MASS SPECTROMETRIC STUDIES OF STEROL METABOLITES TO ELUCIDATE THE BIOSYNTHETIC PATHWAY OF ECDYSONE IN THE DESERT LOCUST

Schistocerca gregaria

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

To my family, who accord me encouragement, support and patience during my studies more so to Fiona, Valerie and Xeldwyn.

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LIST OF ABBREVIATIONS AND ACRONYMS

2, 22-Dideoxyecdysone	Cholesta-7-ene-6-one-3,14,25-triol (3β)
20-hydroxyecdysone	(22R)-2β, 3β, 14, 20, 22, 25-hexahydroxy-5β -cholest-7-en-6-one
2-deoxyecdysone	Cholesta-7-ene-6-one-3,14,20,25-tetrahydroxy (3β)
7-dehydrocholesterol	Cholesta-5,7-dien-3-ol, (3 β)
Cholesterol	Cholest-5-en-3-ol (3 β)
DCM	Dichloromethane
Ecdysone	(2S,3R,5R,9R,10R,13R,14S,17R)-17-[(2S,3R)-3,6-dihydroxy-6-methylheptan-2-yl]-2,3,14-trihydroxy-10,13-dimethyl- 2,3,4, 5,9, 11, 12, 15,16,17-decahydro-1H-cyclopenta[a]phenanthren-6-one .
GC-MS	Gas Chromatography-Mass Spectrometry
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
IS	Internal standard
IUPAC-IUB	International Union of Pure and Applied Chemistry-International Union of Biochemistry

Ketodiol	Cholesta-7-ene-6-one-3,14-diol(3β)-2,22,2-trideoxyecdysone
Lanosterol	Cholesta-8, 24-dien-3-ol, 4, 4, 14- trimethyl,(3β,5 α)
LLE	Liquid–Liquid Extraction
NADPH	Nicotinamide adenine dinucleotide phosphate
ng	nanogram
PG	Prothoracic glands.
QUISTOR	Quadruple ion storage trap mass spectrometer
Rpm	Revolution per minute
SPE	Solid Phase Extraction

ABSTRACT

Ecdysone is a prohormone for 20-hydroxyecdysone, in locusts it is biosynthesized in the prothoracic gland where dietary sterol, in particular cholesterol is sequestered from circulating hemolymph and converted into ecdysone or closely related ecdysteroids via two proposed synthetic pathways. The objective of this work was to a) identify by coupled gas chromatography-mass spectrometry sterols and their trimethylsilyl derivatives present in wheat seedlings, oral secretion, gut, frass, hemolymph, fat bodies and prothoracic glands of the desert locust after feeding on wheat plant, b) propose a pathway for biosynthesis of ecdysone in the locust based on the results. Sterols were identified by comparison of their mass spectral data and those of their trimethylsilyl derivatives with authentic standards if available. Metabolites of cholesterol in the gut were further confirmed through incubation of the gut with cholesterol-[4-^{13}C]. Overall, the distribution of sterols was: wheat 10, oral secretion 15, foregut 15, midgut 15, hindgut 10, frass 10, hemolymph 7, fat body 7 and prothoracic gland 4. This is the first detailed profiling of phytosterols in the rearing diet, body tissues and excreta of locusts. The major discovery from this study is that it identified a metabolite of cholesterol, cholesta-5,7-dien-3-ol,(3β) in the oral secretion, foregut and midgut this compound was initially reported in literature to be synthesized in the prothoracic glands indicating that biosynthesis of ecdysone may start early in the gut system of the desert locust. The incubation studies also revealed other metabolites of cholesterol in the gut system. This study provides opportunities

for further research into sterol profiling in other locust species and ecdysone biosynthesis a knowledge which can be exploited for the desert locust control.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Background information

1.1.1 Ecdysone

Ecdysone is a prohormone of 20-hydroxyecdysone. It belongs to ecdysteroid groups of compounds which are a family of polyhydroxysteroids with a *cis* fused A/B ring junction and 14(hydroxy-7-en-6-one) systems. These compounds exhibit physiological activities in insects and have been found in both invertebrates and plant species (Butenandt and Karlson 1954).

Insects have a rigid exoskeleton (cuticle), which protects them from desiccation and predators. It also acts as the substrate for the development of jointed legs, segments, antennae and wings. Internally, it provides sides that are essential for muscle attachment, leverage and movement. However because the exoskeleton is both rigid and external, insects must periodically shed and replace. This process is called ecdysis, or molting (Gilbert 2004). The molting process is elicited by pulses of the zooecdysteroids, 20-hydroxyecdysone and its precursor ecdysone, and it stops once the insect is fully developed (Thummel 2001; Gilbert *et al*., 2002; Gilbert 2004). 20-hydroxyecdysone is the major molting hormone; however, ecdysone also has morphorgenic functions (Gilbert *et al.,* 2002). The precise mechanism by which 20-

hydroxyecdysone controls or elicits molting and other developmental process is unknown (Warren *et al*., 2006).

The compound, 20-hydroxyecdysone was discovered as the major biologically active insect steroid hormone half a century ago. Much remains to be learned about its biosynthesis and potential use in integrated pest management vis-à-vis its vast role which includes: regulation of molting, effects on polymorphism, regulation of diapause, involvement in reproduction, regulation of metabolic activities and general body functions, regulation of behavior, regulation of preprogrammed cell death, control of harmful insect population, stimulation of reproduction and development of useful species (Thummel 2001; Gilbert *et al*., 2002; Gilbert 2004).

1.1.2 Sites of ecdysone biosynthesis in various organisms

The literature regarding the sites of ecdysteroid synthesis is vast (Rees 1985; Redfern 1989; Delbecque *et al*., 1990). When considering putative ecdysosynthetic tissues it is essential to differentiate between true *de novo* synthesis and release of hormones from stored conjugates (Lafont 1991). Undoubtedly, the prothoracic glands are the major physiologically important source of ecdysteroid during insect post-embryonic development (Rees 1985; Redfern 1989).

A diffusible factor called brain neuropeptide prothoracicotropic hormone (PTTH) stimulates the biosynthesis of zooecdysteroids. PTTH is produced at specific time intervals by a couple of outsized lateral neurons in the brain (corpora allata). Its

release to the hemolymph is under complex control in the brain, which integrates several factors such as time since last molting, nutritional status and physical size. Once the corpus allatum releases PTTH, it stimulates the prothoracic glands to synthesize zooecdysteroids (Gilbert *et al*., 2002).

There is also appreciable evidence of physiological ecdysteroid production in abdomens of the coleopteran, *Tenebrio molitor*; the epidermis apparently functions as a primary source (Delbecque *et al*., 1990). Similarly, the epidermis of the cricket, *Gryllus bimaculatus* can effect ecdysteroid synthesis from [^{14}C] cholesterol in vitro (Hoffmann *et al*., 1992).

In tobacco hornworrn, *Manduca sexta*, marked ecdysteroid synthesis occurs in isolated abdomens devoid of prothoracic glands, testes, or digestive tract, but the identity of the ecdysosynthetic tissue(s) is unknown (Sakurai *et al.,* 1991).

Ecdysteroids have been detected in testes of various species (Koolman *et al.,* 1979), particularly in Lepidoptera and a role in regulation of sperrnatogenesis has been suggested (Loeb *et al.,* 1986). The testes sheath has been implicated as the site of ecdysteroid production under regulation of a brain ecdysotropic factor (Loeb *et al.,* 1988; Loeb *et al.,* 1993). Of various larval tissues of male *Spodoptera littoralis* examined, only the prothoracic glands and testes could effect hydroxylation of [^{3}H]2,22,25-trideoxyecdysone to yield ecdysone (Jarvis *et al.,* 1994a), consistent with, but not establishing, testicular ecdysteroid synthesis. In contrast, in adult female insects, ecdysteroid synthesis has been clearly established in the ovarian follicle cell

epithelium (Golzené *et al.,* 1978; Zhu *et al.,* 1983). The recent demonstration that in several lepidopteran species, 3-dehydroecdysone accompanied by varying amounts of ecdysone, is a major product of the prothoracic glands, being reduced to ecdysone by a haemolymph *reductase* enzyme, has necessitated modification of the classical dogma of hormonal control of post-embryonic development in this order (Warren *et al.,* 1988b; Kiriishi *et al.,* 1990; Grieneisen 1994).

In Crustacea, the Y-organs have been established as an ecdysosynthetic tissue (Lachaise 1990). Although mature ovaries of the shore crab, *Carcinus maenas*, contain high concentrations of ecdysteroids along with potential deoxyecdysteroid precursors (Lachaise *et al.,* 1981), definitive evidence for *de novo* ecdysteroid synthesis in that organ is apparently lacking.

Amongst the Acari, there is a report indicating that the integument is a source of ecdysteroid in nymphs of an argasid tick, *Ornithodoros parkeri* (Zhu *et al.,* 1991). Although this might not be unexpected in view of the epidermal origin of prothoracic glands in insects and the foregoing discussion of ecdysteroid synthesis in insect integument, establishment of *de novo* hormone synthesis by tick integument is awaited.

1.1.3 Biosynthesis of ecdysone in the desert locust

In *Locusta* prothoracic glands, either 5β-ketodiol or 5β-diketol is converted into both ecdysone and 3-dehydroecdysone (scheme 1; Dolle *et al.,* 1991; Roussel 1992). This

is difficult to rationalize, since ecdysone is apparently the sole product of such unlabelled prothoracic glands *in vitro*. However, follicle cells of this species incubated with 5β-ketodiol or 5β-diketol substrates yield ecdysone and 20-hydroxyecdysone after hydrolysis of polar conjugates as expected.

The biosynthesis of ecdysone in the desert locust has been reported to take place in the prothoracic glands where cholesterol from circulating haemolymph is first sequestered and converted into ecdysone or closely related ecdysteroids via two proposed synthetic pathways (Scheme 1, Nation 2002).

The 1st step involves conversion of cholesterol to 7-dehydrocholesterol (**PATH A)** or 3-ketocholesterol **(PATH B)**, this process takes place with the aid of microsomal cytochrome P_{450} monooxygenase requiring NADPH (Gibson *et al*, 1984). The intermediacy of 7-dehydrocholesterol in ecdysteroid biosynthesis, originally suggested by *in vivo* studies (Hom *et al.,* 1974; Milner *et al.,* 1986) has now been firmly established by incubation in vitro of Manduca prothoracic glands (Warren *et al.,* 1988b; Grieneisen *et al.,* 1991); locust follicle-cells and prothoracic glands (Dolle *et al.*, 1990), or crustacean Y-organs (Rudolph and Spaziani 1992) with labelled cholesterol and 7-dehydrocholesterol. Introduction of the Δ^7 bond involves removal of the 7β-hydrogen (Cook *et al.,* 1973) and the cholesterol 7,8-desaturating activity in *M. sexta* prothoracic glands has been shown to be a microsomal cytochrome P450-dependent activity (Grieneisen *et al.,* 1993).

The 7-dehydrocholesterol or 3-ketocholesterol is shuttled from the cytoplasm into the mitochondria where oxidative and hydroxylation steps occur producing a diketol or a ketodiol depending on the sequence of reaction at C-3, mitochondrial membrane shuttle then moves the resulting trideoxyecdysteroid back to the endoplasmic reticulum for hydroxylation at C_{25} by a cytochrome P_{450} enzyme.

Finally the shuttle returns the steroid to the mitochondrial compartment where C_{22} & C_2 hydroxyl groups are added by mitochondrial P_{450}. The end products, depending on the chemistry at C-3, are either 3-dehydroecdysone or ecdysone.

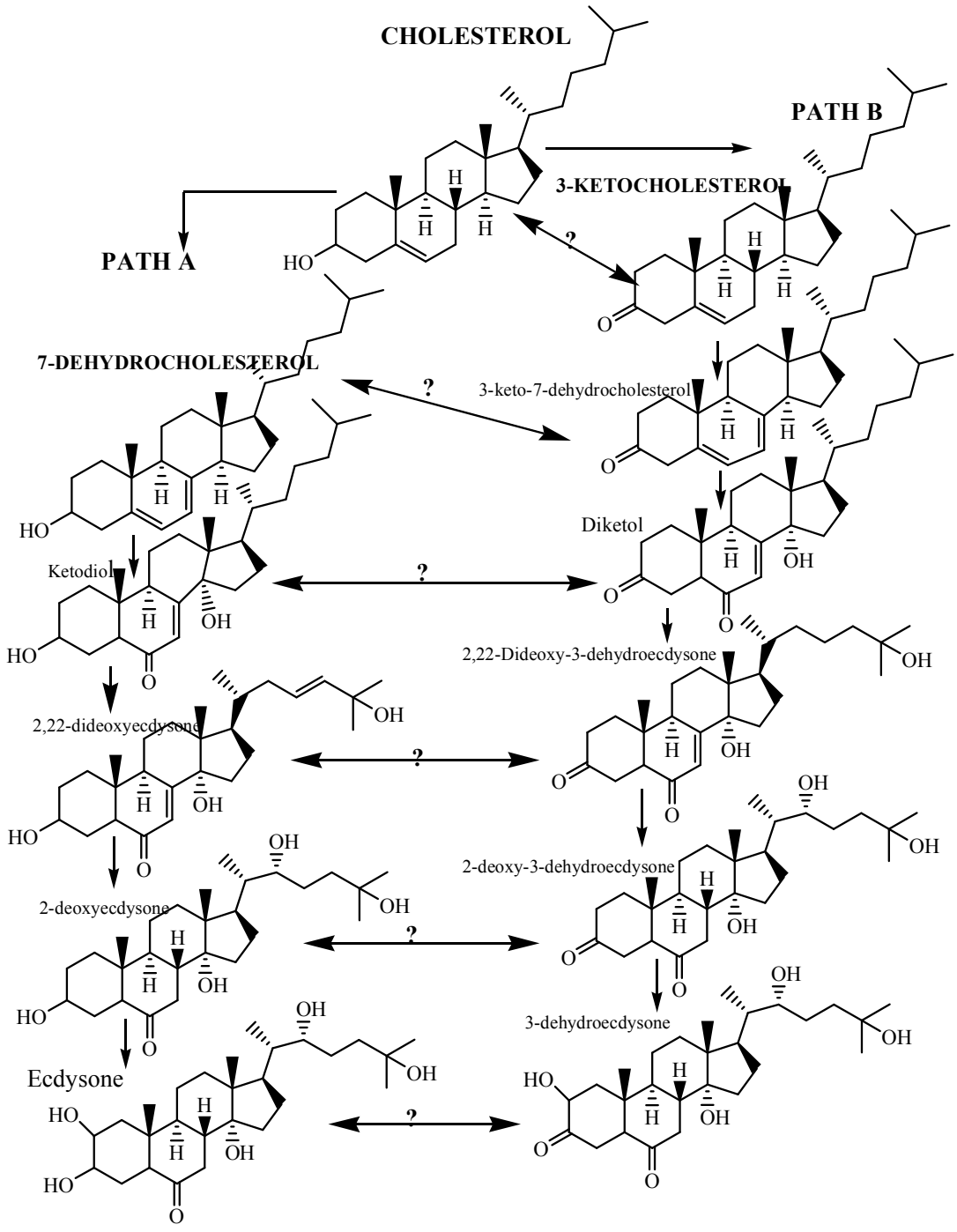
The early steps leading to formation of this compound remain relatively ill-understood, whereas there has been considerable progress in the understanding of the subsequent reactions.

This is largely due to the limited number of sequence permutations of the three hydroxylation involved in conversion of the 5β-ketodiol into ecdysone and the synthesis of high specific radioactivity substrates. At the IVth Ecdysone Workshop held in Strasbourg, the lack of understanding of the initial part of the pathway led Dennis Hom (1974) to designate it the “black box”; unfortunately, despite some progress in that area, the situation is far from clear (Nation 2002).

The prothoracic glands do not store ecdysone, but secrete it into the hemolymph as it is made. Ecdysone appears to have low hormonal activity itself, although this is

difficult to gauge because it is rapidly converted to 20-hydroxyecdysone by the enzyme the enzyme *20-hydoxymonooxygenase* present in most insect tissues.

The malpighian tubules, gut and fat body are especially rich in *20-hydoxymonooxygenase* (Lafont and Koolman 1984). The enzyme is not present in the prothoracic glands. Inactivation by the midgut may protect some insects from ingested phytoecdysteroids (Zhang and Kubo 1993; Blackford *et al.,* 1997). It is also possible that phytophagus insects have a diversity of detoxification pathways that protects them against ingested phytoecdysteroids (Rharrabe *et al*., 2007). Biosynthesis of ecdysone in insects is summarized in Scheme 1, (Nation 2002).



Scheme 1: Two alternative pathways of ecdysone in biosynthesis in insects (Nation 2002)

Arthropods are unable to synthesize ecdysteroids without sterol precursor (cholesterol or sitosterol) in their diet (Gilbert 2004). Herbivorous insects typically obtain sitosterol in their diet, whereas carnivorous insects utilize cholesterol. In either case, cholesterol becomes the precursor of 20-hydroxyecdysone (Warren and Hertu 1990) as plant feeding insects dealkylate sitosterol to cholesterol (Ikekawa *et al*., 1993; Gilbert 2004).

In the following sections, a brief overview is provided on the source of sterols for the insects. A summary is also provided on the bioconversion of C-28, C-29 and C-30 phytosterols to cholesterol (C-27), the biology and economic importance of the desert locust and on the background of gas chromatography coupled to mass spectrometry in structural identification of sterols.

1.1.4 Sources of sterols

Sterols are significant group of primary nutrients for herbivorous insects. They are triterpenoid steroid alcohols that are used by eukaryotic organisms to maintain cell membrane integrity, permeability, and fluidity. Furthermore, they are precursors for steroid hormones and are directly involved in developmental gene regulation (Behmer and Nes 2003). For most insects studied to date, cholesterol**,** (Fig 2) is the primary sterol incorporated into cell membranes and a necessary precursor to steroid hormones search as ecdysteroid in arthropods. However, unlike many other organisms, the inability to synthesize sterols is apparently universal within the phylum Arthropoda and must obtain sterols either directly or indirectly from their

diets (Clayton 1964; Svoboda and Thompson 1985; Rees 1985). Carnivorous insects directly utilize the cholesterol contained from prey's tissues; for herbivorous insects, however, obtaining cholesterol poses a challenge. Plants synthesize hundreds of different sterol molecules (collectively known as phytosterols). Phytosterols share gross structural similarities with cholesterol, but often have ethyl- or methyl groups at carbon position 24 and/or double bonds at positions other than five in the tetracyclic nucleus (B-ring). Consequently, in order to obtain cholesterol from their diet, most herbivorous insects must metabolize ingested phytosterols into cholesterol or other cholesterol-like molecules.

