transfer capillary set to 275 °C. Spectra were internally calibrated using polysiloxane ions at *m/z* 445.12003 and 371.10024. MS1 scans were performed using the Orbitrap detector set at 120 000 resolution over the scan range 350–1650 with the automatic gain control (AGC) at setting 3×10^5 and a maximum injection time of 40 ms. Data were acquired in profile mode. MS2 acquisitions were performed using monoisotopic precursor selection for ions with charges +2 to +6 with an error tolerance set to ±0.02 ppm. Precursor ions were excluded from fragmentation once for a period of 30 s. Precursor ions were selected for fragmentation in higher-energy collisional dissociation (HCD) mode using the quadrupole mass analyser with HCD energy set to 32.5%. Fragment ions were detected in the Orbitrap mass analyser set to 15 000 resolution. The AGC target was set at 1×10^4 and the maximum injection time at 45 ms. The data was acquired in centroid mode. The resulting MS/MS spectra were analysed using the Mascot and Sequest algorithms included in Proteome Discoverer and identification validation was done using the Percolator algorithm. As there is no protein data base for the caracal, all entries in the Uniprot taxonomy felidae data base were used.

The protein with the lowest molecular weight was excised from lane 5 of the gel depicted in Fig. 4.19 and digested. The resulting peptides were separated and analysed as described above.

The resulting TIC and a mass spectrum of peak at 32.13 min are depicted in Figs 4.20 and 4.21, respectively.

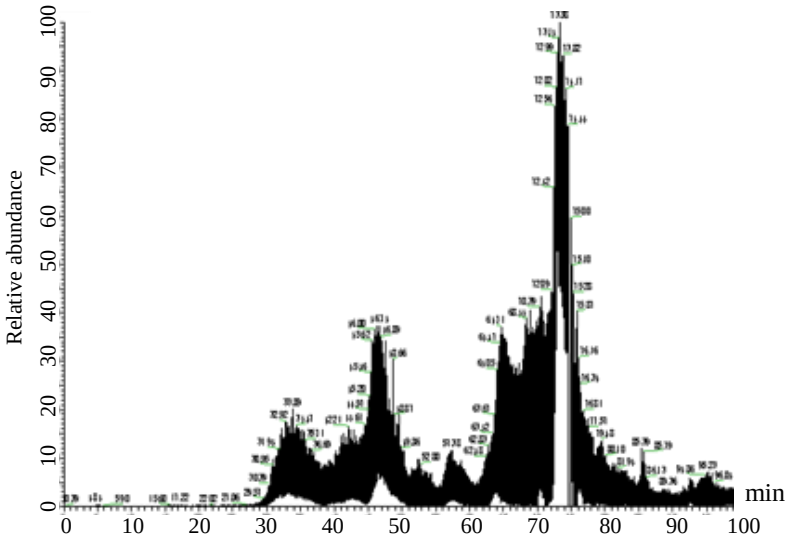


Fig. 4.20: TIC of the peptide digest of the protein band marked on the SDS-PAGE shown in Fig. 4.19.

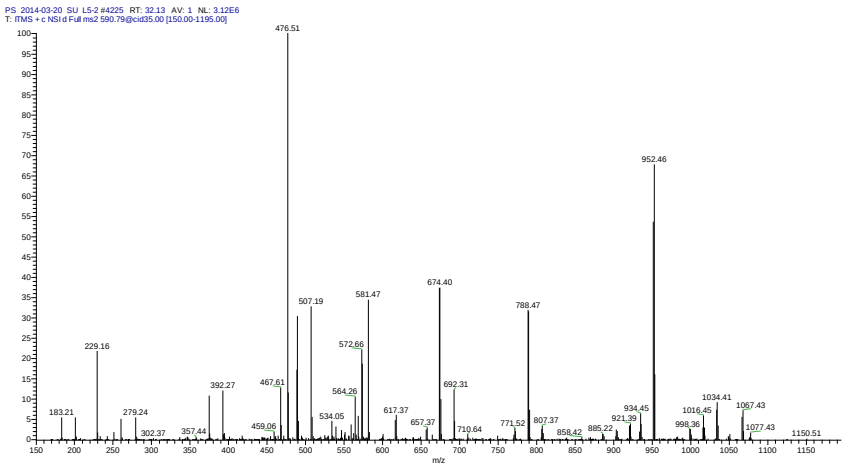


Fig. 4.21: Mass spectrum taken at 32.13 min in the TIC shown in Fig. 4.19.

4.4.5 Data analysis

The raw files generated by the mass spectrometer were imported into Proteome Discoverer version 1.4 (Thermo Scientific, Waltham, USA) and database interrogation was performed. Various search engines are available for protein data analysis. In this particular project, data analysis was carried out on Sequest and Mascot search engines. Database interrogation was performed against

a concatenated database created using the UniProt *P falciparum* database with semi-tryptic cleavage allowing for two missed cleavages. Precursor mass tolerance was set to 10 ppm and fragment mass tolerance to 0.5 Da.

Static and dynamic modifications are important in the protein characterisation. For example, change in the mass of cysteine is usually considered as a static modification. Cysteine can be directly alkylated using various suitable electrophiles (Teerlink, 1994; Chalker *et al*., 2009). For example, cysteine modification can be performed by thiomethylation with *S*-methylmethanethiosulphonate (Lundblad, 2014: 39), or through the use of iodoacetamide (Chalker et al., 2009). Dynamic modifications are more powerful because they are treated as potential modifications to database residues, rather than omnipresent modifications. In dynamic modifications, a database search for modified and unmodified forms of peptides takes place (Meri and Baumann, 2001). Deamidation is an example of dynamic modification because not all asparagine and glutamine residues can be deamidated (Meri and Baumann, 2001). In this project, N-terminal acetylation, deamidation, and oxidation were allowed as dynamic modifications, and thiomethylation of cysteine as static modification. Peptide validation was performed using the peptide validator node set to search against a decoy database with strict false discovery rate (FDR) 1%.

4.4.6 Database search

The tandem mass spectra were extracted. Charge state deconvolution and de-isotoping were not performed. All MS/MS samples were analysed using Sequest (version 1.4.1.14; Thermo Fisher Scientific, San Jose, USA) (Wolters *et al*., 2001). Sequest was set up to search UniProt taxonomy Felidae FASTA (unknown version, 24662 entries) assuming the digestion enzyme to be trypsin. Sequest was searched with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 10.0 ppm. Methylthio derivatisation of cysteine was specified in Sequest as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine, and acetyl derivatisation of the N-terminus were specified in Sequest as variable modifications.

4.4.7 Identification criteria

Scaffold (version Scaffold_4.3.4; Proteome Software Inc., Portland, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they

could be established at greater than 22.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 77.0% probability and contained at least two identified peptides. The peptide and protein decoys were 56.1% and 79.6%, respectively. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al*., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

The molecular mass of the protein excised from lane 5 (Fig. 4.19) was found to be 19.3 kDa. The relevant data are listed in Table 4.2. This protein was identified with high confidence peptide assignments, indicating strong homology, as shown in Table 4.2. Table 4.3 contains additional information regarding its identification.

Table 4.2: Proteins identified in male urine.

Accession	Description	Coverage (%)	Unique peptides	PSMs*	MW (kDa)
M3XEJ0	Uncharacterized protein OS=Felis catus PE=4 SV=1 - [M3XEJ0_FELCA]	9.83%	1	234	19.3

Table 4.3: The peptide sequence, modifications and other information on the 19.3 kDa protein.