Phytophagous species and omnivorous ones derive cholesterol or related C-27 sterol by dealkylation of dietary plant 24-alkyl sterols such as sitosterol, stigmasterol campesterol, or lanosterol (Fig 2). This ability to dealkylate C-28, C-29 and C-30 phytosterols is not confined to insects and has been demonstrated in members of other diverse invertebrate phyla (Rees 1984). With the known exception of molluscs, the invertebrate species which dealkylate phytosterols also apparently lack *de novo* sterol synthesis (Rees 1984). Certain phytophagous and omnivorous insect species, like some *Hymenoptera, Hemiptera* and *Díptera*, as well as a coleoptera, cannot dealkylate the sterol side chain (Svoboda *et al*., 1994). Many such species utilize campesterol as a precursor for the C-28 ecdysteroid, makisterone A (Fig 1). Furthermore, the C-29 ecdysteroid, makisterone C (Fig 1) has been identified as a major embryonic ecdysteroid in *Dysdercus fasciatus* (Hemiptera).

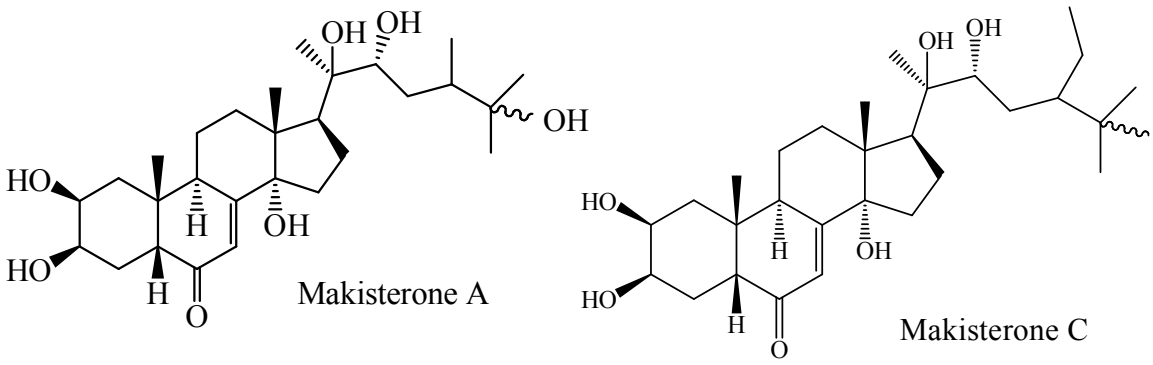
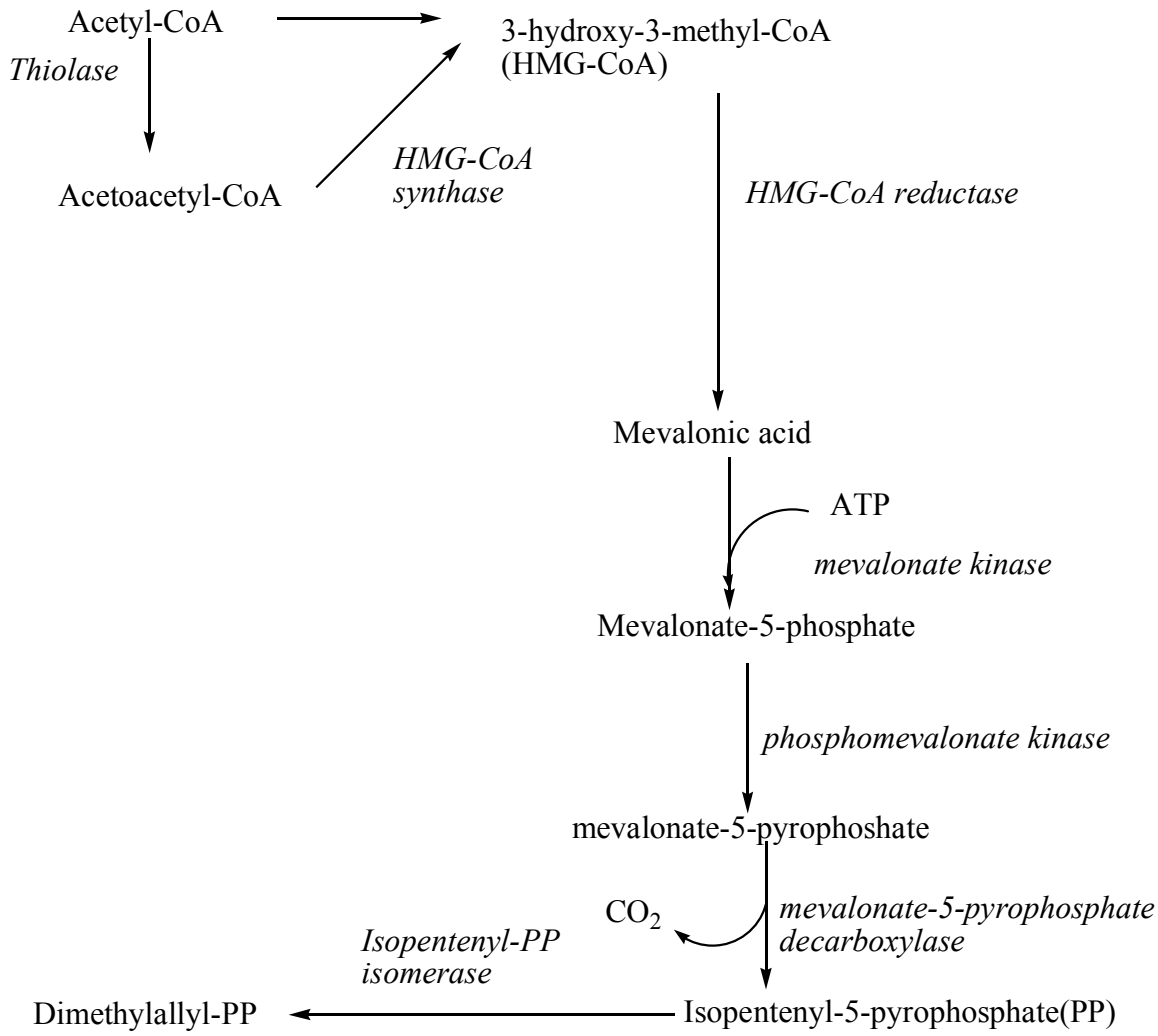


Figure 1: Makisterone A and Makisterone C

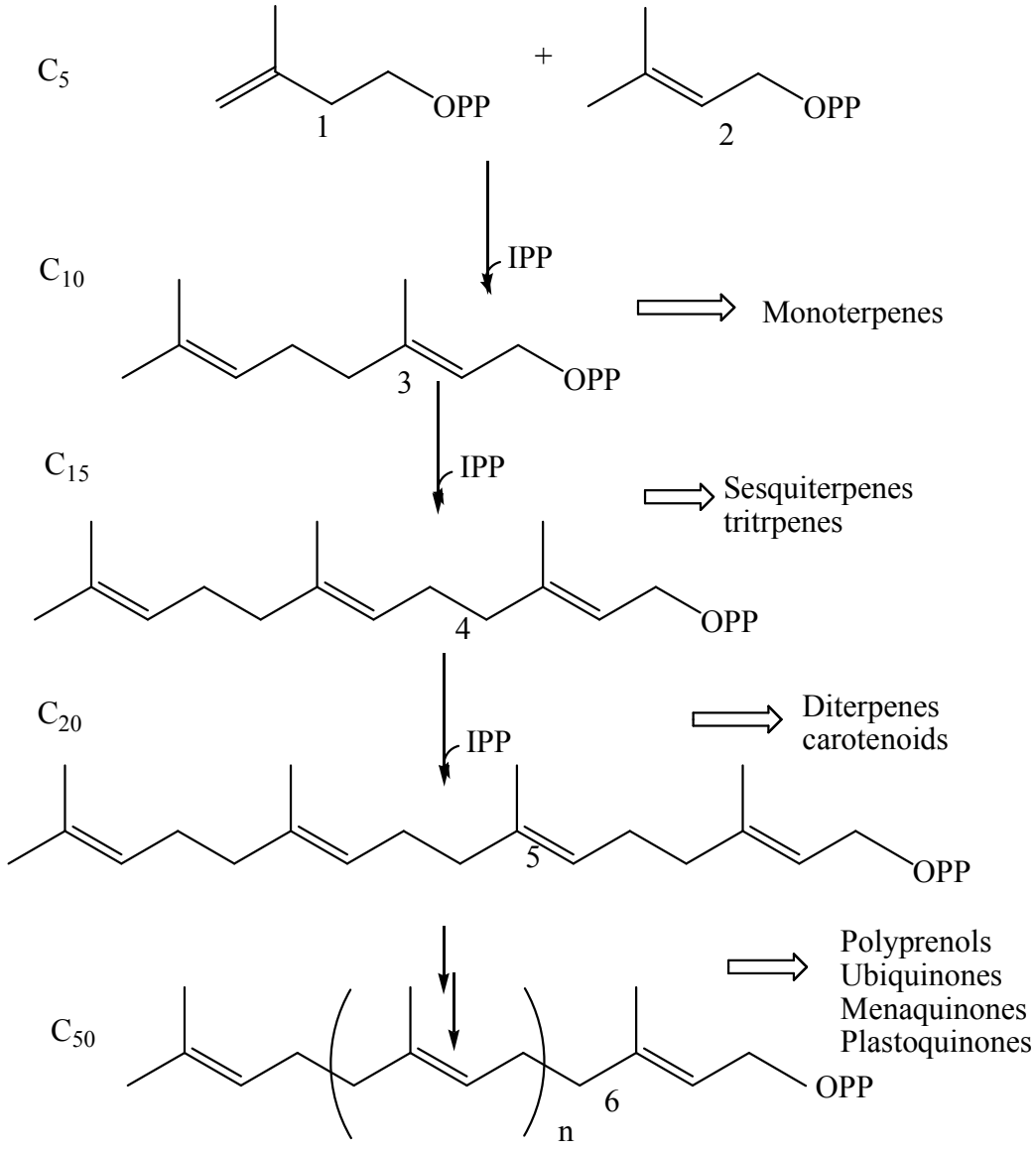
Sterols are terpenes and are biogenetically derived from the isoprene molecule via mevalonate pathway. The structure of the real natural C_5 precursor unit was first discovered by Cornforth (1959) who characterized two forms of isoprene, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) as essential starters for the synthesis of higher plant terpenes. Various enzymes catalyze the incorporation of these units and their intermediates into terpenes (Mann *et a.l,* 1987). The mevalonate pathway is summarized below (Scheme 2) (Ferguson *et al,* 1990).



Scheme 2: Mevalonate pathway (Ferguson *et al,* 1990)

The above biosynthetic pathway (Scheme 2), starting from acetate, activated as acetyl coenzyme A, and yielding IPP 1 (Scheme 3) which is the biological equivalent of isoprene and presents the basic branched C_5 skeleton. 2,3-*prenyltransferases* catalyze the condensation of IPP 1 on to DMAPP 2 (Scheme 3) or on to other prenyl

diphosphates (3 to 6) to give the higher homologues which are the precursors of all isoprenoid series (Scheme 3).



Scheme 3: Biosynthesis of higher isoprenoids from isopentenyl diphosphate 1 and dimethyallyl diphosphate 2: geranyl diphosphate 3, farnesyl diphosphate 4, geranylgeranyl diphosphate 5, polyprenyl diphosphates 6 (Ferguson *et al,* 1990).

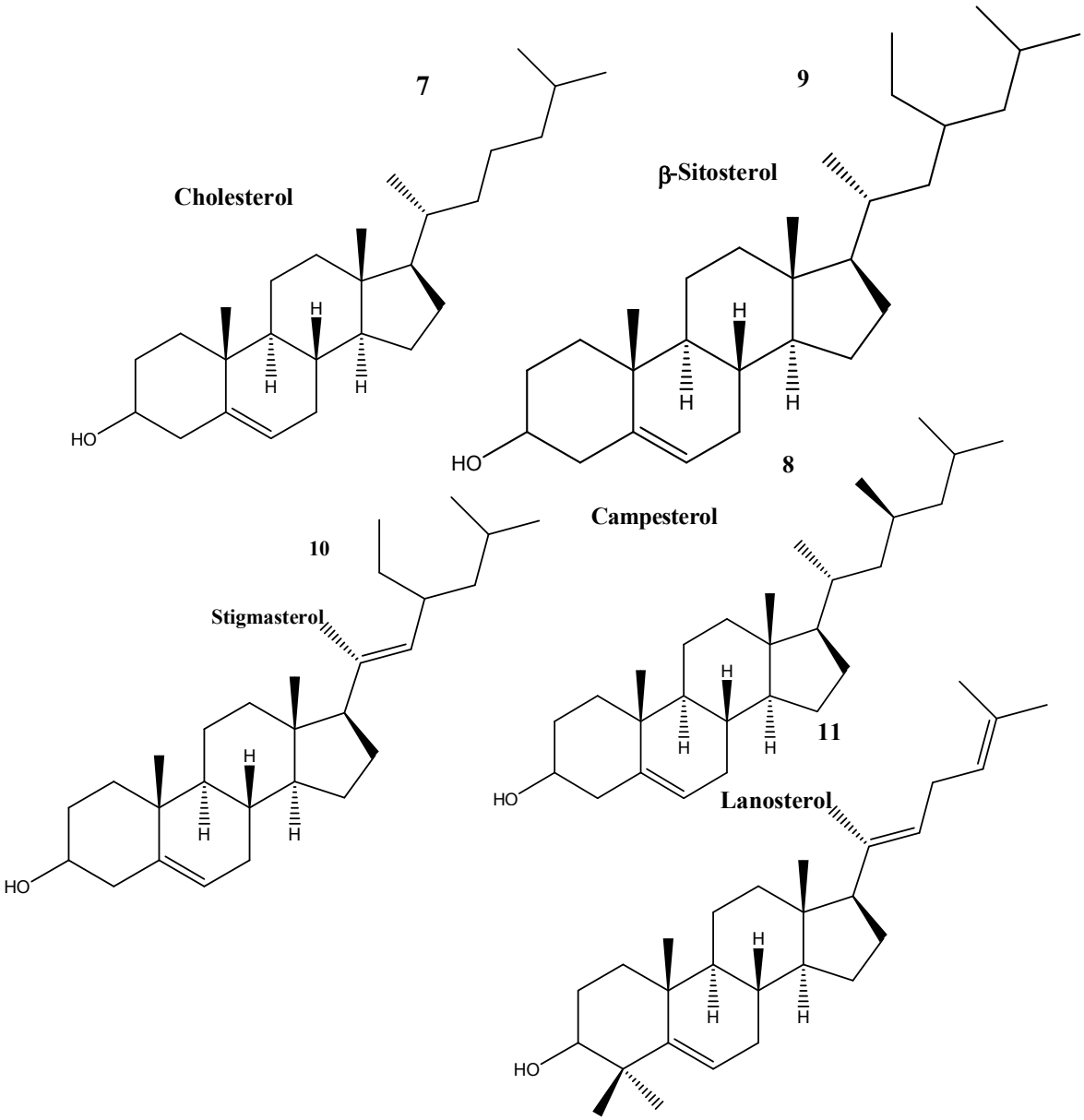
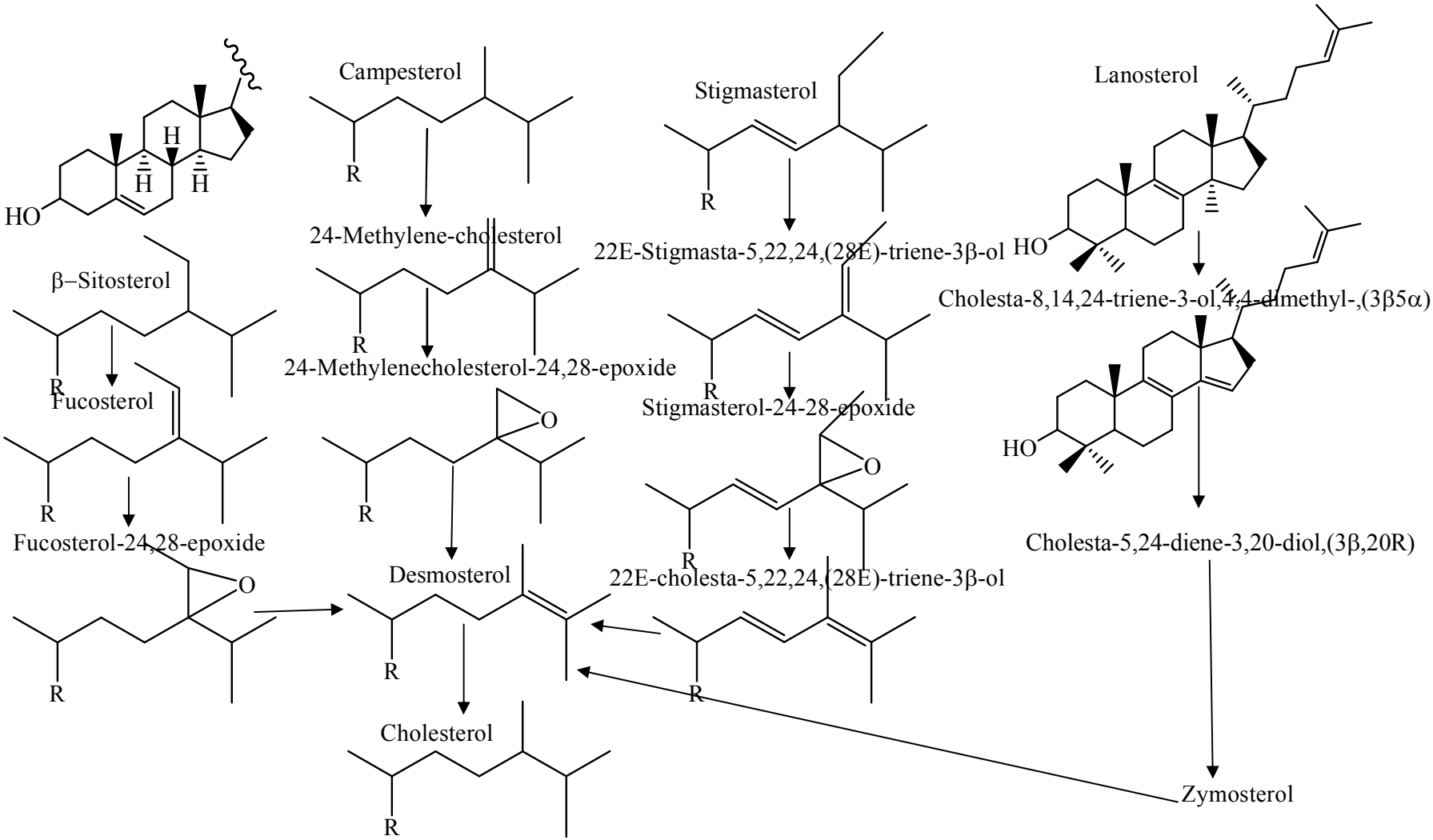


Figure 2: Common phytosterols

Scheme 4 outlines a summary on the bioconversion of C-28, C-29 and C-30 phytosterols to cholesterol (C-27), (Wolf 1994).