Sequence	Modifications	Xcorr	Charge	Precursor *m/z* (Da)	ΔM (ppm)
ENNIPEENLIDmSKTEK	M12 (oxidation)	6.40	2	1010.4794	-1.21
ENNIPEENLIDmSKTEK	M12 (oxidation)	6.54	2	1010.4771	-3.44

4.4.8 Repetition of protein identification

The protein identification discussed in the preceding sections was carried out relatively early in this project and was decided that it might be prudent to repeat the protein identification. The hypothesis that it might be necessary to isolate urinary proteins in their non-denatured state for the identification of those proteins carrying putative protein ligands was explored and discussed above. This made it possible to repeat the identification of the urinary protein(s) with material that had undergone strong anion-exchange chromatography as an additional purification step. Hereafter, SDS-PAGE, tryptic digestion, UPLC, mass spectrometric identification and data processing followed, as described above. Bands from SDS-PAGE gels of the male and female proteins were excised, digested and analysed, and the MS data processed as described above. The

protein bands excised for this purpose are indicated in Figs 4.14 and 4.16. The Orbitrap mass spectrometric analyses and the processing of the resulting data were carried out as described above. The resulting TICs are depicted in Figs 4.22 and 4.23. The proteins identified in male and female urine sample are tabulated in Tables 4.3 and 4.4. The proteins, the respective accession numbers, coverage, the number of unique peptides, and the mass obtained from Mascot database search engine are all included. The male and female urine samples contained the same proteins or fragments, the only differences being the 67.4 and 68.5 kDa proteins which are found only in male and female material, respectively. The 58.3, 64.7, 67.4 and 68.5 kDa proteins listed in Tables 4.3 and 4.4 were identified from their fragments or oligopeptides. These fragments or oligopeptides provide sufficient information about their respective parent protein for identification.

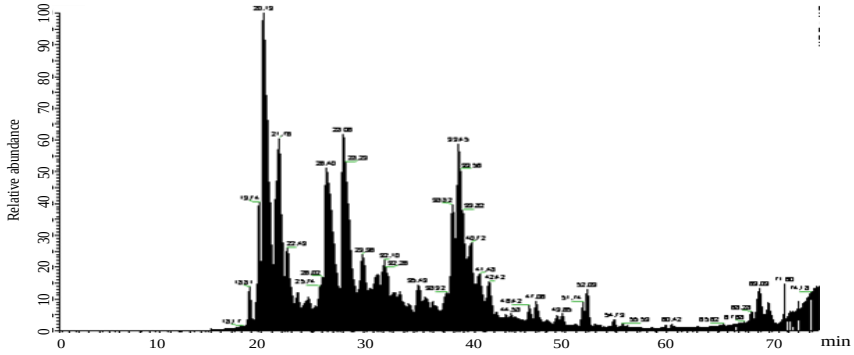


Fig. 4.22: TIC of the digested male protein band (Laingsburg sample LM4).

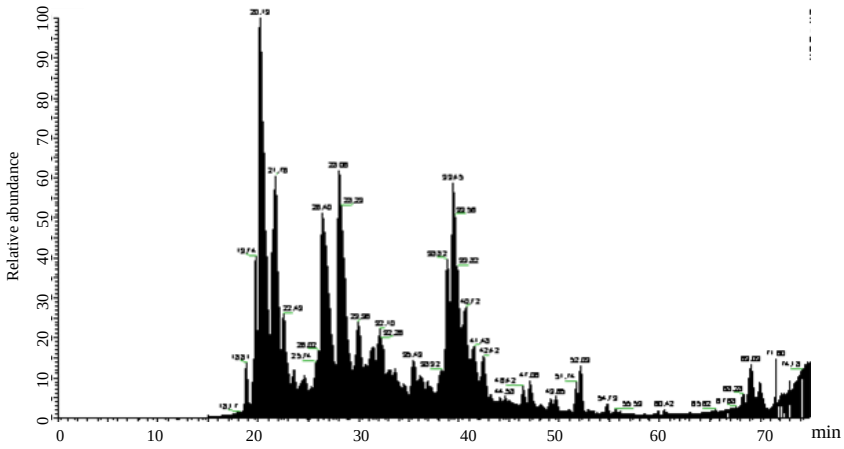


Fig. 4.23: TIC of the digested female protein band (Laingsburg sample LF4).

Six proteins in both the male and female material were identified. They are listed in Tables 4.4 and 4.5.

Table 4.4: Proteins identified in male caracal urine.

Accession	Description	Coverage (%)	Unique peptides	PSMs*	MW (kDa)
M3WJ37	Transgelin (Fragment) OS=Felis catus GN=TAGLN PE=3 SV=1 – [M3WJ37_FELCA]	38.73%	7	30	22.9
M3VXR1	Uncharacterized protein OS=Felis catus GN=KRT10 PE=3 SV=1 – [M3VXR1_FELCA]	16.19%	4	17	58.3
M3XEJ0	Uncharacterized protein OS=Felis catus PE=4 SV=1 – [M3XEJ0_FELCA]	41.62%	3	50	19.3
M3VUG6	Uncharacterized protein OS=Felis catus GN=KRT1 PE=3 SV=1 – [M3VUG6_FELCA]	16.03%	3	23	64.7
M3WP64	Uncharacterized protein (Fragment) OS=Felis catus GN=LOC101085453 PE=3 SV=1 – [M3WP64_FELCA]	8.06%	2	16	26.5
M3WFW6	Serum albumin OS=Felis catus GN=ALB PE=4 SV=1 – [M3WFW6_FELCA]	9.88%	3	50	68.5

*Peptide spectrum matches.

Table 4.5: Proteins identified in female caracal urine.

Accession	Description	Coverage (%)	Unique peptides	PSMs*	MW (kDa)
M3WJ37	Transgelin (Fragment) OS=Felis catus GN=TAGLN PE=3 SV=1 - [M3WJ37_FELCA]	49.02%	6	22	22.9
M3VUG6	Uncharacterized protein OS=Felis catus GN=KRT1 PE=3 SV=1 - [M3VUG6_FELCA]	16.35%	3	25	64.7
M3VZH9	Uncharacterized protein OS=Felis catus GN=KRT2 PE=3 SV=1 - [M3VZH9_FELCA]	18.92%	3	14	67.4
M3VXR1	Uncharacterized protein OS=Felis catus GN=KRT10 PE=3 SV=1 - [M3VXR1_FELCA]	30.40%	3	23	58.3
M3WP64	Uncharacterized protein (Fragment) OS=Felis catus GN=LOC101085453 PE=3 SV=1 - [M3WP64_FELCA]	28.23%	2	16	26.5
M3XEJ0	Uncharacterized protein OS=Felis catus PE=4 SV=1 - [M3XEJ0_FELCA]	52.60%	2	82	19.3

*Peptide spectrum matches

4.4.9 Protein sequences

Many proteins were identified in the urine samples, but only proteins that are unique to the caracal are displayed. The unique peptides that contribute to the highest percentage coverage are underlined in the entire protein sequences. Six proteins were identified in the male and female protein bands excised from the PAGE gel, depicted in Fig. 4.19. The entire protein sequences of the two proteins with the highest percentage coverage are tabulated in Table 4.6.

Table 4.6: Complete sequences of the 19.3 and 22.9 kDa proteins identified the urinary protein of male and female caracal.*

Male

Protein accession number (M3XEJO). Percentage coverage 41.62 %

MKILLLLLGVVLVCDGHLPLPDGHLPLSGEWNTLLVAATNVDKISNGPFHGYMRKVDVDIPNGRMVFK
FSVMMNGLCTEKSAVGTIGRDKFINIGNAGQNNFKILHYTLYSIIVHNVNVDSEGTTTEILGLLGKRLHPD
DNDFAKFRELMRENNIPEENLIDMSKTEKCPKKE

Protein accession number (M3WJ37). Percentage coverage 38.73 %

LSLNMANKGPSYGMSREVQSKIEKKYDEELEERLVEWIIVQCGADVGRPDRGRLGFQVWLKNGVILSK
LVNSLYPDGSKPVKVPENPPSMVFKQMEQVAQFLKAAEDYGVTKTDMFQTVDLFEGKDLAAVQRTLM
ALGSLAVTKNDGYYRGDPNWFMKKAQEHKREFTESQLQEGKHVIGLQMGSNRGASQAGMTGYGRPR
QII

Female

Protein accession number (M3XEJO). Percentage coverage 52.60%

MKILLLLLGVVLVCDGHLPLPDGHLPLSGEWNTLLVAATNVDKISNGPFHGYMRKVDVDIPNGRMVFK
FSVMMNGLCTEKSAVGTIGRDKFINIGNAGQNNFKILHYTLYSIIVHNVNVDSEGTTTEILGLLGKRLHPD
DNDFAKFRELMRENNIPEENLIDMSKTEKCPKKE

Protein accession number (M3WJ37): Percentage coverage 49.02%

LSLNMANKGPSYGMSREVQSKIEKKYDEELEERLVEWIIVQCGADVGRPDRGRLGFQVWLKNGVILSK
LVNSLYPDGSKPVKVPENPPSMVFKQMEQVAQFLKAAEDYGVTKTDMFQTVDLFEGKDLAAVQRTLM
ALGSLAVTKNDGYYRGDPNWFMKKAQEHKREFTESQLQEGKHVIGLQMGSNRGASQAGMTGYGRPR
QII

*The unique peptides identified with high confidence are underlined.

These proteins are lipocalins and found in both domestic cat, *Felis catus* and wildcat, *Felis silvestris catus*. Lipocalins are a family of proteins that are involved in pheromone binding, transporting, enzymatic activity and other activities (North, 1991; Leal *et al*., 2005). Other examples are, M3XEJ0 (19.3 kDa) (pheromone binding and transporting of small hydrophobic molecules), M3WJ37 (epithelial cell differentiation), M3VXR1 (structural molecule activity), M3VUG6 (carbohydrate binding), M3WP64 (serine-type endopeptidase activity) and M3WFW6 (binding of fatty acid, oxygen, etc.).

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CHAPTER 5

DISCUSSION OF RESULTS – CONCLUSIONS – OUTLOOK

5.1 Introduction

Research carried out over more than forty years has shown that marking with urine is a widespread mode of chemical communication in mammals, including several felid species. The present research was based on the premise that the successes that sheep farmers have had in some areas in attempts to control the caracal by catching the animal in traps baited with caracal urine could be interpreted as an indication that urine is possibly used for intraspecific communication and/or territorial marking in this animal. Farmers claim that male and female urine is equally attractive, although, logically, it can be hypothesised that the concentration of the attractive substances in the urine could also be important.

In planning the research, it was hypothesised that the staple diet of the caracal at the time that the urine is collected probably has a profound influence on the composition of its urinary VOCs and, furthermore, that specific diet-related VOCs might possibly be equally important. This imponderable could possibly have been eliminated by using experimental animals fed only on chicken, for example. (A similar approach was followed in an earlier research project successfully carried out by LECUS on the identification of the sex attractant of the red jackal, *Canis mesomelas*). However, if the sex of the animal does indeed play a subordinate role in the case of the caracal as far as the composition of the urinary VOCs are concerned, it could be reasoned that the diet-related VOCs could be responsible for the transmission of other information, for example, the availability of specific prey species.

For this research, urine was collected from caracal living in the wild on some farms in selected areas of the Western Cape Province. A map of the areas where urine was collected is shown in Fig. 5.1. Enormous qualitative and quantitative variations were found in caracal urine from different sheep farming areas.

Within the first week after the research project had been launched, a male and a female caracal were caught together in a trap. An astonishingly large volume of urine was obtained from the male (95 ml); not much was obtained from the female (9 ml). Although several farmers were

initially willing to donate urine for the research, this urine was actually the only material received during the next six months, probably because caracal urine has now become a rather precious commodity in sheep farming areas. The irregular donation of good quality urine was a major constraint throughout this project. Fortunately, as the qualitative analyses were carried out using a headspace analytical method, it was possible to trap VOCs repeatedly from the same urine sample until the VOCs could no longer be reliably determined. Large volumes of urine received from one farmer who kept caracal illegally on his farm could not be used for our research because the concentration of VOCs was very low in these samples, presumably because the animals had practically *ad lib* access to water.

Fig. 5.1: Districts in the Western Cape Province where caracal urine was collected.

No urine was donated also during six-month periods in 2015 and in 2016. Some of the 30 samples of male and female urine donated during the research project were too small for the envisaged analytical work. Eventually, only 11 samples of male and 7 samples of female urine were used. A total of 191 organic compounds were identified in these samples. It is quite likely that additional VOCs would have been identified, had samples from more geologically and biologically diverse areas been available.

5.2 Urinary VOCs

All the alkanes from octane to octacosane were identified in the urine samples that were analysed, although not all of them were present in all samples (Table 3.1). The RIs of the VOCs under investigation were determined with reference to a standard mixture of alkanes from C_7 to C_{40}. All of the analysed urine samples contained at least some unbranched alkanes, which made it possible to check the retention time accuracy of all the analyses. Without any sound motive, it was hypothesised that the simple unbranched hydrocarbons probably do not play any significant role in the caracal's semiochemical communication. Furthermore, some of the long-chain branched and unbranched hydrocarbons have very low vapour pressures and hence were unlikely to be involved in semiochemical communication in this animal. Limonene (probably plant derived), 1-pentadecene, and *cis*-2-phytene were identified in relatively high percentages in either male of female urine and, in a few cases, in the urine of both sexes.

With the exception of 2-ethyl-1-hexanol, which was present in 85% of the female samples, and nerolidol in 55% of the male samples, the aliphatic alcohols are not well represented in caracal urine. More or less the same situation was found in the case of the aldehydes. The most notable exceptions are nonanal in male urine, decanal in male and female urine, and, notably, 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde in both sexes.

The aliphatic ketones are very well represented in caracal urine. However, again, there are only a limited number of ketones that are present in more than 50% of urine samples from males and/or females. In male urine, these ketones are 2-heptanone, 4-heptanone, 2-nonanone, 2-tridecanone, and 3-methylcycloheptanone; in female urine, these ketones are 2-nonanone, and the two branched ketones, *trans*-6,10-dimethyl-5,9-undecadien-2-one, and 2,2,6-trimethylcyclohexanone. It is significant that cyclopentadecanone was identified in the highest percentages: 82% and 86% of the male and female urine samples, respectively. All these ketones were available for behavioural tests.