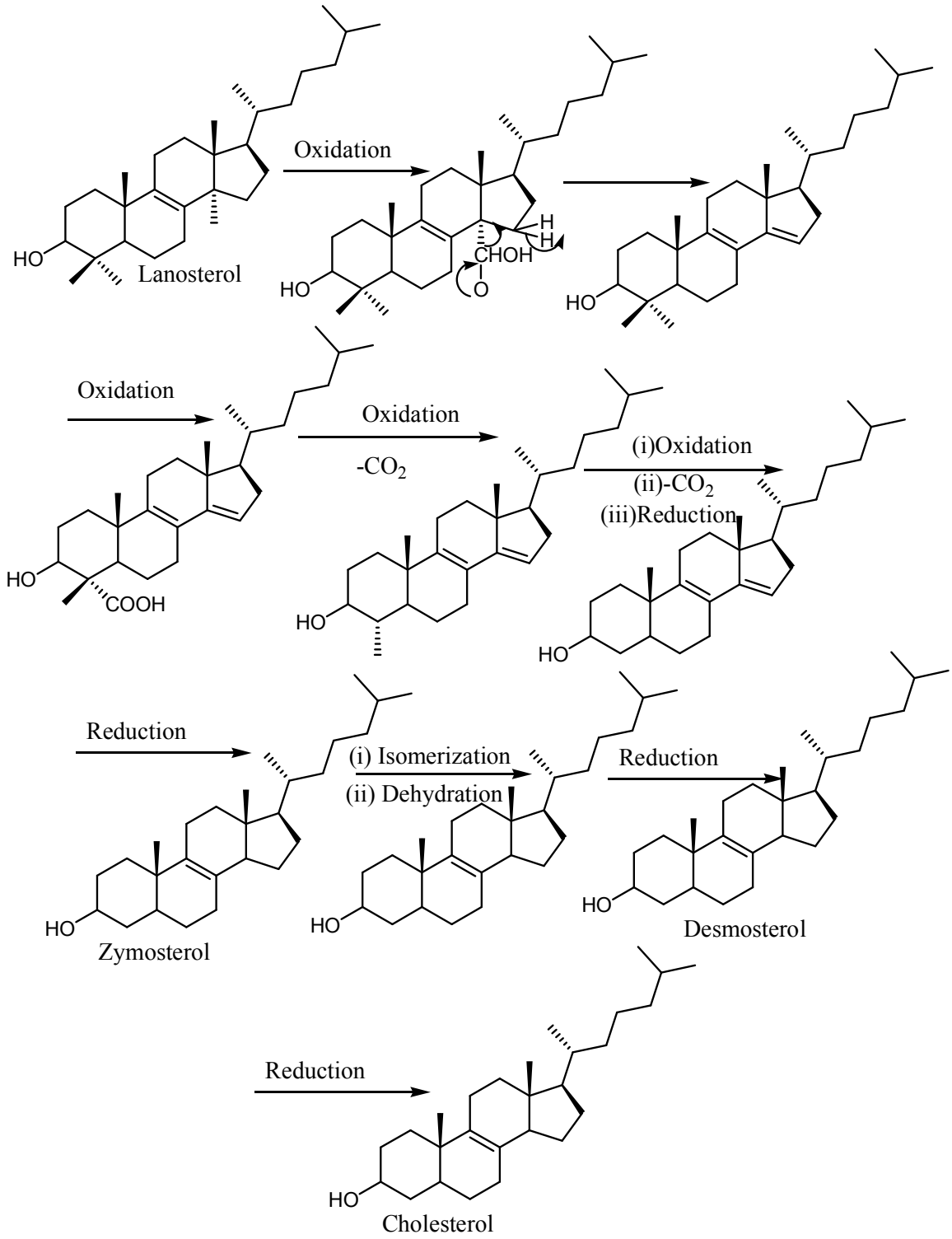


Scheme 4: Pathways of side chain dealkylation of phytosterols in Insects (Wolf, 1994)

1.1.5 Formation of cholesterol from lanosterol

The conversion of the C-30 compound, lanosterol to C-27 steroid, cholesterol, involves oxidative removal of a 4α-, 4β- and 14α- methyl groups. 14α- methyl group is eliminated first and the replacement by hydrogen proceeds with retention of configuration. After oxidation to a 3-ketone, the 14α- methyl group is eliminated. The 4β-methyl group, after the loss of 14α- methyl group, adopts the α-orientation on equilibration of C-4, which is a more stable equatorial substituent. The methyl groups are eliminated after oxidation up to the aldehyde level at 14α, and to the carboxyl level for the 14α- methyl, thus being eliminated respectively as formic acid and carbon dioxide. The oxidations proceed stepwise (methyl to primary alcohol to aldehyde to acid) and are catalyzed throughout by mixed function *microsomal oxygenases* (Svoboda *et al.,* 1994). The decarboxylations are favoured by the presence of a neighboring ketone group at position β- to the point of attack. Thus C-3 is alternatively oxidized to carbonyl and reduced to alcohol during the demethylation process (Scheme 4), (Svoboda *et al.,* 1994)

The next step is reduction of Δ^{14}-double bond in which the 14α-H is derived from NADPH and 15β-H from the medium to form zymosterol. The transfer of Δ^{8} to Δ^{5} double bond involves the isomerisation from Δ^{8} to Δ^{7} double bond. The Δ^{5}-double bond is introduced by dehydrogenation of the Δ^{7}-steroid to the $\Delta^{5,7}$-diene. In the final step the Δ^{7}-double bond is reduced by the two proteins enzyme to form desmosterol. In the last step, the Δ^{24}-double bond of desmosterol is reduced by a trans- process to form cholesterol. (Scheme 5), (Manitto *et al.,* 1985).



Scheme 5: Formation of cholesterol from lanosterol (Manitto *et al,* 1985

1.2 The desert locust

Locusts are members of the grasshopper family Acrididae, (FAO 1992) and their species include: migratory locust (*Locusta migratoria*), red locust (*Nomadracis septemfasciata*), Australian plague locust (*Chortoicetes terminifera*), American desert locust (*Schistocerca americana*), desert locust (*Schistocerca gregaria*) and rocky Mountain locust (*Melanoplus spretus*) in North America had some of the largest recorded swarms, but mysteriously died out in the late 19th century.

For this study, the Desert locust is of special interest because:

1. It is polyphagous and has a wide range of food plants hence pose a threat to food security (Uvarov 1977; Steedman 1988).
2. It consumes daily between the equivalents of half their own weight (which varies from 100 mg for the nymph, 2-3 g for the adult (Steedman 1988; Meinzingen 1993).
3. In the gregarious phase, locusts form dense bands of nymphs and swarms of adults. A typical swarm of locust can be of 300 km^2 in size with 40 million individuals per km^2 (Steedman 1988).
4. The mature adults of *Schistocerca gregaria* can fly up to 1000 km a week (Steedman 1988; Meinzigen 1993).

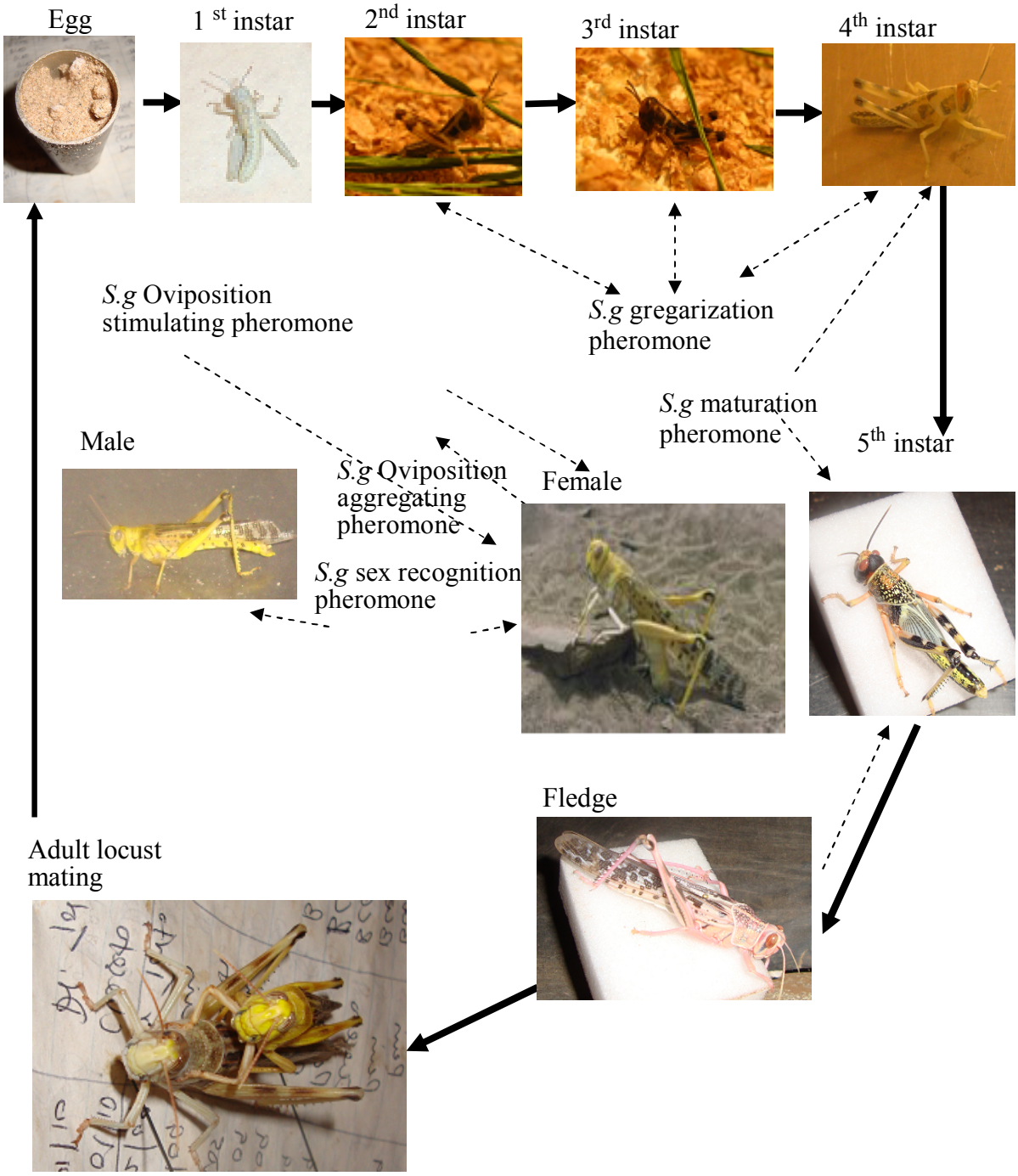
Scientific classification of the desert locust

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Orthoptera
Suborder	Caelifera
Super family	Acridoidea
Subfamily	Cyrtacanthacridinae
Family	Acrididae
Genus	*Schistocerca*
Species	*gregaria*

Plate 1: Desert locust (Photo: by Cheseto)

1.2.1 The biology of the locust

The life cycle of the desert locust comprises of three stages namely; egg, hopper, and adult. Experimental evidence of pheromone effects on various stages of locust are indicated by dashed lines and arrows in Scheme 6.



Scheme 6: Life cycle of the gregarious desert locust

The development of locusts follow either solitary or gregarious pathway, which differ from each another in the developmental rate, morphology, behavior, viability, and reproductive potential of the insects (Uvarov 1966).

Major ecdysteroids have been identified and their haemolymph titres quantified in the gregarious phase of both *S. gregaria* and *L.migratoria* (Morgan *et al.*, 1975; Morgan and Poole 1974; Gande *et al*., 1979; Baehr *et al.*, 1979; Hirn *et al.*, 1979). It is possible that diverse ecdysteroid titres affect the morphological, behavioral, and other phase characters indirectly, via changes in instar duration. Other effects include control the production of aggregation pheromones that were recently identified in the gregarious phase of *S. gregaria* (Torto *et al*., 1996).

The gregarious immature desert locust adults are pinkish, lighter, or darker depending upon whether the locusts have bred under high or low temperature, respectively. The immature adults of solitarious locusts are usually pale grayish or beige. The soil temperature influences the incubation period of eggs. In summer, it takes 10-14 days, while in cold weather the incubation can take 60-80 days. Hatching takes place either shortly before or within 3 hours after sunrise, and all the hoppers from one egg pod normally hatch on the same morning. When hatching is over and hoppers are a day old, they start to feed.

The gregarious nymphs undergo four molts, thus five instars, while the solitarius ones undergo five molts (six instars). Gregarious nymphs have black coloration on yellow or orange background, while solitarius individual are green and become

brown in the last two instars. In favorable conditions, the larval development duration is 25 and 30 days, respectively, for *gregaria* and *solitaria* nymphs .The last hopper molts into fledges and finally adults (Uvarov 1966).

1.2.2 Locust control methods

The gregarious desert locust is a serious pest of both cultivated and wild plant species (Uvarov 1966; Steedman 1988). Various methods have been used or tested for the control of locusts, the methods include: traditional methods, natural enemies, chemicals, insect growth regulators (IGRs), botanicals and semiochemicals

1.2.2.1 Traditional methods

Traditional locust control methods are still in use and they include: ploughing the areas infested with egg pods, crop rotation, cultivating grasslands, fallowing agricultural land, postponement of planting, burning roosting locusts at night, herding hopper band into deep trenches and burying them (Han 1997), crushing the locust early in the morning while still roosting (Anon 1990) and exploiting locusts as food.

1.2.2.2 Natural enemies

Enemies including insects, birds, nematodes, reptiles, mammals, and pathogens attack locusts (Steedman 1988). Amongst locust egg parasites and predators are; *Salio spp*. (Hymenoptera: Scelinonidae), *Stromorhina lunata* (Diptera: Calliphoridae), *Systoechus somali* (Diptera: Bomlyliidae) and Troxpropcerus (Coleoptera: Trogidae). Insects such as *Blaesoxipha filipjeui* (Diptera: Sarcophagidae), *Symmictus costatus* (Diptera: Nemestrinidae) are ecto-parasites of

desert locust hoppers and adults (Hinks and Ewen 1986). These enemies are not numerous enough to reduce the locust population significantly, but can locally play an important role in reducing the number of *S. gregaria* and *Locusta migratoria* eggs and/ or individuals.

Use of entomopathogens in biological control started more than a century ago (Prior and Greathhead 1989). The efficacy of several entomopathogenic agents on desert locusts have been evaluated including protozoan *Melamoeba locustae* (Hinks and Ewen 1986) and fungi such as *Beaveria bassiana* (Johnson and Groettel 1986). The *Metarhizium spp*. has proven most effective for locust control (Milner, Staples and Prior 1996).

1.2.2.3 Use of synthetics

After the Second World War, synthetic insecticides became the sole solution to control of the desert locust; in particular, the long lasting chlorinated hydrocarbons. They were used as bait and/or spray.

After a few decades of extensive use of these organochlorine pesticides, many drawbacks were identified, such as contamination of the environment and toxicity to non-target organisms (predators, ecto-parasites, and pollinators) harmful residues in food, pesticide resistance, and bioaccumulation in the food chain.

New synthetic pesticides with shorter periods of persistence, namely organophosphates, carbamates, and pyrethroids appeared more suitable, but with

these, blanket coverage applications were necessary, thus causing negative impacts on the environment and increased cost of control. Five main groups of chemical pesticides have been used in locust control programs:

1. **Organochlorines**: this group includes Dieldrin and BHC (benzene hexachloride). Its main characteristic feature is that the chemicals are highly persistent in the environment and are very effective against adult locusts and hoppers. They act by contact and as stomach poisons (FAO 1992). Until 1986 Dieldrin and BHC were used for the control of the desert locust. But their use has since been abolished.
2. **Organophosphates**: this group includes Malathion, Sumithion and Diazinon. The members of this group are known to have less residual effect than the organochlorine pesticides. They also have fast knockdown effect and act as contact poisons FAO 1992).
3. **Carbamates**: this group includes Bendiocarb, Carbaryl, Propoxur, and others. This group is very similar to the organophosphates group because insecticides have fast knockdown effect but short residual effect and they act both as contact and stomach poisons (FAO 1992).
4. **Synthetic pyerthroids**: this group includes Decis, Cypermethrin and Deltamethrin, which are considered relatively safe, compared to the other commonly used locusticides. They are also known to have rapid and fast knockdown effect on insects and act as contact poisons (Paster *et al.* 1989).

5. **Phenyl pyrazoles**: this is a relatively new group, which includes Fipronil which has a reasonably long residual effect and acts as a contact and stomach poison, and is considered to be relatively safe on non-target organisms (FAO 1989).