Aliphatic acids have been identified in the secretions and excretions of many mammals (e.g., Albone and Shirley, 1984: 177, 187, 192; Burger, 2005: 231-278). Although a total of 28 fatty acids were identified in the available urine samples, only dodecanoic acid and tetradecanoic acid were present in the majority of the urine samples (64% and 54% of the male and female samples, respectively). The majority of the acids were present in fewer than 3 male or female urine samples.

The aliphatic esters were even less well represented in the urine of both sexes. Many of the esters were identified in only one urine sample.

Regarding sulphur and sulphur-containing compounds, only S_8 and benzothiazole were present in the urine of both sexes in percentages higher than 50%. Although the cheetah, *Acinonyx jubatus*, is thought to be the fastest mammal, it is not very strong and would stand a very slim chance of defending itself and its offspring against larger and stronger carnivores. It was hypothesised (Burger *et al*., 2006) that the absence of malodorous (to the human nose) sulphur compounds and the presence of high concentrations of S_8 in the cheetah's urine could be ascribed to a defence strategy of the animal in which easily detectable sulphur-containing compounds are converted into elemental sulphur. Sulphur does not have a 'strong' smell and hence, secreted in this form, it is probably not as easily detectable as sulphur compounds. Large predators could thus find it more difficult to detect and to find the lair of a female cheetah with offspring (Burger *et al.*, 2006). On the other hand, S_8 was also identified in the marking fluid of the Bengal tiger, *Panthera tigris* (Burger *et al.*, 2008), an animal that is capable of defending itself against almost any other carnivore. Voiding urine without a strong odour could, however, benefit females of all of these species with young cubs.

It was surprising to find that about 50% of the compounds grouped together as 'miscellaneous compounds' were identified only in one urine sample, or in one male and one female sample. Acetophenone, phenol, 2-phenylethanol, and 2-piperidone are the only compounds in this group that were present in more than 50% of the samples from one of the sexes.

The 191 compounds listed in Table 3.1 do not represent the total number of constituents present in caracal urine. Selected ion chromatograms constructed for ions present as impurities in many of the mass spectra of the identified VOCs, and also for ions detected in the valleys between the peaks in the TICs, suggest the presence of trace quantities of many unidentified constituents. If it is taken into consideration that only 20 samples of acceptable quality were analysed, the impression is gained that the availability of additional samples could have contributed more VOCs to the list. Statistical analysis of the available data was not considered viable.

In retrospect, had it been possible, a comparison of the quantitative composition of the urinary VOCs of male and female caracal fed only chicken with that of wild ranging animals would have been helpful. However, a fortunate coincidence enabled us to obtain some idea of the influence that the diet of male and female caracal could have on the VOC profiles of their urine.

The very first two samples of urine that were donated for this research were collected, in my presence, from a pair of caracal caught in the same trap in the district of Porterville. They had possibly shared prey that one of them had caught. It was thought that quantitative analysis and a comparison of the urinary VOCs of these two animals, although tenuous, would provide some information on the degree of similarity of the VOC profiles that could be attained for males and females on the same diet. The required data were obtained by SEP-GC-MS analyses, which, provided that the sample enrichment was carried out under strictly reproducible conditions, produce credible results. Compared to GC analysis, GC-MS analysis yields less reliable quantitative data. However, this is not a serious shortcoming if, as in the present case, the concentrations of the same compounds in different samples are compared. In any case, it is practically impossible to assign and accurately integrate peaks in complex chromatograms recorded on different instruments without supporting qualitative information.

In the present project, quantitative data were thus obtained by peak integration of the TICs of the GC-MS analyses of the urine obtained from these two animals, mentioned above. The quantitative results (raw data) of these analyses are tabulated in Table 5.1.

Table 5.1: Relative concentrations of VOCs in the urine collected from a male and a female caracal caught together in the same trap

Compound	Peak area	
	Male urine	Female urine
2-Butanone	128315	12121450
2-Pentanone	729863	6982266
3-Hexanone	82318	757411
2-Hexanone	41054	
Undecane	56095	207170
Dodecane		338442
4-Heptanone	6898340	
2-Heptanone	1854311	4442886
Pyridine		333139
3-Methylcyclopentanone		1600985
3-Methyl-2-heptanone	2383241	
1-Pentanol		3733313
2-Pentyl-furan	102083	
6-Methyl-2-heptanone	122297	
5-Methyl-2-heptanone	32192	
2-Octanone	129039	758984
Octanal		312691
3-Methylpyridine		1011369
trans-3-Hepten-2-one		2453087
3-Nonanone	524883	
2,2,6-Trimethylcyclohexanone	136586	4348404
3-Methylcycloheptanone		1246196
2,6-Dimethylpyrazine		3074107
2-Methyl-3-octanone	194847	19025
6-Methyl-5-hepten-2-one	112359	2758058
3-Methylcycloheptanone		1448917
1-Hexanol		896576
2,4,6-Trimethylpyridine		941414
2-Nonanone	1625539	2550187
Decane	459574	
4-Undecanone	284895	
Acetic acid	312195	3019434
2-Decanone	287537	1289602
Isophorone		1013805
Decanal	677910	4477107
Tetradecane		669409
Trimethylpyrazine		3075490
1-Heptanol		541606
Tetramethylpyrazine		398560
2-Ethyl-1-hexanol		4819408
Pentadecane	801999	1338036
trans-3-Nonen-2-one	151371	864754
Benzaldehyde	1082241	2759761
1-Pentadecene	195131	
Linalool		938453
1-Octanol	196794	1164387
Formic acid		263386
trans-6-Me-3,5-heptadien-2-one* (**3.92**)		863749
2-Undecanone	491797	5952977
2-Acetylpyridine		1610772
Hexadecane		844528

Table 5.1: *Contd.*

3-Dodecanone	437955	3924343
Cyclic aldehyde derivative* (**3.58**)	410454	11142600
Acetophenone	342105	952320
5,9-Dimethyl-2-decanone	190889	
1-Nonanol	489228	1103832
trans-6,10-Dimethyl-2-undecanone	1015235	
Salicylaldehyde		4984085
2-Dodecanone	479596	212849
Heptadecane	140961	385702
1-Phenyl-2-propanone	433959	
Icosane	338368	803579
1-Decanol		303619
Acetamide		1413550
cis-2-Phytene	6153841	
Octadecane	344324	
2-Tridecanone	2875997	184722
trans-6,10-Dime-5,9-undecadien-2-one* (**3.92**)	123171	3707232
Hexanoic acid		519942
Nonadecane		452178
Dimethylsulphone	708226	
2-Phenylethanol	766525	5903765
trans-2-Phytene	22926464	
Methyl 6-methylheptanoate	606633	
Nerolidol	93058	278497
Methyl octanoate	271222	441847
2-Piperidone	2115968	
1-Dodecanol	276472	704622
β-Ionone		1551368
Benzothiazole		1870770
Heptanoic acid		725282
Octanoic acid		7140573
Nonanoic acid	416683	12823893
Methyl nonanoate	665927	1780477
Cyclopentadecanone	387939	917245
Decanoic acid	894985	14365815
Methyl decanoate	1761747	6806020
3-Methyldecanoic acid	7728484	16428515
1-Tetradecanol	313198	1527777
2-Tetradecanol		2267774
Undecanoic acid	190678	1651842
2-Methylundecanoic acid	1874630	2956740
Dodecanoic acid	1843968	29316848
Methyl undecanoate		5737946
Methyl dodecanoate	732665	3401161
2(4H)-Benzofuranone-derivative* (**3.189**)		2053705
Phenol		5280243
Isopropyl teradecanoate		1112798
1-Hexadecanol		2545349
Tridecanoic acid	925516	7008841
Tetradecanoic acid	3556625	66088228
Heneicosane		1144813
Docosane		935312
Tetracosane		2493780
Hexacosane		4397860