1.2.2.4 Insect growth regulators (IGRs)

Benzoylphenyl ureas are synthetic growth regulators that disrupt the molting process of insects by inhibiting chitin synthesis (Nasseh *et al,* 1992; Wilps and Nasseh 1994). This often leads to deformation of treated nymphs. They also adversely affect insect metabolism and oogenesis, hence can be employed in intergrated pest management strategy.

1.2.2.5 Semiochemicals

A semiochemical or infochemical is a chemical that in the natural context conveys information between two individuals, evoking in the receiver a behavioral or physiological response that is an adaptive to or the interactants or both (Dicke and Sabelis 1988). Depending on the source and the recipient, there are several groups of semiochemicals: pheromones, kairomones, allomones and synomones.

Bashir and Hassanali (2010) explored the effects of longer-term contact of field gregarious hopper bands and laboratory crowd-reared nymphs with the major constituent of the adult pheromone. They observed that during the first few days, hoppers in treated bands became relatively hyperactive. Their movements became random and they stopped marching as coherent groups, they started to roost for longer periods on vegetations, and they fragmented into smaller and smaller

groupings and individuals. When attacked by birds, they demonstrated subdued levels of collective defensive behavior compared to normal hoppers, and there were clear signs of increased predation and cannibalism at the roosting sites. In cage experiments, crowd-reared nymphs treated with the pheromone component behaved the same as above. Their observations showed that the major adult pheromone constituent has a solitarising effect on gregarious hoppers; the ideas generated in their study can be capitalized in locust control.

1.3 Structural characterization of sterols

Sterols are steroids carrying a hydroxyl group at C-3 (Fig 3). Steroids are compounds possessing the skeleton of cyclopenta[a]phenanthrene or a skeleton derived there from by one or more bond scissions or ring expansions or contractions. Methyl groups are normally present at C-10 and C-13. An alkyl side chain may also be present at C-17. Sterols are numbered and rings are lettered as in Figure1. If one of the two methyl groups attached to C-25 is substituted it is assigned the lower number (26); if both are substituted, that carrying the substituent is cited first in the alphabetical order is assigned the lower number.

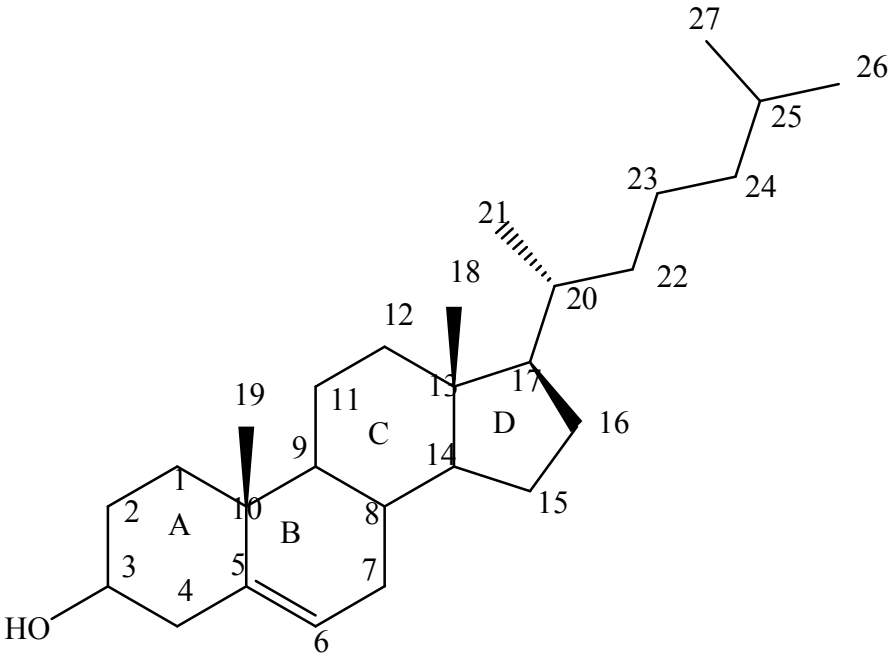


Figure 3: Numbering and ring letters of sterols (IUPAC –IUB 1976)

1.3.1 Gas chromatography - mass spectrometry (GC-MS)

Gas chromatography-Mass chromatography (GC-MS) is the synergistic combination of two powerful analytical techniques. The GC separates the components of a mixture in time and the MS provides information that aids in structural identification of each component (Fulton *et al* 1996).

GC-MS in the present work has been used as a technique for identification and quantification of sterols from various parts analyzed. This is because the mass spectra of the various components eluting during GC separation are recorded, and the mass spectrum of a compound is characteristic of the identity of the compound confirmed by derivatization. So long as there is a library equipped with the mass spectra of all

the compounds, any sterol in the sample analyzed can be identified. Hence identification is not limited to the availability of authentic samples.

1.3.2 Sample Introduction

Once the GC separates the constituents of the original sample, the individual components enter the mass spectrometer through an interface between the GC and the MS. The MS is held at extremely low pressure by use of a specialized vacuum which is coupled to the MS manifold. Within the manifold is housed the ion source. As the compounds elute from the GC column, the stream of molecules enters this source, where a metallic filament discharges electrons into the oncoming path (Raymond 2003). Several different interface designs are used to connect these two instruments as listed below:

1.3.3 Interfacing techniques

Interfacing techniques include:

- Direct interface: The end of GC column is fitted directly into the ion source
- Open slit interface: The capillary transfer column is split in the middle and purge gas used to introduce the effluent to MS.
- Molecular jet separator: The GC carrier gas passes through a jet and expands into a partial vacuum. Only the heavier molecules pass through into the capillary jet.

- Effusion separator: Consist of a tube through which the carrier gas flows at slightly reduced pressure. It then diffuses through the porous material and is removed.
- Diffusion separator: The carrier gas passes through a thin membrane supported on a fine metal mesh .The inorganic carrier gas passes onto the exit while organic analyte is attracted to the membrane and diffuses through to the low pressure ion source.

The most common GC/MS interface now uses a capillary GC column. Since the carrier gas flow rate is very small for these columns, the end of the capillary is inserted directly into the source region of the mass spectrometer. The entire flow from the GC enters the mass spectrometer.

1.3.4 Ion Sources

A variety of ionization techniques are used for mass spectrometry. Most ionization techniques excite the neutral analyte molecule which then ejects an electron to form a radical cation (M^{+})*. Other ionization techniques involve ion molecule reactions (MH+)** The most important considerations are the physical state of the analyte and the ionization energy. Electron ionization and chemical ionization are only suitable for gas phase ionization. The ionization energy is significant because it controls the amount of fragmentation observed in the mass spectrum. Although this fragmentation complicates the mass spectrum, it provides structural information for the identification of unknown compounds (Ernnst 2004).

1.3.4.1 Electron ionization

The electrons used for ionization are produced by passing a current through a wire filament. The amount of current controls the number of electrons emitted by the filament.

An electric field accelerates these electrons across the source region to produce a beam of high energy electrons. When an analyte molecule passes through this electron beam, a valence shell electron can be removed from the molecule to produce an ion. EI produces positive ions by knocking a valence electron off the analyte molecule. As the electron passes close to the molecule the negative charge of the electron repels and distorts the electron cloud surrounding the molecule. This distortion transfers kinetic energy from the fast-moving electron to the electron cloud of the molecule.

If enough energy is transferred by the process; the molecule will eject a valence electron and form a radical cation (M^+). A mass spectrum is produced by ionizing many molecules; the spectrum is a distribution of the possible product ions. Intact molecular ions are observed from ions produced with little excess energy. Other molecular ions have more energy and undergo fragmentation in the source region. The abundance of the resulting fragments, often called product ions is determined by the kinetics of the fragmentation pathways and the ionization energy. This distribution provides the structural information for interpreting mass spectra

1.3.4.2 Chemical Ionization

Chemical Ionization (CI) is a "soft" ionization technique that produces ions with little excess energy. As a result, less fragmentation is observed in the mass spectrum. Only slight modifications of an EI source region are required for CI experiments. In Chemical Ionization the source is enclosed in a small cell with openings for the electron beam, the reagent gas and the sample. In the CI source, analyte molecules undergo many collisions with a reagent gas which is ionized with an electron beam to produce a cloud of ions. The reagent gas ions in this cloud react and produce adduct ions which are excellent proton donors (Ernst 2004).

When analyte molecules (M) are introduced to a source region with this cloud of ions, the reagent gas ions donate a proton to the analyte molecule and produce MH^+ ions. The energetic proton transfer is controlled by using different reagent gases. The most common reagent gases are methane, isobutene and ammonia. Methane is the strongest proton donor commonly used. For softer ionization, isobutane and ammonia are frequently used. The reagent gas must be a strong enough Brønsted acid to transfer a proton to the analyte. Fragmentation is minimized in CI by reducing the amount of excess energy produced by the reaction. Because the adduct ions have little excess energy and are relatively stable, CI is very useful for molecular mass determination.

Other ionization techniques include: field ionization, fast atom bombardment and liquid secondary ion mass spectrometry, field desorption, plasma desorption, laser

desorption, matrix-assisted laser desorption ionization, thermospray, atmospheric pressure ionization, electrospray, atmospheric pressure chemical ionization, atmospheric pressure photoionization, atmospheric pressure secondary ion mass spectrometry; (desorption electrospray ionization, direct analysis in real time), inorganic ionization sources; (spark source, glow discharge source, inductively coupled plasma source) and gas-phase ion-molecule reactions.

1.3.5 Mass Analyzers

After ions are formed in the source region they are accelerated into the mass analyzer by an electric field. The mass analyzer separates these ions according to their m/z value. The selection of a mass analyzer depends upon the mass range, scan rate and detection limits required for an application. There are two common mass analyzers or separators commercially available for GC/MS these are: quadruple analyzer and the ion trap analyzer.

1.3.5.1 The Quadruple

The quadruple mass spectrometer is the most common mass analyzer. Their compact sizes, fast scan rate, high transmission efficiency, and modest vacuum requirements are ideal for small inexpensive instruments. Most quadruple instruments are limited to unit m/z resolution. In the mass spectrometer, an electric field accelerates ions out of the source region and into the quadruple analyzer. The analyzer consists of four rods or electrodes arranged across from each other (Scott 1998).

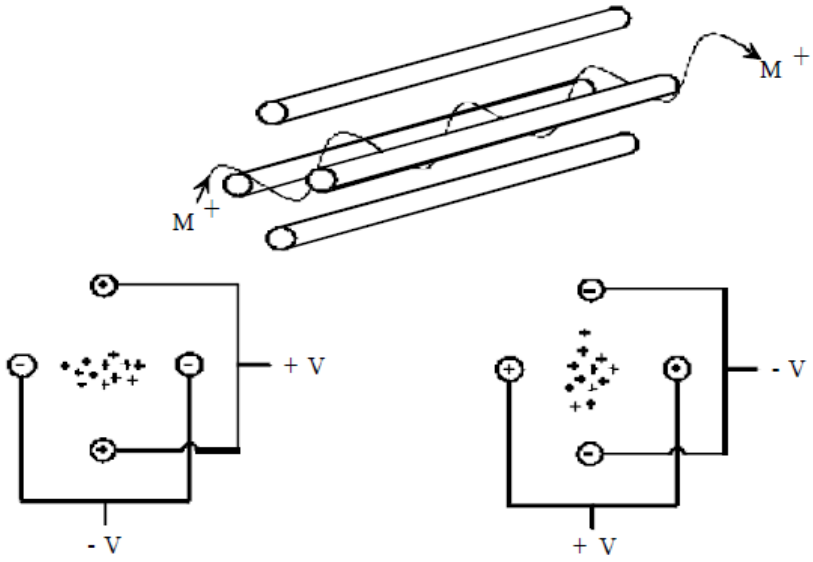


Figure 4: Quadruple Mass Analyzer

As the ions travel through the quadruple they are filtered according to their m/z value so that only a value ion can strike the detector single m/z. The m/z value transmitted by the quadruple is determined by the Radio Frequency (RF) and Direct Current (DC) voltages applied to the electrodes. These voltages produce an oscillating electric field that functions as a band pass filter to transmit the selected m/z value. The RF voltage rejects or transmits ions according to their m/z value by alternately focusing them in different planes. The four electrodes are connected in pairs and the RF potential is applied between these two pairs of electrodes. During the first part of the RF cycle the top and bottom rods are at a positive potential and the left and right rods are at a negative potential. This squeezes positive ions into the horizontal plane.

During the second half of the RF cycle the polarity of the rods is reversed. This changes the electric field and focuses the ions in the vertical plane. The quadruple

field continues to alternate as the ions travel through the mass analyzer. This causes the ions to undergo a complex set of motions that produces a three dimensional wave. The quadruple field transmits selected ions because the amplitude of this three dimensional wave depends upon the m/z value of the ion, the potentials applied, and the RF frequency. By selecting an appropriate RF frequency and potential, the quadruple acts like a high pass filter, transmitting high m/z ions and rejecting low m/z ions.

1.3.5.2 Quadruple Ion Trap.

The Quadruple ion storage trap mass spectrometer (QUISTOR) is a recently developed mass analyzer with some special capabilities. Several commercial instruments are available and this analyzer is becoming more popular. QUISTORs are very sensitive, relatively inexpensive, and scan fast enough for GC/MS experiments. The sensitivity of the QUISTOR results from trapping and then analyzing all the ions produced in the source.

Other analyzers include: electrostatic trap or 'orbitrap', time-of-flight analyzers, linear time-of-flight mass spectrometer, magnetic and electromagnetic analyzers, and ion cyclotron resonance and fourier transform mass spectrometry

1.3.6 Ion Detection

Detection of ions is based upon their charge or momentum. For large signals a faraday cup is used to collect ions and measure the current. Most detectors currently used amplify the ion signal using a collector. The gain is controlled by changing the

high voltage applied to the detector. A detector is selected for its speed, dynamic range gain, and geometry. Some detectors are sensitive enough to detect single ions (Thomas 1998).

1.3.7 Library analysis

The most popular computerized method for determining the structure of unidentified, but not novel compound is to search through a library of low-resolution mass spectra for a match between its spectrum and that of the compound in the library usually stores as many as 50,000 electron impact mass spectra (Rose and Johnstone 1982).

The matching routine involves calculation by the data system of a similarity index, match factor or purity between the unknown spectrum and the library (reference) spectra. Common scales are 0 for complete mismatch to 1, 100 or 1000 for a perfect match. To avoid time-consuming calculations for the purity against all entries in a large library, there is usually a pre-search or a filter which rapidly eliminates dissimilar spectra. Often at each *m/z* value in turn, the ratio (R) of the mass peak heights in the normalized unknown and reference spectra is calculated. The individual R values are summed and the average value is normalized to produce the final purity for a particular reference spectrum.

The entries with the highest figures are displayed as the results of the library search. The entries are ranked in order of highest purity, and the fit values for each match calculated. Whatever the fit or purity values, the result of library search should be assessed by careful visual examination of the matching spectra when presented for

the highest ranked library entry. All peaks in the reference spectrum should be present also in the spectrum of the sample.

1.4 Statement of the Problem

Ecdysone and in particular 20-hydroxyecdysone is a potential insect growth regulator and its biosynthetic pathway in the locust fed on wheat seedling is not well known. GC-MS studies of biosynthesized metabolites in the mouth and gut of the desert locust, fed on wheat seedling, will help understand 20-hydroxyecdysone biosynthesis.

1.5 Justification

The biosynthetic pathway for ecdysone in the desert locust is believed to follow two paths A and B (Scheme 1, p 8). Major ecdysteroids have been identified and their haemolymph titres ascertained in the gregarious phase of both S. *gregaria* and *L. rnigratovia* (Morgan *et al.,* 1975; Morgan and Poole 1974; Gande *et al*., 1979; Baehr *et al.*, 1979; Hirn *et al.*, 1979). It is possible that diverse ecdysteroid titres affect the morphological, behavioral, and other phase characters only indirectly, via changes in instar duration. Other effects have also been suggested for example, ecdysteroids could control the production of aggregation pheromones that were recently identified in the gregarious phase of *S. gregaria* (Torto *et al.,* 1994). To improve our understanding of the role of ecdysone in the desert locusts its biosynthetic pathway must first be ascertained. The results from this study would serve as a basis for similar studies in other insects and can further be exploited for locust control.

1.6 Hypothesis

All the sterols found in the wheat plants are converted to cholesterol by the desert locust *Schistocerca gregaria* and path A (scheme 1, p 8) is the preferred pathway for the biosynthesis of ecdysone.

1.7 Objectives

1.7.1 General objective

The aim of the proposed project is to identify the preferred pathway for ecdysone synthesis in desert locusts by GC-MS

1.72 Specific objective

- To identify sterols found in wheat seedlings and in the oral secretion, gut, frass, hemolymph, fat body and prothoracic gland of the desert locust after feeding on wheat plant by GC-MS.
- To propose a pathway for biosynthesis of ecdysone in the desert locust based on the results from the above studies

CHAPTER TWO

2.0 Materials and methods

2.1 General experimental procedures

All recyclable glass-wares used were washed in hot water and soap and rinsed with distilled acetone and dried at 110^0C for at least 12 hour.

2.2 Reagents and reference compounds

All the solvents and reference compounds used were obtained from Aldrich Chemical Co. ltd., California, USA, unless otherwise stated. Reagents used had purities ranging 95-100% and were used as supplied by Aldrich Chemical Co. Ltd., California, USA unless otherwise stated. Standard compounds cholesterol was purchased from BDH chemicals England, lupeol was a gift from B. Torto *(icipe)* a compound, which he isolated from a plant *Fagara tessmanii*. Hydrosil was purchased from chrompack, Holland. Cholesterol-[4-^{13}C] was a gift from Peter E.A Teal (USDA) a compound, which he purchased from CND-isotopes Pointe-Claire, Quebec, Canada.

2.3 Camera

All Plates were taken at *icipe* using Canon Powershot A530 digital camera, USA.

2.4 Study Area

Laboratory study was carried out at the International Centre of Insect Physiology and Ecology (*icipe*) campus in Nairobi (B Fig 5) (01^{o} 13' 25.3" S, 36^{o} 53' 49.2"E), at ~ 1600 m above sea level (Kenya Web, http://www.kenyaweb.com).

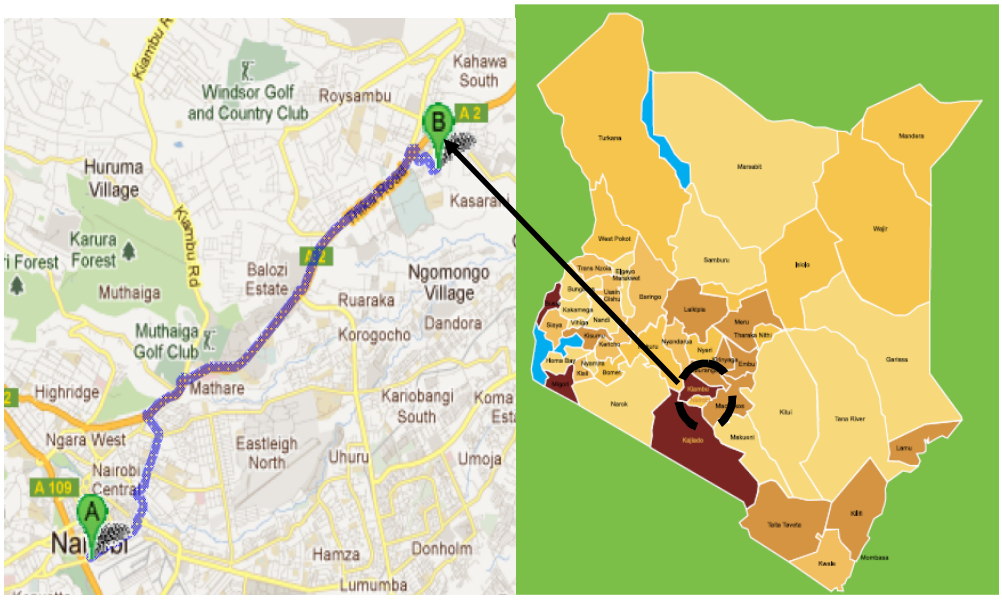


Figure 5: Map of Kenya showing icipe in Nairobi County (Google maps 2012)

2.5 Rearing of locusts

The gregarious desert locust used in this study, were obtained from *icipe* colony which originated from a stock colony reared by the Desert Locust Organization for Eastern Africa (DL CO-EA) in Sudan. Two insect cages (Plate 4) were thoroughly cleaned using hot water and soap and allowed to dry before the introduction of sand which contained egg pods of gregarious desert locust (Plate 2 and 3) .

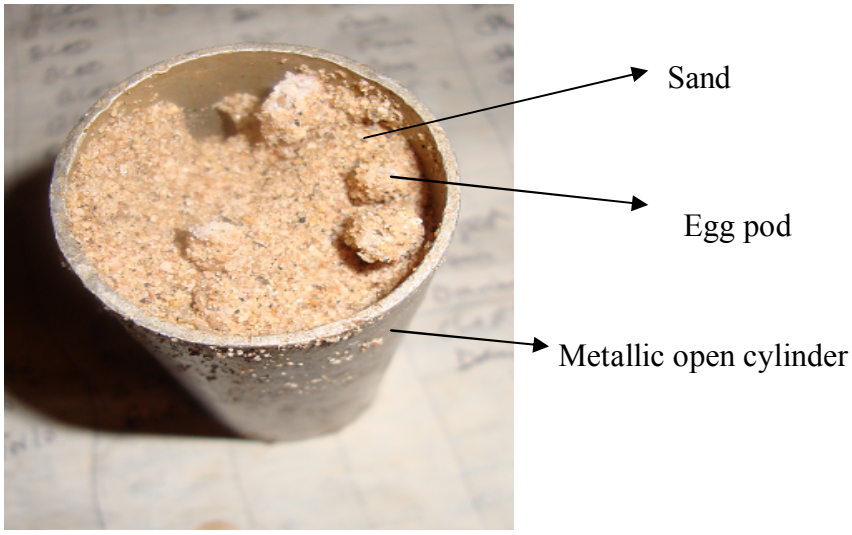


Plate 2: Sand plus eggs of gregarious desert locust (Photo: by Cheseto)

Plate 3: Hoppers emerging from sand (Photo: by Cheseto)

The gregarious desert locusts were reared in a cage (Plate 4), on a diet of wheat seedling and wheat bran at 28-37^{0}C, 50% humidity and 12:12 h photoperiod.

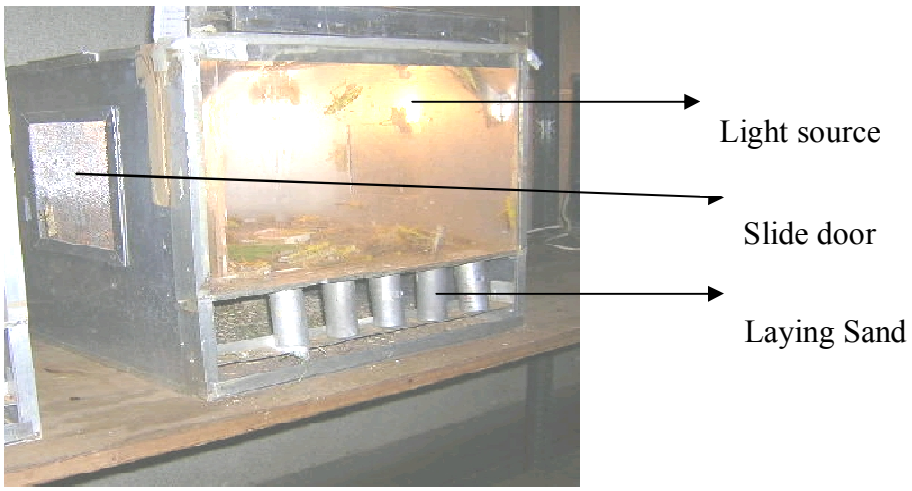


Plate 4: The cage used for rearing the desert locusts (Photo: by Cheseto)

2.3 Preparation and analysis of oral secretion from 5th instar desert locust fed on wheat seedlings and wheat bran

Sample preparation was done in two stages; Liquid-liquid extraction followed by Solid Phase extraction as shown below:

2.3.1 Liquid-liquid extraction

Total volume of 200 µl of oral secretion was collected from 5th instars desert locust as described previously by Turlings *et al.*, (1993). Each locust was hand captured and its mouth placed at the tip of a 200 µl micro capillary with ring mark. The oral secretion was then allowed to flow by adhesion, cohesion and free flow as shown in Plate 5. This was repeated severally until the 200 µl mark was reached (averagely 20, 5th instar desert locusts were required to fill the 200 µl capillary tube).

The oral secretion was then transferred into a 5.0 ml clear glass vial containing 200 µl of phosphate buffer pH 6.8 and 300 µl of Pentane and agitated in a super mixer (Lab-line instruments Inc, Meleuse Park, III, USA) under fast for five minutes. The content of the vial was transferred into an amber glass centrifuge vial, corked and centrifuged at 13000 rpm for five minutes at 20^0 C using analytical centrifuge (Biofuge (Fresco) Heraeus Kendro lab products, California, USA. Using a 100 µl glass syringe the supernatant was sucked and drained into a clean vial onto which 300 µl of pentane (≥99%) was added, the sample was then cleaned using the following procedure.

Plate 5: Collection of oral secretion (Photo: by Cheseto)

2.3.2 Solid Phase Extraction

Basing on the amount of oral secretion (200 µl) that was used for the experiment a 1ml tube size was selected for purification of the centrifuged sample. The tube has the following diagrammatic appearance (Fig 6).

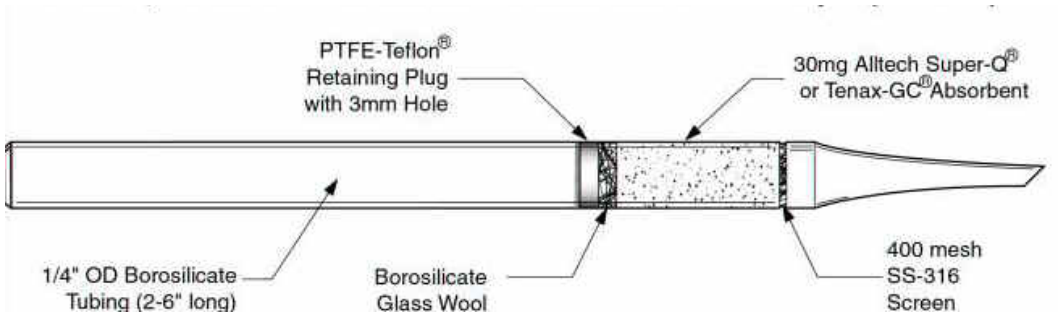
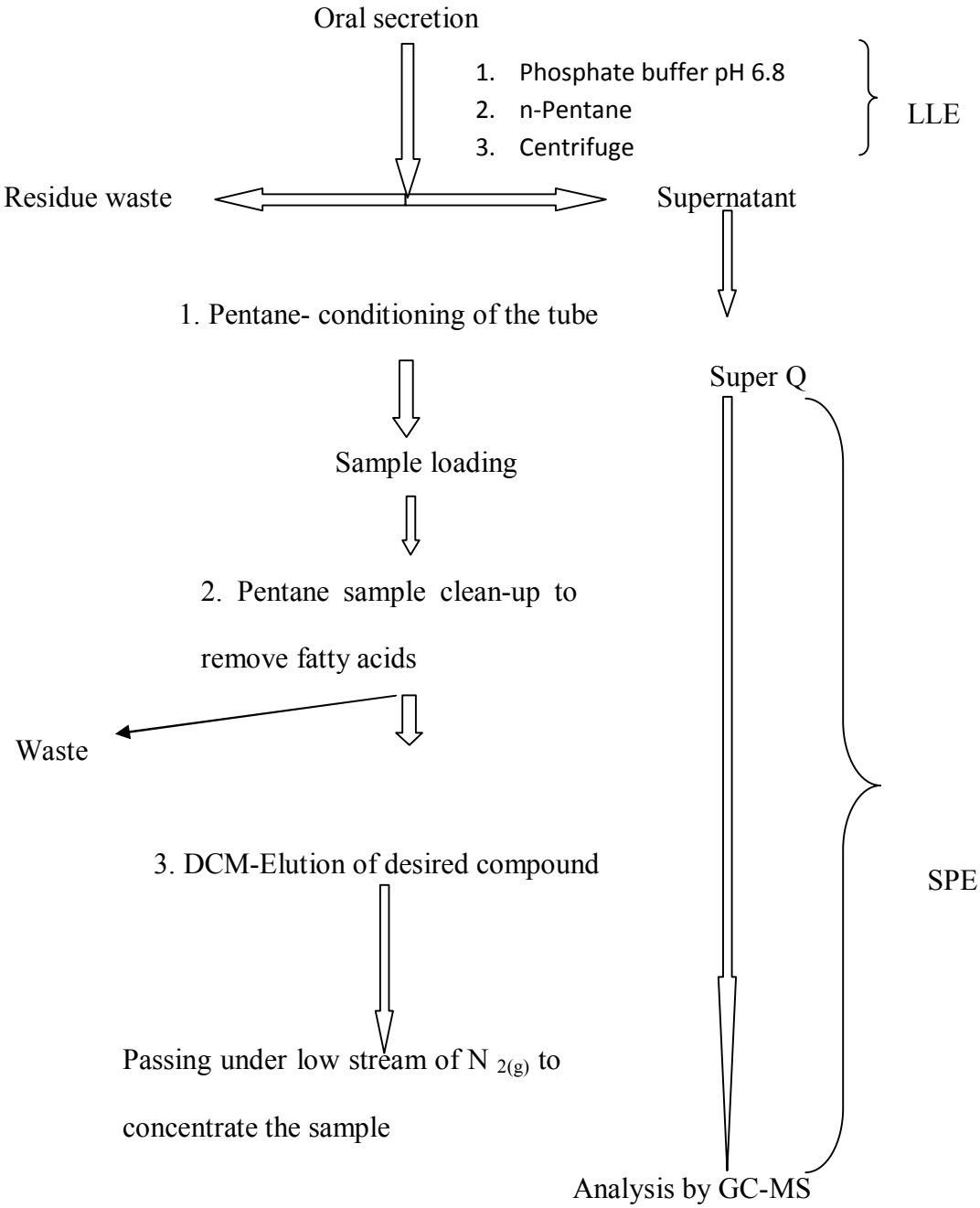


Figure 6: Components of a Super Q extraction tube

The tube was thoroughly cleaned using dichloromethane and dried by passing a stream of nitrogen gas through it. The Super Q tube was then conditioned by rinsing it with one tube-full of pentane and the supernatant loaded onto it. The sample was washed with 50 µl of pentane, eluted with 400 µl of dichloromethane and pre-concentrated by passing a gentle flow of $N_{2\,(g)}$ to initial volume of 200 µl. Triplicate of 40 µl of the collected sample was then transferred into auto sampler vials, 1 µl (200 pg) of lupeol internal standard (IS), was added to each vial and analyzed using GC-MS. The GC-MS chromatographic profiles of the analyzed sample and a summary of sterols identified are shown in (Fig 10) and the corresponding Tables respectively. The above procedure used for sample clean out is summarized in Scheme 7 below:



Scheme 7: Collection and clean-up of oral secretion from the desert locust after feeding on wheat seedling and wheat bran

2.4 Preparation and analysis of oral secretion from newly emerged unfed 1st instar desert locust

Sample volume100 µl of oral secretion was collected from newly emerged unfed 1st instars desert locust as described previously by Turlings *et al.*, (1993). Each locust was hand captured and its mouth placed at the tip of a 100 µl micro capillary with ring mark. The oral secretion was then allowed to flow by adhesion, cohesion and free flow as shown in Plate 5. This was repeated severally until the 100 µl mark was reached (averagely 70, 1th instar desert locusts were required to fill the 100 µl capillary tube).

The oral secretion was then transferred into a 5.0 ml clear glass vial containing 200 µl of phosphate buffer pH 6.8 and 300 µl of pentane and agitated in a super mixer under fast for five minutes. The content of the vial was transferred into an amber glass centrifuge vial, corked and centrifuged at 13000 rpm for five minutes at 20^0 C. Using a 100 µl glass syringe the supernatant was sucked and drained into a clean vial onto which 300 µl of pentane (≥99%) was added, the sample was then cleaned by passing through Super Q as described earlier. Triplicate of 40 µl of the collected sample was then transferred into auto sampler vials, 1 µl (200 pg) of lupeol internal standard (IS), was added to each vial and analyzed using GC-MS. The tic of the analyzed sample and a summary of sterols identified (Fig 11) and the corresponding Tables respectively.

2.5 Wheat seedlings

Wheat (*Triticum aestivum* L) caryopses shown in Plate 6 were purchased from Rift Valley. The caryopses were allowed to germinate in vermiculite in a planting pot (Fig 7; 20-white planting pots, tumbler in shape each with 4 drainage holes). The pots were maintained in a screen house at 22°C in a 12:12 h 1ight: dark regime. 3-4 weeks old seedlings were fed to newly emerged locust hoppers.

Plate 6: Assortment of wheat caryopses (Photo: by Cheseto)

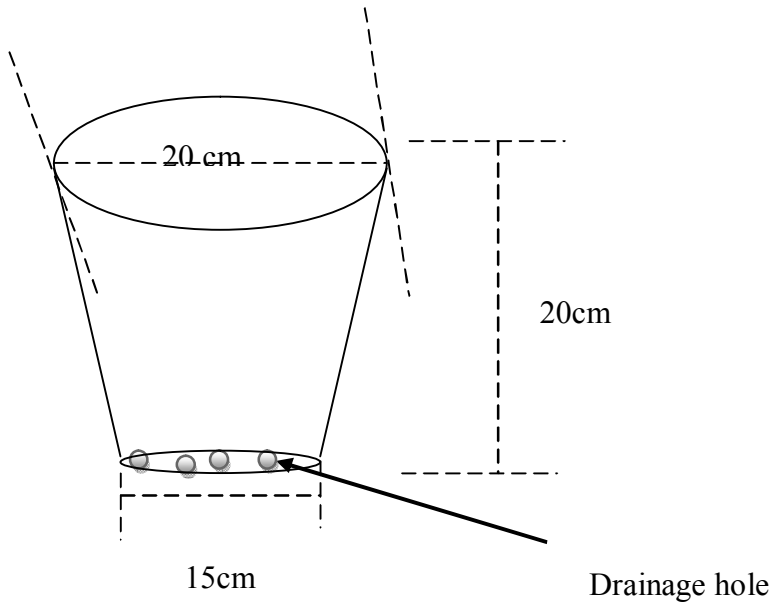


Figure 7: Planting pot (not drawn to scale)

2.5.1 Planting of wheat seedling

Seed selection: well formed seeds void of mechanical and insect damage were hand selected a day prior to planting.

Vermiculite type of soil obtained from *icipe*, crushed, sieved (1mm i.d holes) to obtain fine tilth, thoroughly mixed with diamonium phosphate fertilizer (DAP) and rabbit droppings as manure. The prepared soil was transferred to the twenty planting pots up to three-quarter full. 2cm deep and 1cm wide lines were made across twenty planting pots which were used for planting wheat seeds. The wheat seeds were lightly covered with soil. The twenty planted pots were placed inside a big wire meshed cage to protect them from rodents attack (Plate 7).

Plate 7: Caged planting pots (Photo: by Cheseto)

The plants were irrigated once a day using tap water. Two weeks after germination Calcium Ammonium Nitrate fertilizer was applied to the plants.

2.5.2 Preparation and analysis of wheat seedlings extract

The three weeks old wheat seedlings (Plate 8) were cut using a sterilized razor blade and bench dried for one week at room temperature to remove moisture. The dried seedlings were crushed to fine powder. The powder was then transferred into a glass vial containing pentane and agitated using super mixer.

The solution was cleaned by passing through a glass wool. The eluent was further purified by passing it through a Super Q conditioned using pentane as described earlier. The final elution was done using dichloromethane. Triplicate of 40 µl of the

collected sample was then transferred into auto sampler vials; 1µl (200 pg) of lupeol (IS); was added to each vial and analyzed using GC-MS. Tic of the analyzed sample and a summary of sterols identified are shown in (Fig 12).