Table 5.1: *Contd*

Octacosane		4255581
Nonacosane		2823166
Benzoic acid		1983902
iso-Pentadecanoic acid		4826043
anteiso-Pentadecanoic acid		3174055
Pentadecanoic acid	465774	31388652
Hexadecanoic acid	10196064	237612416
cis-9-Hexadecenoic acid	3344801	4521080
trans-9-Hexadecenoic acid		62345264
Heptadecanoic acid		709190
Squalene (isomer 1)	1975575	57455984
Squalene (isomer 2)	2817260	122677232
Octadecanoic acid	1858220	45635880
Oleic acid		125013032
Linoleic acid		9773356

*Chemical names abridged

It is practically impossible to interpret the mass of information in Table 5.1 in terms of what the presence of some compounds and the absence of others could communicate to a caracal; even when presented in graphical form, it is difficult to draw conclusions regarding the possible semiochemical significance of urinary VOCs of the animal.

An attempt was made to simplify the interpretation of the results by comparing the quantitative VOC composition of the urine of these two animals with respect to one or two of the most abundant compound classes present in the urine. The ketones, carboxylic acids and esters were chosen for this purpose. Quantitative data for the ketones are presented in a bar graph shown in Fig. 5.2. To simplify interpretation of this bar graph and to allow for the inclusion of quantitative data in the bar graph, the raw quantitative data (Table 5.1) were divided by 1×10^4.

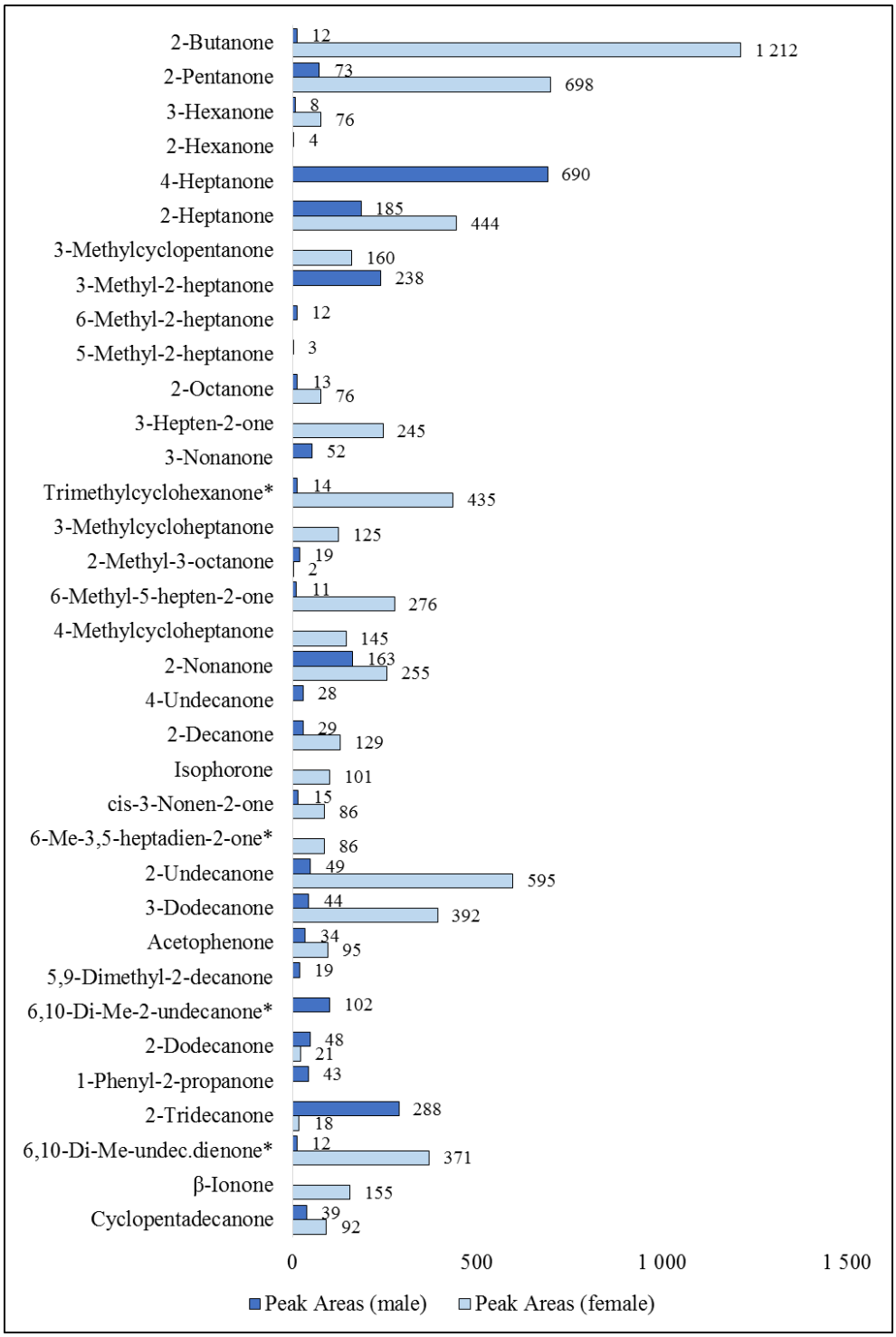

Fig. 5.2: Bar graph presentation of a comparison of the quantitative composition of the urinary ketones from a male and female caracal caught together in the same trap. *Chemical names abridged.

The volume of urine that was drained from the male's bladder suggested that the male had taken in a large volume of water shortly before he was caught. Compared to the volume of urine

collected from other females, this female yielded about the volume of urine typically collected from female caracal in this project. The concentrations of the VOCs were therefore expected to be much lower in the male than in the female urine. The general appearance of the bar graph in Fig. 5.2 confirms this assumption. Although it must be taken into consideration that some of the ketones could have been present in the urine samples in concentrations lower than the detection limit of the analytical method that was used, 9 of the 35 ketones under discussion were present only in the male urine and 7 only in the female urine. Even if the male data were to be multiplied to compensate for the presumed dilution of the male animal's urine, the VOC profiles of the two animals are totally different. On the other hand, if the ketones play any role in the semiochemical communication of this species, the composition of these two urine samples are sufficiently complex and different to convey a considerable volume of information.

A first possible explanation of these results could be that the two animals never had any contact with each other until one of them was enticed, for some reason, into entering the trap, in which case the different ketone profiles of their urine could conceivably have contained information on the prey available in the animals' home range. This information would most likely benefit only the receiver of the information. The second possibility is that they were a breeding pair that shared all or most of their meals, and that their ketone profiles are determined by a combination of the composition of their food and each animal's metabolism. In this case, the ketones could serve *inter alia* as an identification cue, in addition to carrying information about the animal's diet. In view of the uncertainty concerning the two animals' relationship, all of this is probably mere speculation.

These explanations are even more applicable regarding the quantitative data on the carboxylic acids and esters present in the male and female urine, as displayed in the bar graph in Fig. 5.3.