Plate 8: Cut wheat seedlings (Photo: by Cheseto)

2.6 Preparation and analysis of the gut extracts from 5th instar desert locust fed on wheat seedling and wheat bran

Five 5th instar desert locusts were collected and frozen overnight in a freezer to make them rigid and easy to cut. The locusts were separately pinned on to the dissecting board, mounted on the dissecting microscope and incised cephalocaudially (Plate 9) using standard procedures of dissection.

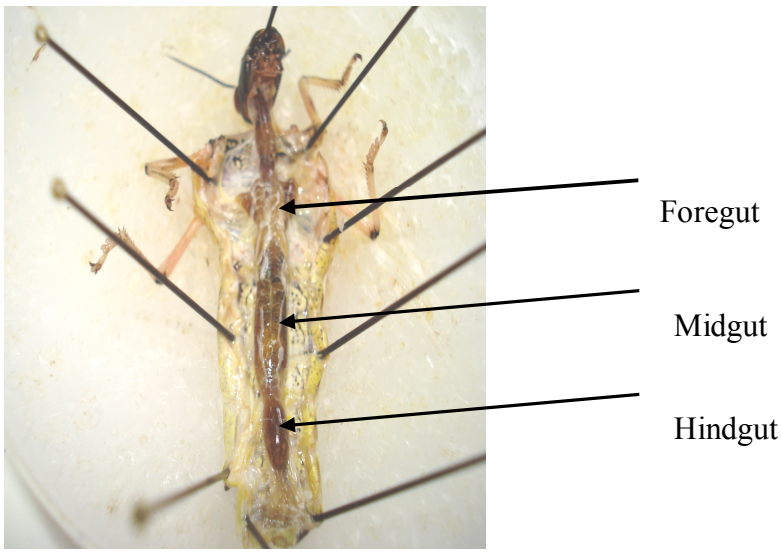


Plate 9: Cephalocaudially incised 5th instar desert locust (Photo: by Cheseto)

The three guts were separately cut open, sliced to small pieces and the gut content transferred into 5 ml clear glass vial containing 100-µl of pentane. The mixture was agitated using a super mixer and sieved by passing through a glass wool. The collection was further purified by passing it through a Super Q conditioned using pentane. The final elution was done using 500µl of DCM and pre-concentrated to 200µl by slowly passing a flow of nitrogen over it.

Triplicate of 40 µl of the collected sample was then transferred into auto sampler vials; 1 µl of lupeol (IS); 200 pg was added to each vial and analyzed using GC-MS. Tic of the analyzed sample and a summary of sterols identified are shown in (Fig 12,13,14) and the corresponding Table.

2.7 Preparation and analysis of the 5th instar desert locust gut after incubation with cholesterol

The three guts were separately cut open under normal saline B.P (Nairobi, Kenya). The guts were thoroughly cleaned in the saline after which they were transferred in a 10 ml vial containing Cholesterol dissolved in a mixture of distilled water and saline 1:5. The guts were then incubated in an oven at 32˚ C for 24 hrs with periodic vortexing. After 24 hrs, 1 ml of distilled water and 4 ml of pentane was added vortexed for 10 min and then centrifuged at 13 000 rpm for 5 min. The supernatant was drawn and moisture removed by addition of anhydrous Na_2SO_4. The supernatant was further purified by sieving through glass wool to remove particulate matter before concentrating under $N_{2\,(g)}$ to dryness. This was reconstituted by addition of 30 µl pentane before analysis by GC-MS. Tic of the analyzed sample and a summary of sterols identified are shown in (Fig 15).

2.8 Preparation and analysis of the 5th instar desert locust gut after incubation with cholesterol-[4-^{13}C].

The three guts were separately cut open under normal saline B.P (Nairobi, Kenya). The guts were thoroughly cleaned in the saline after which they were transferred in a 10 ml vial containing cholesterol-[4-^{13}C] dissolved in a mixture of distilled water and saline 1:5. The guts were then incubated in an oven at 32˚C for 24 hrs with periodic vortexing.

After 24 hrs, 1 ml of distilled water and 4 ml of pentane was added vortexed for 10 min and then centrifuged at 13 000 rpm for 5 min. The supernatant was drawn and moisture removed by addition of anhydrous Na_2SO_4. The supernatant was further purified by sieving through glass wool to remove particulate matter before concentrating under $N_{2\ (g)}$ to dryness. This was reconstituted by addition of 30µl pentane before analysis by GC-MS. Tic of the analyzed sample and a summary of sterols identified are shown in (Fig16).

2.9 Preparation and analysis of the hemolymph from the 5th instar desert locust fed on wheat seedling and wheat bran

The junction between the coxa, trochantin and the metathorax of the 5th instar desert locust (Plate 10) was punctured using a scalpel and hemolymph collected using a 200 µl micropipette (approximately 15, 5th instar desert locust were used). The haemolymph was transferred into a vial containing 500 µl, 70% methanol: water, agitated for 5 min and centrifuged 13 000 rpm for 5min at 5°C to remove precipitated protein.

The supernatant was then transferred to a clean vial containing 300 µl pentane, vortexed for 5min and centrifuged at 13 000 rpm for 5 min at 5°C. The collection was further purified by passing it through a Super Q conditioned using pentane as described earlier. The supernatant was then analyzed by GC-MS as above. Tic spectrum of the analyzed sample and a summary of sterols found are shown in Figure 18

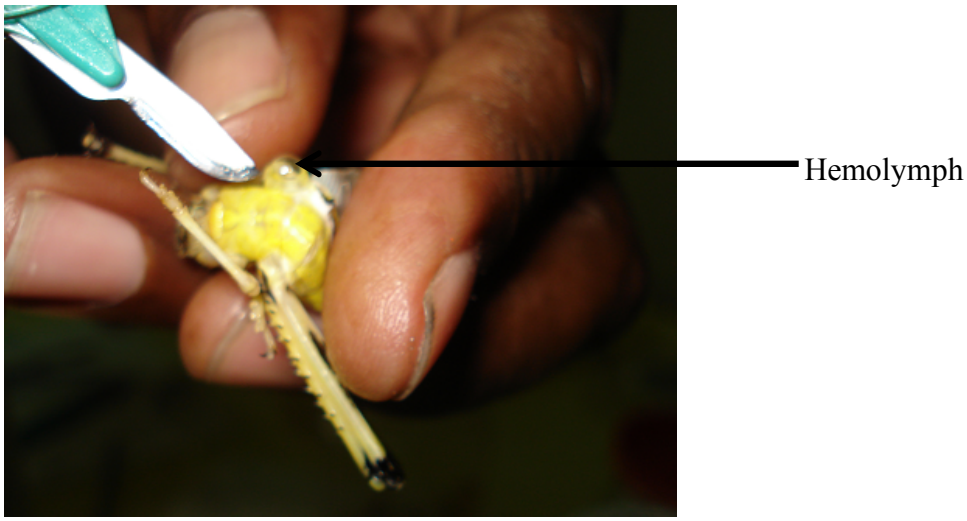


Plate 10: Punctured 5th instar desert locust (Photo: by Cheseto)

2.10 Preparation and analysis of the fat bodies from 5th instar desert locust fed on wheat seedling and wheat bran

Five 5th instar desert locusts were collected and frozen overnight in a deep freezer to make them rigid and easy to cut. The locusts were separately pinned on to the dissecting board, and incised cephalocaudially (Plate 12) using standard procedures of dissection. The abdominal fat bodies content was separately sliced into small pieces and transferred into 5ml clear glass vial containing 100 µl of pentane, vortexed for 5 min and centrifuged 13000 rpm for 5min at 5° C. The collection was further purified by passing it through a Super Q conditioned using pentane as described earlier. The supernatant was then analyzed using GC-MS as above. Tic spectrum of the analyzed sample and a summary of sterols found are shown in Figure 19.

2.11 Preparation and analysis of the prothoracic glands from 5th instar desert locust fed on wheat seedling and wheat bran.

Five 5th instar desert locusts were separately dissected using standard procedures. The pg was sliced into small pieces and transferred into 5 ml clear glass vial containing 100 µl of pentane, vortexed for 5 min and centrifuged at 13 000 rpm for 5 min at 5°C. The collection was further purified by passing through Super Q conditioned using pentane as described earlier. The supernatant was then analyzed using GC-MS. Tic spectrum of the analyzed sample and a summary of sterols found are shown in Figure 20.

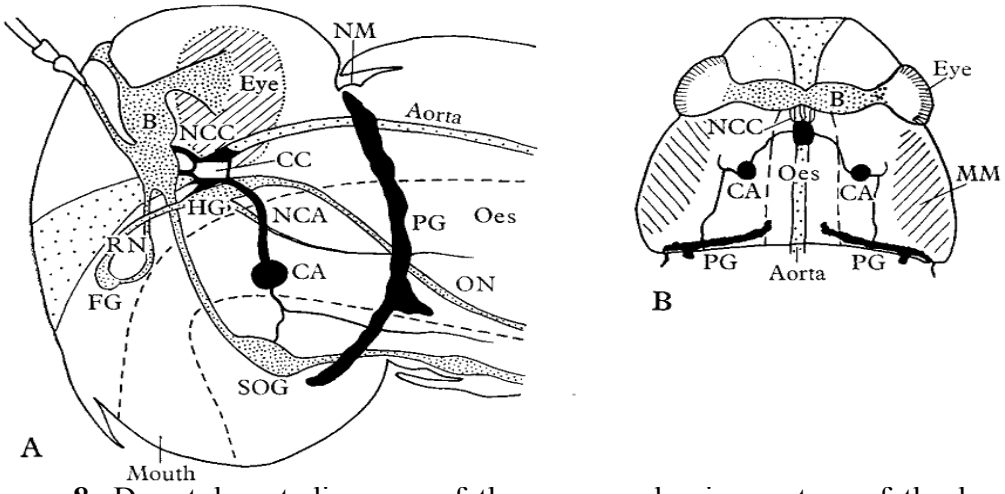


Figure 8: Desert locust diagrams of the neuro-endocrine system of the head A, lateral view; B, dorsal view

B, brain: NM, neck membrane; NCC, nerve of the corpus cardiacum: CC, corpus cardiacum: HG, hypocerebral ganglion: NCA, nerve of the corpus allatum: RN, recurrent nerve: FG, frontal ganglion: PG, prothoracic gland: Oes, oesophagus: ON, oesophageal nerve: MN, mandibular muscles.

2.12 Preparation and analysis of the frass from the 5th instar desert locust fed on wheat seedling and wheat bran

Frass (Plate 11) was collected from the cage where 5th instar desert locust were reared. The frass was crushed to fine powder. 1g from the bulk was weighed and transferred into a vial containing 300 µl of pentane. The mixture was vortexed and sieved by passing through a glass wool. The collection was further purified by passing it through a Super Q conditioned using pentane and washed using 500µl of pentane. The final elution was done using 500 µl of DCM and concentrated to 200µl by slowing passing $N_{2\,(g)}$ over it. Triplicate of 40 µl of the collected sample was then transferred into auto sampler vials; 1-µl of lupeol (IS); 200 pg was added to each vial and analyzed using GC-MS. Tic spectrum of the analyzed sample and a summary of sterols found are shown in Figure17 and Table 8.

Plate 11: Frass

2.13 Ex-situ derivatization

The sterols from each stage of analysis (wheat plant, oral secretion, the gut, haemolymph, fat bodies, prothoracic glands and frass) were concentrated to 1 ml and derivatized using TMS (Hydrox-sil) as the derivatizing agent. Derivatizations were carried out in 5 ml Reacti-vial^{TM} small reaction vial for 1 hour at 100ºC. The derivatized sample was then transferred into inserts placed in auto sampler glass vial with Teflon caps and analyzed using GC-MS.

2.14 Identification of compounds

The extracts were injected directly into GC- MS analysis. Cholesterol was identified by comparison of gas chromatographic retention time with that of the standard cholesterol analyzed by GC-MS. Sterols were identified through a confirmatory test using ex-situ–derivatization procedure. Library–MS searches using NIST/EPA/NIH MASS SPECTRAL LIBRARY NIST 05, NIST 08, Chemcol.l and Adams 2.l data bases and NIST MASS SPECTRAL SEARCH PROGRAM Version 2.0d, used for characterization purposes in the GC-MS data system. The area was used for quantification based on the amount of internal standard added.

Sterols were identified and quantified by GC-MS using lupeol as an internal standard for all sterols. Sterols were analyzed by GC-MS on a 7890A stand-alone gas chromatograph (Agilent Technologies, Inc., Beijing, China) and a 5975 C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA) by using the

following conditions: Inlet temp 270°C, transfer line temp of 280°C, and column oven temperature programmed from 35 to 285°C with the initial temperature maintained for 5 min then 10 °C/min to 280 °C for 10.5 min and the final one for 29.9min 50 °C/min to 285 °C. The GC was fitted with a HP-5 MS low bleed capillary column (30 m × 0.25 mm i.d., 0.25-μm) (JandW, Folsom, California, USA). Helium at a flow rate of 1.25 ml/min served as carrier gas. The Agilent 5973 mass selective detector maintained an ion source temperature of 250°C and a quadruple temperature of 180°C. 230°C was set as the MS ion source temperature. Electron impact (EI) mass spectra were obtained at acceleration energy of 70 eV. A 1.0 μL aliquot of extract was automatically injected in the split/ splitless mode using an auto sampler 7683 (Agilent Technologies, Inc., Beijing, China). Fragment ions were analyzed over 40-550 m/z mass range in the full scan. The filament delay time was set at 3.3 min for underivatized sample and 8.6 min for derivatized sample.

2.15 Data management

Data collected were processed using excel. The means presented in Tables of results were computed from the original untransformed data. Analysis of variance was carried out for sterols found in wheat, oral secretion, gut system, frass, hemolymph, fat bodies and prothoracic gland of the desert locust and means were separated using Tukey's studentized Honestly Significant Difference at 5% level of significance. R-statistical program 2.12.0 was used for the data analyses.

CHAPTER THREE

3.0 RESULTS

3.1 Introduction

This chapter presents the experimental findings of the study in relation to the research objectives in the order shown below. A summary of sterols in relation to biosynthesis of ecdysone in the desert locust is provided in the section after the introduction in Table 1.

- The sterol profile of wheat seedling.
- The sterol profile of the oral secretion from 5th instars desert locust after feeding on wheat seedling and wheat bran.
- The sterol profile of the foregut from 5th instars desert locust after feeding on wheat plant and wheat bran.
- The sterol profile of the midgut from 5th instars desert locust after feeding on wheat plant and wheat bran.
- The sterol profile of the hindgut from 5th instars desert locust after feeding on wheat seedling and wheat bran.
- The sterol profile of foregut, midgut and hindgut of the 5th instar desert locust after incubation with cholesterol-[4-^{13}C] and normal cholesterol.
- The sterol profile of the frass from 5th instars desert locust after feeding on wheat seedling and wheat bran.

- The sterol profile of the haemolymph from 5th instars desert locust after feeding on wheat seedling and wheat bran.
- The sterol profile of the fat bodies from 5th instars desert locust after feeding on wheat seedling and wheat bran.
- The sterol profile of the prothoracic glands from 5th instars desert locust after feeding on wheat seedling and wheat bran.
- Ms data analysis.

Table 1: A summary of sterols identified in wheat seedling, oral secretion, gut, frass, hemolymph, fat bodies and prothoracic glands of 5th instars desert locust in relation to ecdysone biosynthesis

Sterol name	W S	OS	GE			F	H	FB	PG
			FG	MG	HG				
Lanosterol	✓	✓	✓	✓	✓	✓	✓	✓	×
Cholesta-8,14,24-triene-3-ol, 4, 4-dimethyl,(3β 5α)	×	✓	✓	✓	×	×	✓	✓	×
Cholesta-5,24-diene-3,20-diol, (3 β,20R)	×	✓	✓	✓	×	×	✓	✓	×
β-Sitosterol	✓	✓	✓	✓	✓	✓	✓	✓	×
Fucosterol	×	✓	✓	✓	×	×	✓	✓	×
Desmosterol	×	✓	✓	✓	×	×	✓	✓	×
Cholest-5-en-3-ol (3 β)	✓	✓	✓	✓	✓	✓	✓	✓	✓
Cholesta-5,7-dien-3-ol,(3 β)	×	✓	✓	✓	×	×	✓	✓	✓
Cholesta-7-ene-6-one-3,14-diol(3β)	×	×	×	×	×	×	×	×	✓
Cholesta-7-ene-6-one-3,14,25-triol (3β)	×	×	×	×	×	×	×	×	✓

WS-wheat seedling; OS- oral secretion; G- gut extract; FG-foregut; MG-midgut; HG-hindgut; F-frass; H-Hemolymph; FB- fat bodies; PG- prothoracic glands

3.2 Results for wheat seedling extracts

3.2.1 Sterols profile for wheat seedling extract

Retention time comparison for cholesterol standard aided in identification of cholesterol in the sample while TMS derivatives were used as confirmatory indicators in the identification of other sterols. Literature comparison of mass spectra was used in the identification process. Table 2, tic Figure 9 and concentrations Figure 9.1 show sterol profile for wheat seedling. A total of ten sterols were identified.

From the results, there was a significant ($P \leq 0.001$) difference in the mean amounts of sterols in wheat seedlings, with the highest (351.04 pg / g) being cholest-5-en-3-ol (3β) and the lowest (14.19 pg/g) being cholest-8(14)-en-3-ol,2,2-dimethyl-, (3β,5α). There were no significant differences in the amounts of sterols; cholest-4-en-6-on-3-ol & ergosterol, campesterol & stigmasterol, cholest-8(14)-en-3-ol,2,2-dimethyl-, (3 β,5 α)- & cholesterol, 7-oxo.

Basing on the mean amounts of sterols calculated the following can be deduced: 239.44 pg/g of the total sterol found in the wheat plant contain double bond at position 7 in the tetracyclic nucleus (B-ring) and/or position 22 ($\Delta^{7,\ 22}$) and 741.23 pg/g of the total sterol found in the wheat plant contain double bond at position 5 and or position 24 ($\Delta^{5,\ 24}$).