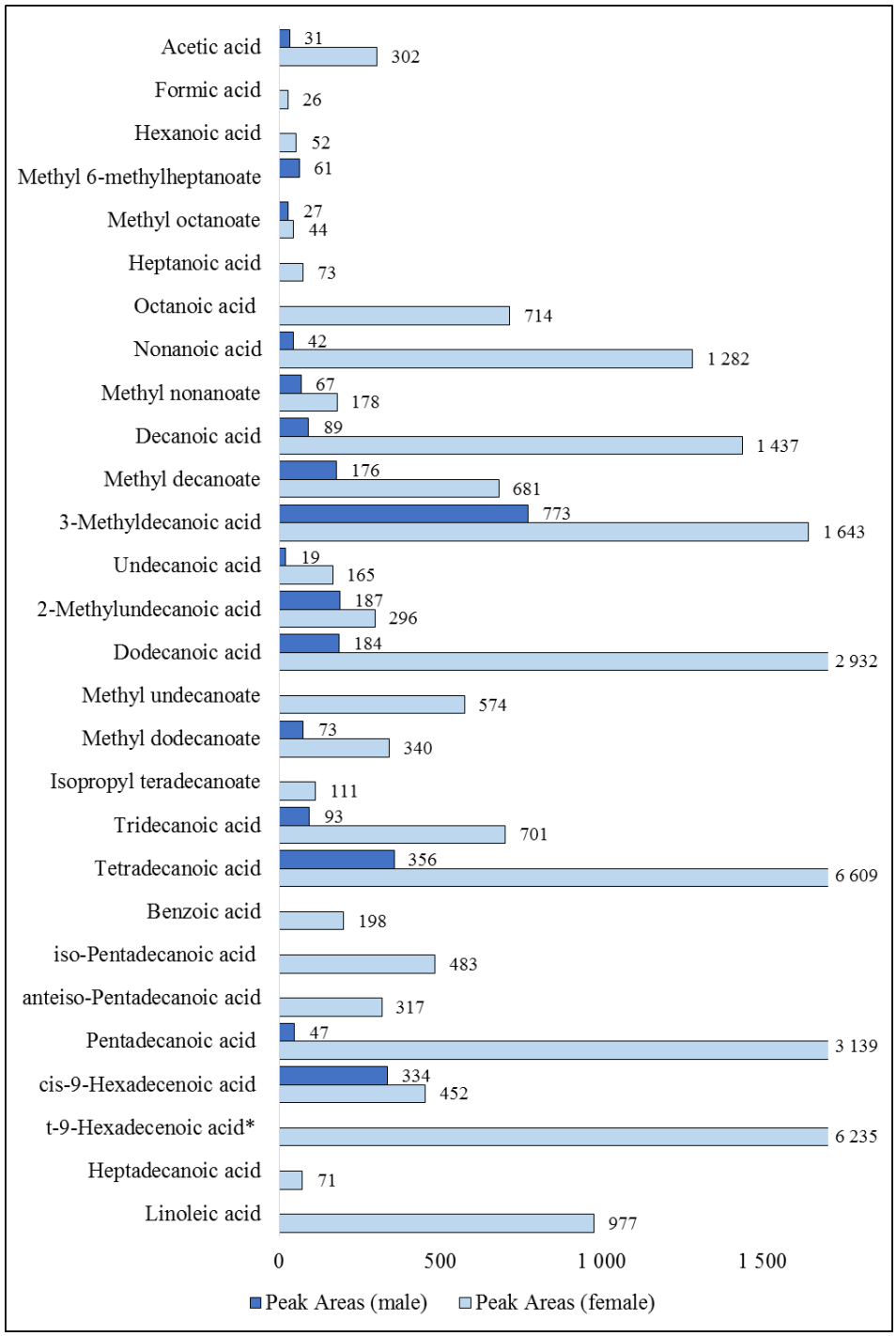

Fig. 5.3: Bar graph presentation of a comparison of the quantitative composition of the urinary carboxylic acids and esters from a male and female caracal caught together in the same trap. *Chemical names abridged.

Here, the high dilution of the male's urine is particularly evident. The relative concentrations of dodecanoic, tetradecanoic, pentadecanioc acid, *trans*-9-hexenoic, and heptadecanoic acid in the female urine were so high that it was impossible to compare the information contained in the bar graph in the lower VOCs concentration range. In Fig. 5.3, therefore, only the concentration range 0–1700 is displayed.

In the light of the concentration differences between the urine samples and the ambiguity of the relationship between the two animals, these observations, unfortunately, contribute very little towards determining whether the urinary VOCs play a significant role in the chemical communication of the caracal.

5.3 Urinary steroids

Only one sample of male urine and one of female urine were analysed for the presence of steroids. Androstenedione and testosterone were found to be present only in male urine at concentrations of 0.004 and 0.003 ppm, respectively (Table 3.2). Aldosterone, cortisol, and cortisone were identified in both male and female urine. With the exception of cortisone, which was present in the male urine sample at a concentration of 0.14 ppm, these three steroids were present in the male and female urine in concentrations ranging from 0.03 to 0.09 ppm. Although research has revealed that steroids, including urinary steroids, play an important role in the semiochemical communication of many mammalian species (e.g., Albone and Shirley, 1984: 85, 130, 183, 237), practically no attention was devoted to this aspect in the present investigation. Due to time constraints, resulting from an unreliable supply of urine and the discovery of ligand-carrying proteins in the urine of male and female caracal, indicating that the urinary protein of both male and female caracal releases several macrocyclic ketones when the protein undergoes denaturation, the research was focused mainly on the role of the macrocyclic ketones in the semiochemical communication of the caracal.

5.4 Proteomics

Healthy mammals excrete small amounts of urinary proteins; usually, proteinuria is an indicator of renal abnormality (Raila *et al*., 2005). Several studies have revealed that urinary proteins are involved in chemical communication in mammals. Over the past two decades, the role of the major urinary proteins (MUPs) in the semiochemical communication of the house mouse, *Mus*

domesticus, has been studied in great detail (e.g., Novotny 2003). For example, it was found that MUPs bind dehydro-*exo*-brevicomin and 2-(*sec*-butyl)-4,5-dihydrothiazole (Bacchini *et al.*, 1992), which elicit male aggression in the house mouse (Novotny *et al*., 1985). It has also been suggested that the pattern of MUPs present in male urine may act as a type of individuality barcode that signals the identity of the owner of a particular scent mark (Beynon and Hurst 2003). On the other hand, Miyazaki *et al*. (2006b) suggest that the excreted MUPs of the domestic cat act as enzymes in the synthesis of putative pheromone precursors and not as carrier proteins.

According to Miyazaki *et al*. (2006a), the carboxylesterase-like urinary excreted protein, also known as cauxin, is not present in the urine of *Panther*a species. The function of cauxin is unknown, but Miyazaki *et al*. (2006b) suggest that it might regulate the production of felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid), a putative pheromone precursor in the domestic cat. However, cauxin was identified as one of the proteins in the urine fraction of marking fluid of the Bengal tiger (*Panthera tigris*). The Bengal tiger's marking fluid separates into two layers upon standing. The lower layer contained 0.318 mg protein (molecular mass 16–180 kDa) per millilitre of urine (Burger *et al*., 2008). In the present research project, with the exception of only a few small samples, the caracal urine available for this project was generally clear. An exploratory investigation was nevertheless undertaken into the possibility that protein(s) could be involved in the caracal's intraspecific communication. In fact, it was found that all the samples of caracal urine that were analysed contained in the order of 10 mg protein per millilitre of urine, i.e., much higher concentrations than those found in the urine fraction of the tiger's marking fluid. Exploratory UPLC-MS/MS analysis of fresh whole urine indicated that only one protein, or a protein complex, with a molecular mass of ca. 20 kDa was present in male and female caracal urine.