Table 2: Sterol profile of wheat plant extract

	Peak no.	Sterols identified	RT (Min)	Mean Conc.(pg/ g)	Δ position
[b]	1	Cholest-8(14)-en-3-ol,2,2-dimethyl, (3 β,5 α)-	34.008	14.19 ± 1.33	$\Delta^{8(14)}$
[b]	2	Cholest-4-en-6-on-3-ol	34.860	50.94 ± 2.13	Δ^{4}
[a]	3	Cholest-5-en-3-ol (3 β)-	35.453	351.04 ± 2.00	Δ^{5}
[b]	4	Cholest-7-en-3-ol, (3 β,5α)-	35.916	74.93 ± 2.23	Δ^{7}
[b]	5	Campesterol	36.507	103.54 ± 2.04	Δ^{5},24-methyl
[b]	6	Stigmasterol	37.001	95.06 ± 2.54	$\Delta^{5,22}$,24-ethyl
[b]	7	Ergost-5-en-3-ol,(3β)-	38.279	47.27 ± 0.42	Δ^{5},24-methyl
[b]	8	Lanosterol	39.003	253.40 ± 1.30	$\Delta^{8,24}$
[b]	9	Cholesterol, 7-oxo-	39.999	22.18 ± 1.09	Δ^{5}
[b]	10	Stigmasterol, 22,23-dihydro-(β-Sitosterol)	41.887	33.25 ± 0.81	Δ^{5},24-ethyl

[a] Identified by comparison with authentic sample, [b] Identified by comparison with literature spectra, concentration values expressed as means ± SE

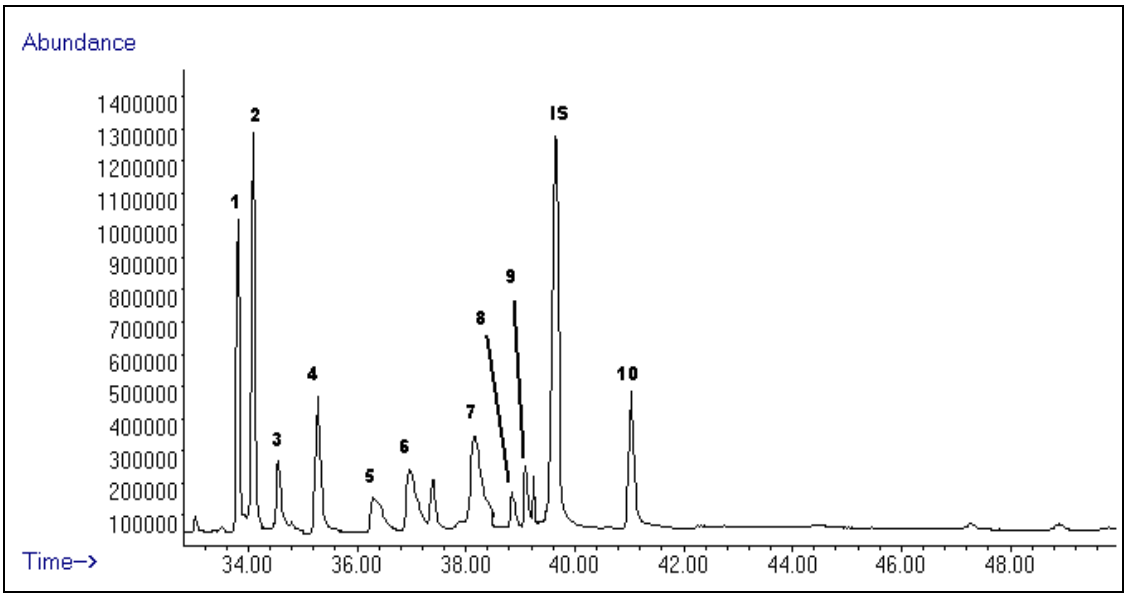


Figure 9: Total ion chromatogram showing sterol profile from wheat seedling extract

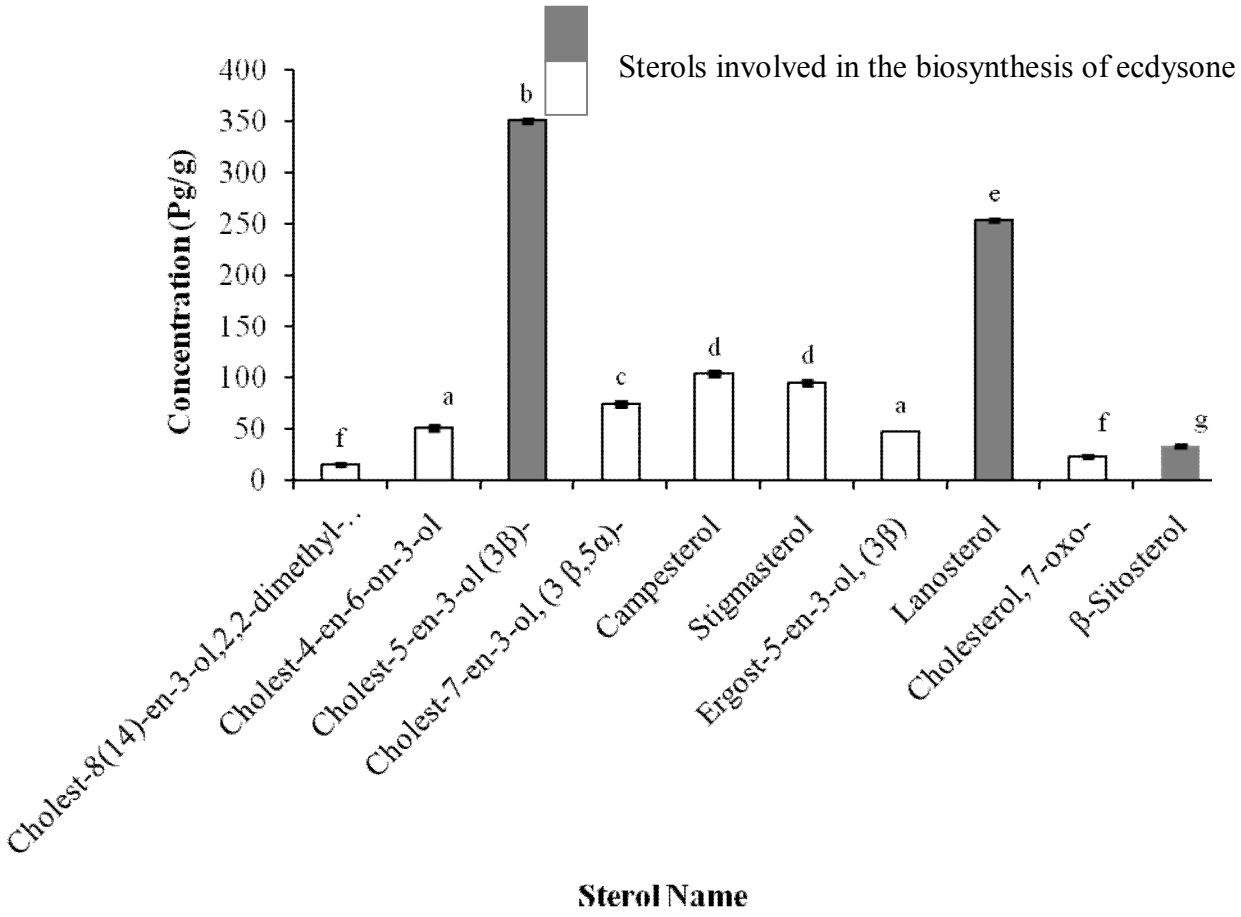


Figure 9.1: Sterol concentration from wheat seedling. Bars bearing the same letters are not significantly different (P≤0.05, Tukey's, HSD test)

3.3 Results for oral secretion

3.3.1 Sterols profile for oral secretion from the 5th instars desert locust fed on wheat seedlings and wheat bran.

Retention time comparison of cholesterol standard aided in the identification of cholesterol in the sample while TMS derivatives were used as confirmatory indicators in the identification of other sterols. The following sterols shown in Table 3 tic Figure 10 and mean concentration Figure 10.1 gives the sterol profile. A total of fifteen sterols were identified. The number increased by five sterols compared to wheat seedling extract, indicating the formation of new metabolites by the desert locust from the consumed phytosterols. The new metabolites were: cholesta-5,7-dien-3-ol,(3β), cholesta-8,14,24-triene-3-ol,4,4-dimethyl-,(3β,5α), cholesta-5,24-diene-3,20-diol,(3β,20R), desmosterol and fucosterol which are dealkylation products as explained in Chapter Four.

From the results, there was a significant ($P \leq 0.001$) difference in the mean amounts of sterols in the oral secretion, with the highest (2.87±0.007 pg/µl) being cholest-5-en-3-ol(3β) and the lowest (0.09 ±0.009 pg/µl) being cholest-8(14)-en-3-ol,2,2-dimethyl, (3β,5α). There were no significant differences in the amounts of sterols; cholesta-5,7-dien-3-ol,(3β) & cholest-7-en-3-ol,(3β,5α), cholest-7-en-3-ol,(3β,5α)- & campesterol , β-sitosterol & fucosterol.

From the results, 3.92 pg/µl of the total sterol found in the oral secretion contain double bond at position 7 in the tetracyclic nucleus (B-ring) and/or position 22 ($\Delta^{7,22}$) and 5.91 pg/µl of the total sterol found in the oral secretion contain double bond at position 5 and or position 24 ($\Delta^{5,24}$).

Table 3: Sterol profile of oral secretion from the 5th instars desert locust fed on wheat seedlings and wheat bran

	Peak no.	**Sterols identified**	**RT (Min)**	**Mean Conc. (pg/µl)**	**Δ position**
b	1	Cholest-8(14)-en-3-ol, 2,2-dimethyl-,(β,5 α)-	34.008	0.09 ± 0.009	$\Delta^{8(14)}$
a	2	Cholest-5-en-3-ol (3β)-	35.553	2.87 ± 0.007	Δ^{5}
b	3	Cholesta-5,7-dien-3-ol, (3β)-	35.853	0.46 ± 0.009	$\Delta^{5,7}$
	4	Cholest-7-en-3-ol,(3 β,5α)-	36.034	0.43 ± 0.013	Δ^{7}
b	5	Campesterol	36.507	0.40 ± 0.000	Δ^{5},24-methyl
b	6	Stigmasterol	37.001	0.60 ± 0.000	$\Delta^{5,22}$,24-ethyl
b	7	Ergost-5-en-3-ol,3β)	38.279	2.23 ± 0.011	$\Delta^{5,7,22}$,24-methyl
b	8	Cholesta-8,14,24-triene-3-ol, 4, 4-dimethyl-, (3 β, 5α)	38.999	0.51 ± 0.010	$\Delta^{8,14,24}$
b	9	lanosterol	39.003	1.08 ± 0.003	$\Delta^{8,24}$
	10	Cholesterol, 7-oxo-	39.999	0.25 ± 0.003	Δ^{7}
b	11	Stigmasterol,22,23-dihydro-(β-Sitosterol)	41.887	0.56 ± 0.007	Δ^{5},24-ethyl
b	12	Cholesta-5,24-diene-3,20-diol,(3 β,20R)-	42.003	0.21 ± 0.007	$\Delta^{5,24}$
b	13	Desmosterol	44.203	0.14 ± 0.007	$\Delta^{5,24}$

[b]	14	Ergost-8(14)-en-3-ol, (3 β)-	46.009	0.40 ± 0.006	$\Delta^{8(14)}$
[b]	15	Fucosterol	48.003	0.54 ± 0.004	Δ^{5},24-ethyl-1-ene

[a] Identified by comparison with authentic sample, [b] Identified by comparison with literature spectra, concentration values expressed as means ± SE

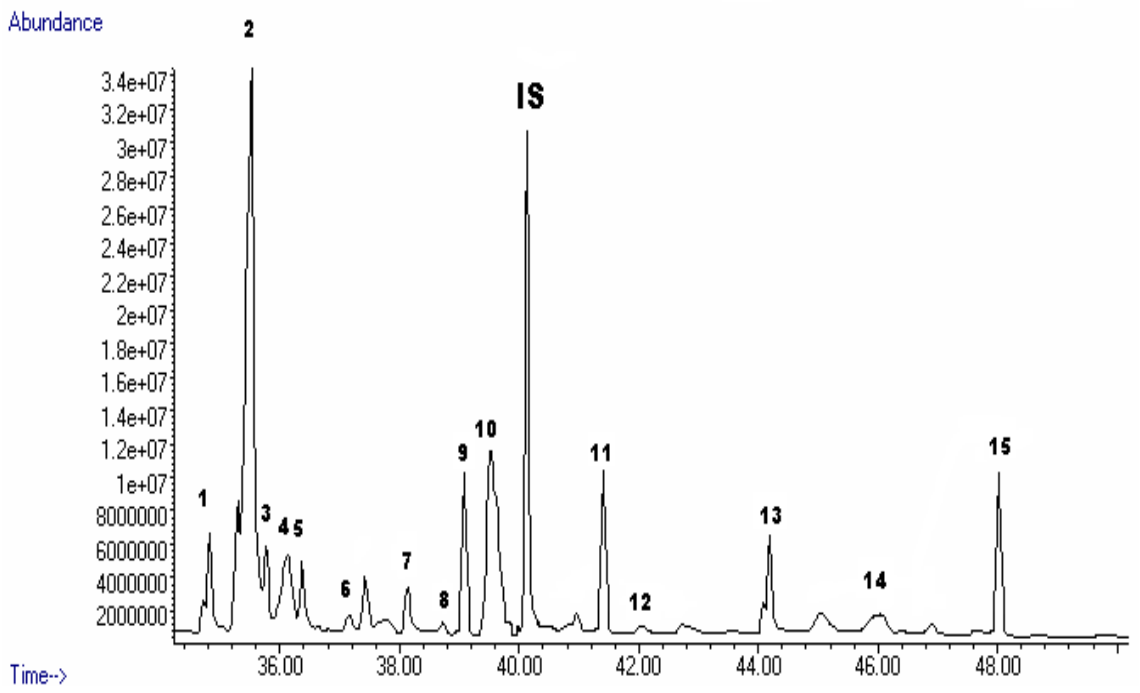


Figure 10: Total ion chromatogram showing sterol profile from oral secretion extract of 5th instars desert locust fed on wheat seedlings and wheat bran

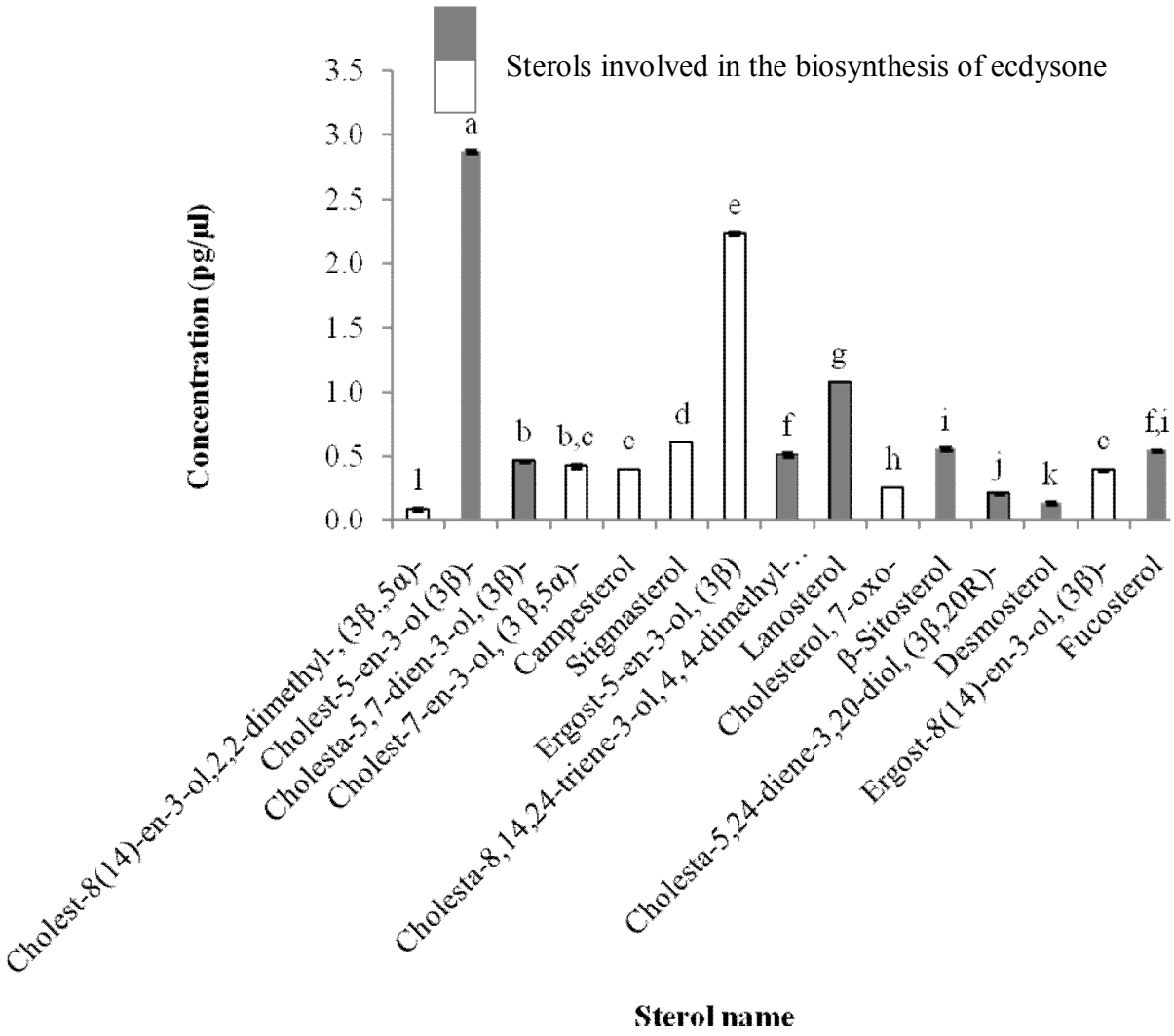


Figure 10.1: Mean concentration of sterols from oral secretion of 5th instars desert locust fed on wheat seedlings and wheat bran. Bars bearing the same letters are not significantly different (P≤0.05, Tukey's, HSD test).

3.3.2 Results for the oral secretion of newly emerged unfed 1st instars desert locust

Retention time comparison of cholesterol standard aided in the identification of cholesterol in the sample. Literature comparison of mass spectra was used in the identification process. The sterol present is shown in Table 4, tic Figure 11 gives the sterol profile. From the results, only cholesterol was found to be present.