Using a powerful mass spectrometer, further protein analyses were carried out on dialysed and electrophoretically separated male and female proteins. In further analyses, dialysed male and female proteins were first subjected to strong ion exchange chromatographic fractionation before SDS-PAGE gels were submitted for MS analysis. Cauxin was not identified as a caracal urinary protein, but the proteins M3WJ37 (Transgelin (fragment) OS=Felis catus) and M3XEJ0 (Uncharacterized protein OS=Felis catus) were identified with high confidence.

As mentioned in the Introduction (§ 1.7), cauxin is a carboxylesterase that regulates the production of felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid), a putative

pheromone precursor. Cauxin hydrolyses the felinine precursor 3-methylbutanolcysteinnyl-glycine to felinine and glycine. Cauxin and felinine are excreted age dependently after three months of age. Cats spray urine with a species-specific odour for territorial marking. The urine contains 3-mercapto-3-methyl-1-butanol, 3-mercapto-3-methylbutyl formate, 3-mercapto-3-methylthio-1-butanol and 3-methyl-3-(2-methyldisulfanyl)-1-butanol (Miyazaki *et al*., 2006b), compounds that contribute to the catty odour of cat urine.

The fact that caracal urine is practically odourless is consistent with the qualitative results showing that caracal urine does not contain cauxin.

5.5 Protein ligands

To answer the question as to whether any of the VOCs identified in caracal urine could be ligands of the urinary proteins of the caracal, the VOCs present in the headspace of male and female urine were sampled by SEP-GC-MS in fresh urine samples and again after heating the urine samples to 95 °C, at which temperature the proteins were expected to undergo denaturation and to release any putative ligands. The results of these experiments varied, but clear evidence was found that denaturation of the urinary protein resulted in a marked increase in the headspace concentration of, specifically, cyclopentadecanone. More pronounced increases in the headspace macrocyclic ketones were observed when dialysed and concentrated urinary protein was thermally denatured.

Credible results indicated that cyclohexadecanone and cycloheptadecanone could also be urinary protein ligands in the caracal. Initially, cyclotridecanone and cyclotetradecanone were not detected in the headspace gas of undialysed urine. They were, however, found to be present in the headspace gas of denatured urinary protein samples. Based on the observed retention times of the latter two ketones, cyclotetradecanone was subsequently located and quantitated also in extracts of untreated urine.

5.6 Quantitation of the protein ligands

In view of the identification of the macrocyclic ketones as ligands of the urinary proteins of the caracal, it seemed prudent to compare the relative concentrations of these ketones in caracal urine. Urine of satisfactory quality was available from 5 males and 1 female. In the laboratory, about 6–7 ml urine was also drained from each of three quite small caracal bladders (ones that were considered to contain too little urine for SEP-GC-MS analyses). This urine was stored at –23 °C

for possible future use. Because the bladders were very small, they were presumed to be those of females. Analyses of these nine urine samples were carried out as described in § 2.3.3. The urine obtained from the small bladders was divided into two 3-ml samples each. The VOCs were extracted with DCM from one of each pair of samples, and the other samples were subjected to thermal denaturing before the VOCs were extracted with DCM. Two aliquots from a larger sample of male urine were treated and extracted likewise. This experiment was carried out to determine whether the urinary proteins undergo complete denaturation upon extraction with DCM. No significant quantitative difference was observed between extracts of fresh urine and thermally denatured urine. The resulting quantitative data are tabulated in Table 5.2 and are displayed graphically in the bar graph in Fig. 5.4.

Table 5.2: Comparison of the relative quantitative ratios in which the macrocyclic ketones are present in unprocessed urine of male and female caracal[a]

Urine extract[b]	C_{13}[c]	C_{14}	C_{15}	C_{16}	C_{17}
UE1			1039518 (100 %)	1081666 (104 %)	245235 (24 %)
UE2		27106 (3 %)	900774 (100 %)	297754 (33 %)	155982 (17 %)
UE3			387329 (100 %)	98775 (26 %)	10106 (26 %)
UE4		12069 (4 %)	310636 (100 %)	89124 (29 %)	
UE5		23115 (5 %)	512447 (100 %)	106495 (21 %)	175613 (34 %)
UE6		28489 (5 %)	591475 (100 %)	142630 (24 %)	200831 (34 %)
UE7			241641 (100 %)	99271 (41 %)	122943 (51 %)
UE8			164066 (100 %)	39814 (24 %)	69332 (42 %)
UE9		453036 (2 %)	26784186 (100 %)	1949833 (7 %)	6153619 (23 %)

[a]The quantitative data were normalized with respect to C_{15} = 100%. [b]UE1, male urine; UE2–UE4, female urine; UE5–UE8, male urine; UE9, female urine. [c]Cyclotridecanone was detected only in the headspace of denatured urinary protein.

As the concentrations of the ketones varied greatly between individual urine samples, (again, possibly due to differences in the concentration of VOCs), the results were normalised with respect to the concentration of cyclopentadecanone = 100 for the construction of the bar graph shown in Fig. 5.4.

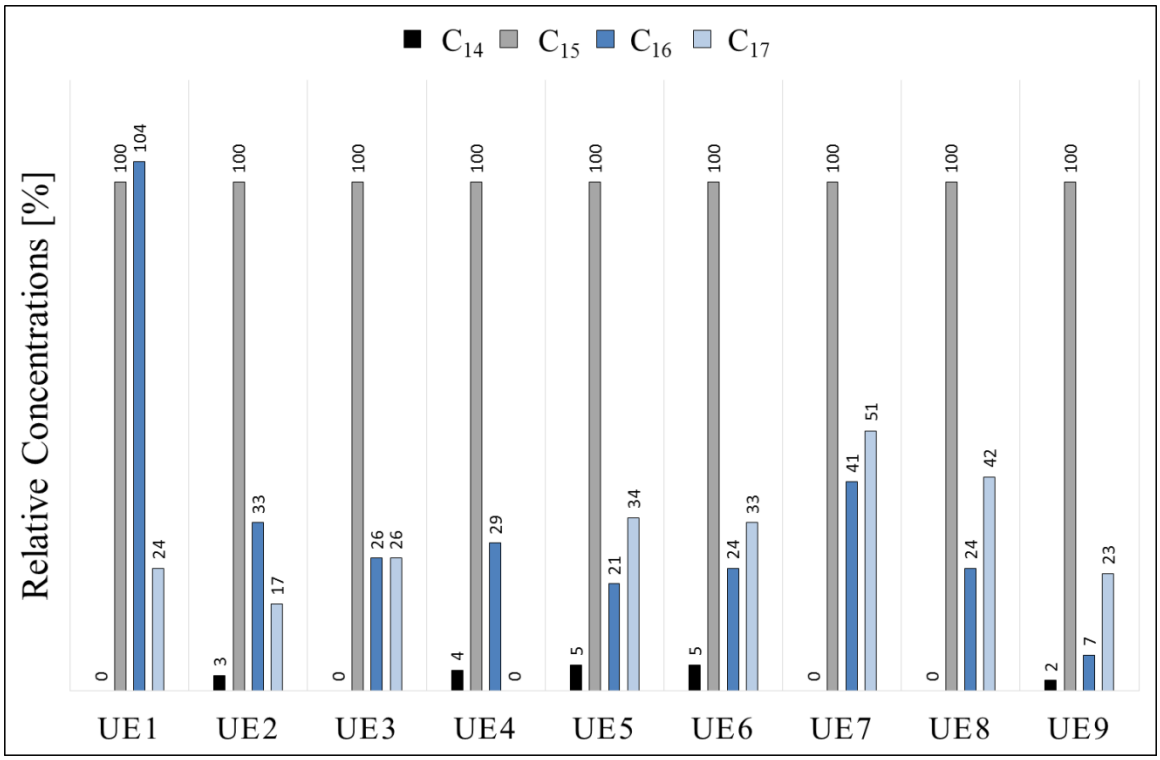

Fig. 5.4: Comparison of the macrocyclic ketone profiles of individual caracal. UE1, male urine; UE2–UE4, female urine; UE5–UE8, male urine; UE9, female urine.

When these analyses were carried out, only sample UE1 (80 ml), collected from a male caracal, and sample UE9 (25 ml), collected from a female, in the presence of a zoologist in this case, had not already been used for SEP headspace analyses. Samples UE2–UE4 (*ca*. 6–7 ml) were drained from very small bladders that were presumed to be those of female caracal. It should be taken into consideration that it is possible that the farmers who collected the male urine (samples UE5–UE8) could have made a mistake when labelling the sample bottles as urine from male caracal. Nevertheless, it was clearly evident that the macrocyclic ketone profiles of these animals were very different.

As credible conclusions cannot be based on such a small sample of experimental results, obtained from animals of uncertain sex, it does not appear likely that these macrocyclic ketone profiles could communicate information on the sex of the animal. Nonetheless, these profiles do suggest that the macrocyclic ketones could somehow be involved in individual recognition in this species.

5.7 Bioassays

According to current legislation, it is illegal to keep caracal privately. Only two male caracal at the Wildlife Awareness Centre, Giraffe House, were available for behavioural tests with synthetic analogues of the VOCs identified in caracal urine. However, it was considered unlikely that successful behavioural experiments would be possible in a facility where the two experimental caracal are kept together in the same enclosure and in close proximity to many other animals. In fact, even a lure with female caracal urine initially elicited barely any response.

Although caracal are also active during the early morning and the late afternoon, they are essentially night-active animals. Better results were therefore expected when a movement-activated day/night camera became available with which the behaviour of the two caracal could be recorded during the night. In each of the following experiments two lures were compared (for experimental details, see § 2.6):

1. Ketones *vs.* female urine.
2. Cyclopentadecanone *vs.* female urine.
3. Mixtures of two groups of the 'miscellaneous compounds' listed in Table 3.1 were compared with each other.
4. Synthetic C_{13}–C_{16} macrocyclic ketones *vs.* male urine.
5. Synthetic C_{13}–C_{16} macrocyclic *vs.* male urine from another male.

In our evaluation of the results of these experiments, the following criteria (a–g below) were applied, in order of increasing significance of the animals' response to the attraction of the lure. Wording included in brackets below clarifies wording used in the table of results (Table 5.3).

a. Sniffing at a lure (Sniffing).
b. Looking around after sniffing at a lure (Observing).
c. Kneeling down and sniffing at a lure (Kneeling).
d. Playing with a lure in typical domestic cat manner (Playing).
e. Lying down with the head towards the lure (Lying).
f. Rubbing a side of the face on the lure (Rubbing).
g. Squatting with the back towards the lure and with the tail lifted straight up, in typical marking behaviour (Over-marking).

In each recorded video, the number of observed instances of each of these behavioural response patterns towards each of the lures was counted. The results are tabulated in Table 5.3.

Table 5.3: Comparison of the responses of two caracal males when offered the choice between two types of lure in each experiment

		Behavioural response patterns						
Experiment	Lure	Sniffing	Observing	Kneeling	Playing	Lying	Rubbing	Over marking
1. 14–15 Dec.	Ketones[a]	6	5	7	3	5	1	-
	Female urine ♀	-	8	6	1	3	3	1
2. 15–16 Dec.	Cyclopentadecanone	5	-	-	1	-	-	1
	Female urine	8	3	2	1	1	-	1
3 16–17 Dec.	Miscellaneous VOCs 1[b]	3	1	-	-	-	1	-
	Miscscellaneaous VOCs 2	3	-	1	-	-	1	-
4. 17–18 Dec.	Macrocyclic ketones[c]	1	1	-	-	-	-	-
	Male urine 1	3	2	-	1	2	3	-
5. 18–19 Dec.	Macrocyclic ketones/Squalene[d]	6	5	1	1	8 (7 min)	-	-
	Male urine 2	2	1	-	-	1	1	-

[a]Available synthetic ketones as in Table 5.1, including cyclopentadecanone (C_{15}); [b]Available synthetic VOCs as in Table 5.1, but classified as in Table 3.1;[c]Synthetic macrocyclic ketones C_{13}, C_{14}, C_{15} and C_{16}; [d]The same macrocyclic ketones in admixture with squalene vs the urine of a different male.

5.8 Conclusions

Discussing the results of this research, it should be noted that one could barely try to draw conclusions from bioassays that were carried out over a period of only a few days, under less than optimal environmental (experimental) conditions. Nonetheless, the results obtained could be valuable in terms of providing direction for future research on the semiochemistry of the caracal.

In light of the presence of cyclopentadecanone in the mixture of ketones tested in experiment 1, the large range and number of repetitions of behavioural patterns that were observed could be significant, particularly when the results of this experiment are compared with those obtained in experiments 2, 4 and 5, in which the lures also contained cyclopentadecanone, but none of the many other ketones identified in caracal urine. This could be an indication that the caracal reacted more strongly towards the protein ligand in the presence of the other ketones, i.e., as 'a signal in context'. On the other hand, the over marking of cyclopentadecanone in experiment 2 could be interpreted as a very drastic reaction to an 'out of context' signal.

The relatively mild reaction of the caracal to the miscellaneous VOCs in experiment 3 indicates that these compounds probably do not play an essential role in the semiochemical communication of this species.

Two explanations are offered for the lack of response in experiment 4. It is possible that the lure contained too little cyclopentadecanone, or that the ketone evaporated too quickly from the cloth. (At the end of the experiment this ketone was no longer detectable by the human nose).

In a final experiment (experiment 5), five times more ketone was applied to the cloth. However, the addition of squalene, which is a controlled-release substance, probably also contributed to the positive outcome of this experiment.

Considering the 'less-than-optimal' conditions under which the experiments were carried out, quite interesting information was obtained, although all results need to be considered with circumspection.

5.9 Outlook

A successful series of behavioural bioassays would have been a most desirable conclusion to this research project. It is evident that any future research should be focused on thoroughly planned and executed bioassays. The following approach could be considered: A pair of caracal that has lived together under as natural conditions as possible, ideally housed in separate enclosures within

visible distance from each other, but without direct contact, could be used. Urine samples should be drained by catheter from the bladders of the animals, under sedation. A test mixture should be composed with qualitatively and quantitatively exactly the same composition as the urine of these two animals. (This should not be difficult with our experience of the analysis of caracal urine). A study could then be carried out of the behavioural responses of the experimental animals to lures containing these VOCs, using a motion-activated camera, as in the closing stages of the present research project. If successful, such a study could perhaps also serve as a starting point for similar studies on other mammals.

5.10 References

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