Table 4: Sterol profile of oral secretion from the newly emerged unfed 1st instars desert locust fed

	Peak no.	Sterols identified	RT (Min)	Mean Conc. (pg/µl)	Δ position
[a]	1	Cholest-5-en-3-ol (3 β)-	35.553	0.07 ± 0.01	Δ^5

[a] Identified by comparison with authentic sample

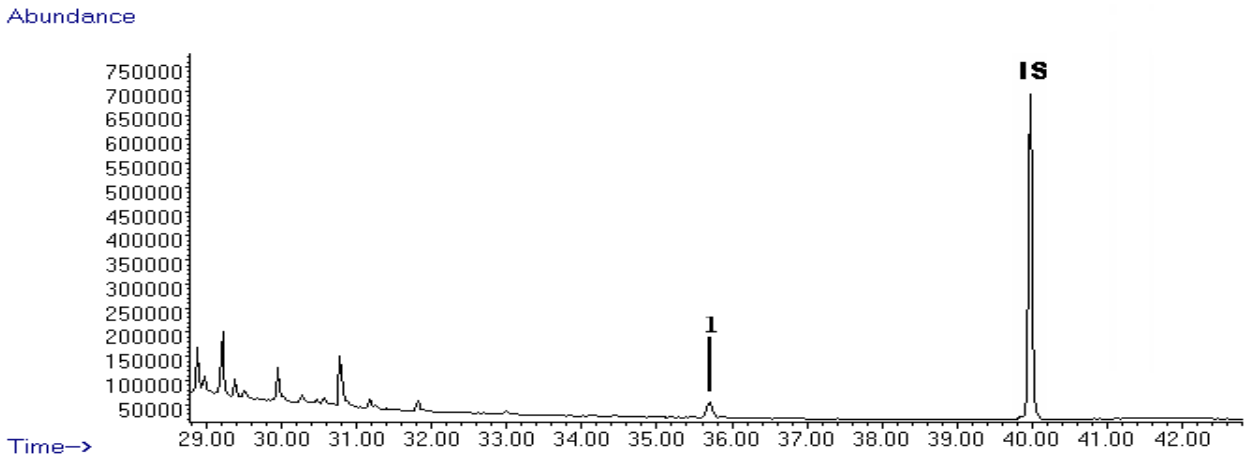


Figure 11: Total ion chromatogram showing sterol profile from oral secretion extract of newly emerged unfed 1st instars desert locust fed

3.4 Results for the gut extracts

3.4.1 Sterol Profile for the foregut from 5th instars desert locust fed on wheat seedling and wheat bran

Retention time comparison of cholesterol standard aided in the identification of cholesterol in the sample while TMS derivatives were used as confirmatory indicators in identification of other sterols. Literature comparison of mass spectra was used in the identification process. The following sterols shown in Table 5, tic Figure 12 and mean concentration Figure 12.1 show sterol profile for the foregut extracts from the 5th instars desert locust fed on wheat seedling and wheat bran. A total of fifteen sterols similar to the oral secretion were identified.

From the results, there was a significant ($P \leq 0.001$) difference in the mean amounts of sterols in the foregut, with the highest (9.76 ± 0.02 pg/mg) being cholest-5-en-3-ol (3β) and the lowest (0.31 ± 0.02 pg/mg) being ergost-5-en-3-ol, (3β). There were no significant difference in the amounts of sterols; campesterol & stigmasterol.

From the results, 3.55 pg/mg of the total sterol found in the foregut contain double bond at position 7 in the tetracyclic nucleus (B-ring) and/or position 22 ($\Delta^{7,\ 22}$) and 33.14 pg/mg of the total sterol found in the foregut contain double bond at position 5 and or position 24 ($\Delta^{5,\ 24}$).

Table 5: Sterol profile for foregut extracts from 5th instars desert locust fed on wheat seedling and wheat bran.

	Peak no.	Sterols identified	RT (Min)	Mean Conc.(pg/mg)	Δ position
[b]	1	Cholest-8(14)-en-3-ol, 2,2-dimethyl-, (3 β,5 α)-	34.008	1.18 ± 0.02	$\Delta^{8(14)}$
[a]	2	Cholest-5-en-3-ol (3 β)-	35.553	9.76 ± 0.02	Δ^{5}
[b]	3	Cholesta-5,7-dien-3-ol, (3 β)-	35.853	6.21 ± 0.01	$\Delta^{5,7}$
[b]	4	Cholest-7-en-3-ol, (3 β,5α)-	36.034	1.50 ± 0.04	Δ^{7}
[b]	5	Campesterol	36.507	1.71 ± 0.03	Δ^{5},24-methyl
[b]	6	Stigmasterol	37.001	1.74 ± 0.02	$\Delta^{5,22}$,24-ethyl
[b]	7	Ergost-5-en-3-ol, (3β)	38.279	0.31 ± 0.02	$\Delta^{5,7,22}$,24-methyl
[b]	8	Cholesta-8,14,24-triene-3-ol, 4, 4-dimethyl-, (3 β, 5α)	39.003	2.86 ± 0.02	$\Delta^{8,14,24}$
[b]	9	Lanosterol	39.999	1.00 ± 0.01	$\Delta^{8,24}$
[b]	10	Cholesterol, 7-oxo-	38.999	2.93± 0.01	Δ^{7}
[b]	11	Stigmasterol,22,23-dihydro- (β-Sitosterol)	41.887	0.83 ± 0.01	Δ^{5},24-ethyl
[b]	12	Cholesta-5,24-diene-3,20-diol, (3 β,20R)-	42.003	4.58 ± 0.02	$\Delta^{5,24}$
[b]	13	Desmosterol	44.203	8.42 ± 0.03	$\Delta^{5,24}$
[b]	14	Ergost-8(14)-en-3-ol, (3 β)-	46.009	1.19 ± 0.01	$\Delta^{8(14)}$
b	15	Fucosterol	48.003	7.76 ± 0.02	Δ^{5},24-ethyl-1-ene

[a] Identified by comparison with authentic sample, [b] Identified by comparison with literature spectra, concentration values expressed as means ± SE

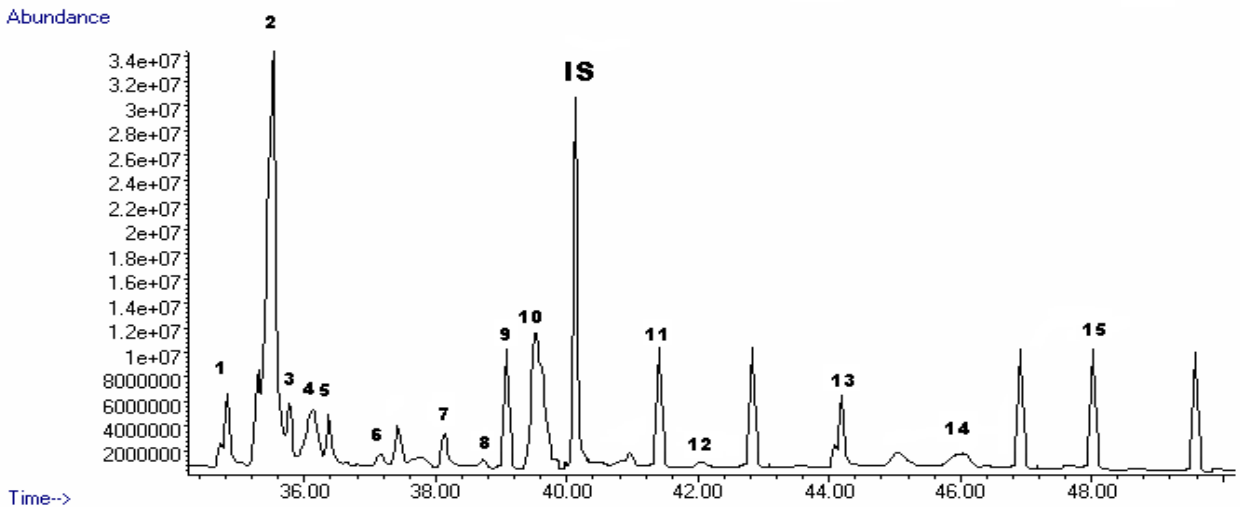


Figure 12: Total ion chromatogram showing sterol profile from the foregut of 5th instars desert locust fed on wheat seedling and wheat bran

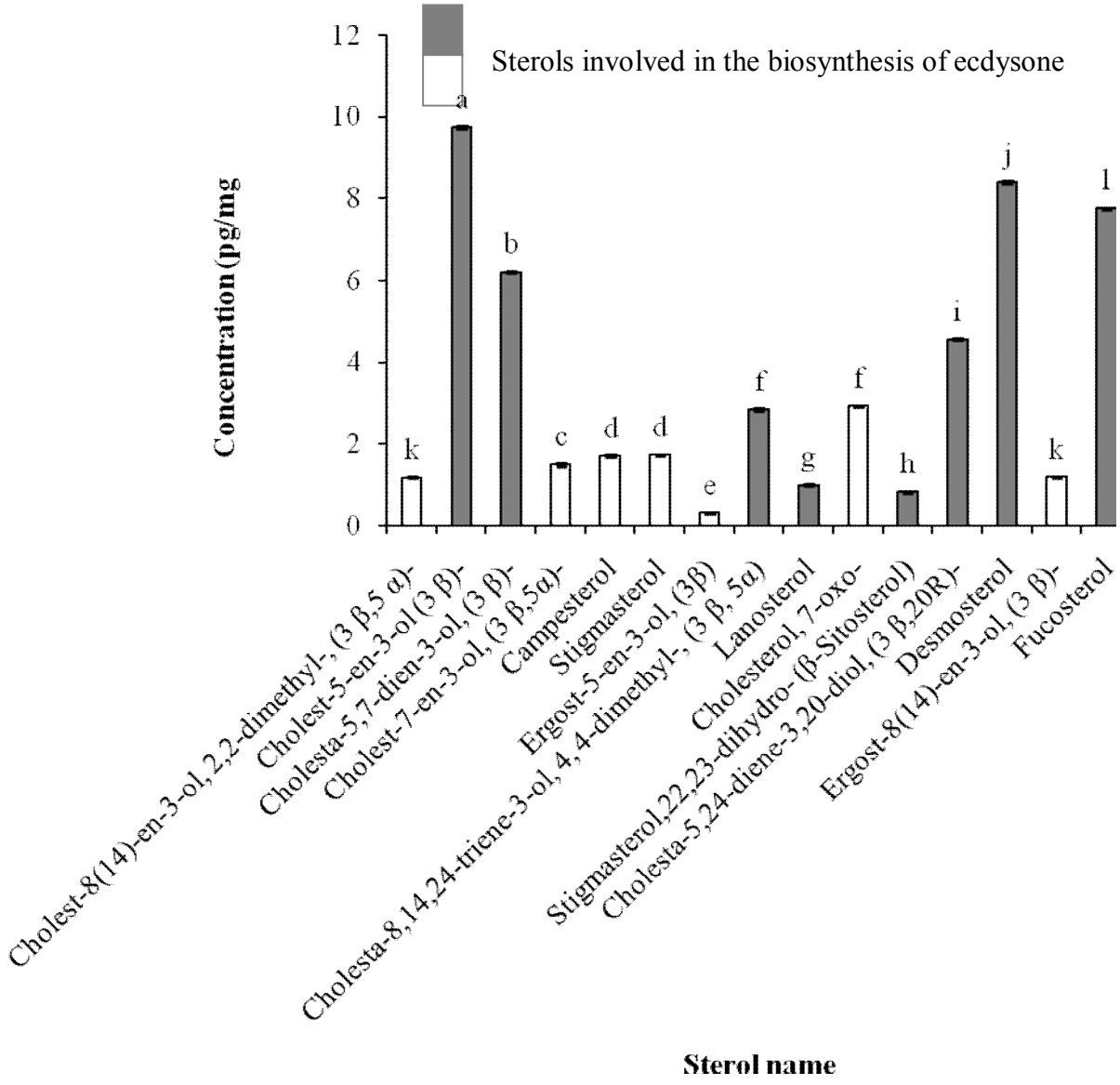


Figure 12.1: Mean concentration of sterols from foregut of 5th instars desert locust fed on wheat seedling and wheat bran. Bars bearing the same letters are not significantly different (P≤0.05, Tukey's, HSD test).

3.4.2 Sterol profile for the midgut extracts from 5th instars desert locust fed on wheat seedling and wheat bran.

Retention time comparison of cholesterol standard aided in the identification of cholesterol in the sample while TMS derivatives were used as confirmatory indicators in identification of other sterols. Literature comparison of mass spectra was used in the identification process. The following sterols shown in Table 6 tic Figure 13 and mean concentration Figure 13.1 show sterol profile for the midgut extracts from the 5th instars desert locust. A total of fifteen sterols were identified, the sterols were similar to oral secretion and foregut.

From the results, there was a significant ($P \leq 0.001$) difference in the mean amounts of sterols in the midgut, with the highest (5.93 ± 0.004 pg/mg) being cholest-5-en-3-ol (3β) and the lowest (0.22 ± 0.014pg/mg) being ergosterol. There were no significant differences in the amounts of sterols; campesterol & stigmasterol, ergost-8(14)-en-3-ol, (3 β) & cholest-8(14)-en-3-ol, 2,2-dimethyl,(3β,5α), cholesta-8,14,24-triene-3-ol, 4, 4-dimethyl, (3 β,5α) & cholesterol,7-oxo.

Based on the amounts found the following can be deduced; 2.85 pg/mg of the total sterol found in the midgut contain double bond at position 7 in the tetracyclic nucleus (B-ring) and/or position 22 ($\Delta^{7,\ 22}$) and 20.75 pg/mg of the total sterol found in the midgut contain double bond at position 5 and or position 24 ($\Delta^{5,\ 24}$).

Table 6: Sterol profile for midgut extracts from 5th instars desert locust fed on wheat seedling and wheat bran.

	Peak no.	Sterols identified	RT (Min)	Mean Conc.(pg/mg)	Δ position
[b]	1	Cholest-8(14)-en-3-ol, 2,2-dimethyl, (3 β,5 α)-	34.008	0.73 ± 0.015	$\Delta^{8(14)}$
[a]	2	Cholest-5-en-3-ol (3 β)-	35.553	5.93 ± 0.004	Δ^{5}
[b]	3	Cholesta-5,7-dien-3-ol, (3 β)-	35.853	3.81 ± 0.015	$\Delta^{5,7}$
[b]	4	Cholest-7-en-3-ol, (3 β,5α)-	36.034	0.91 ± 0.012	Δ^{7}
[b]	5	Campesterol	36.507	1.08 ± 0.008	Δ^{5},24-methyl
[b]	6	Stigmasterol	37.001	1.07± 0.027	$\Delta^{5,22}$,24-ethyl
[b]	7	Ergost-5-en-3-ol, (3β)	38.279	0.22 ± 0.014	$\Delta^{5,7,22}$,24-methyl
[b]	8	Cholesta-8,14,24-triene-3-ol, 4, 4-dimethyl-, (3 β, 5α)	39.003	1.77 ± 0.019	$\Delta^{8,14,24}$
[b]	9	Lanosterol	39.999	0.62 ± 0.012	$\Delta^{8,24}$
[b]	10	Cholesterol, 7-oxo-	38.999	1.80 ± 0.020	Δ^{7}
[b]	11	Stigmasterol,22,23-dihydro-(β-Sitosterol)	41.887	0.50 ± 0.015	Δ^{5},24-ethyl
[b]	12	Cholesta-5,24-diene-3,20-diol, (3 β,20R)-	42.003	2.80 ± 0.023	$\Delta^{5,24}$
[b]	13	Desmosterol	44.203	5.15 ± 0.019	$\Delta^{5,24}$
[b]	14	Ergost-8(14)-en-3-ol, (3 β)-	46.009	0.74 ± 0.019	$\Delta^{8(14)}$
b	15	Fucosterol	48.003	4.77 ± 0.030	Δ^{5},24-ethyl-1-ene

[a] Identified by comparison with authentic sample, [b] Identified by comparison with literature spectra, concentration values expressed as means ± SE

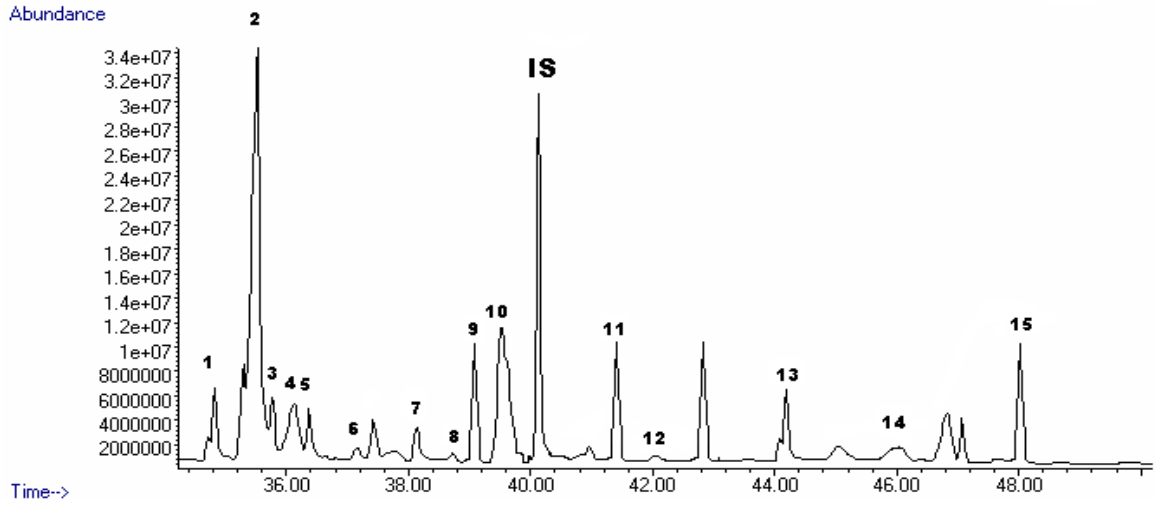


Figure 13: Total ion chromatogram showing sterol profile for midgut extracts of 5th instars desert locust fed on wheat seedling and wheat bran

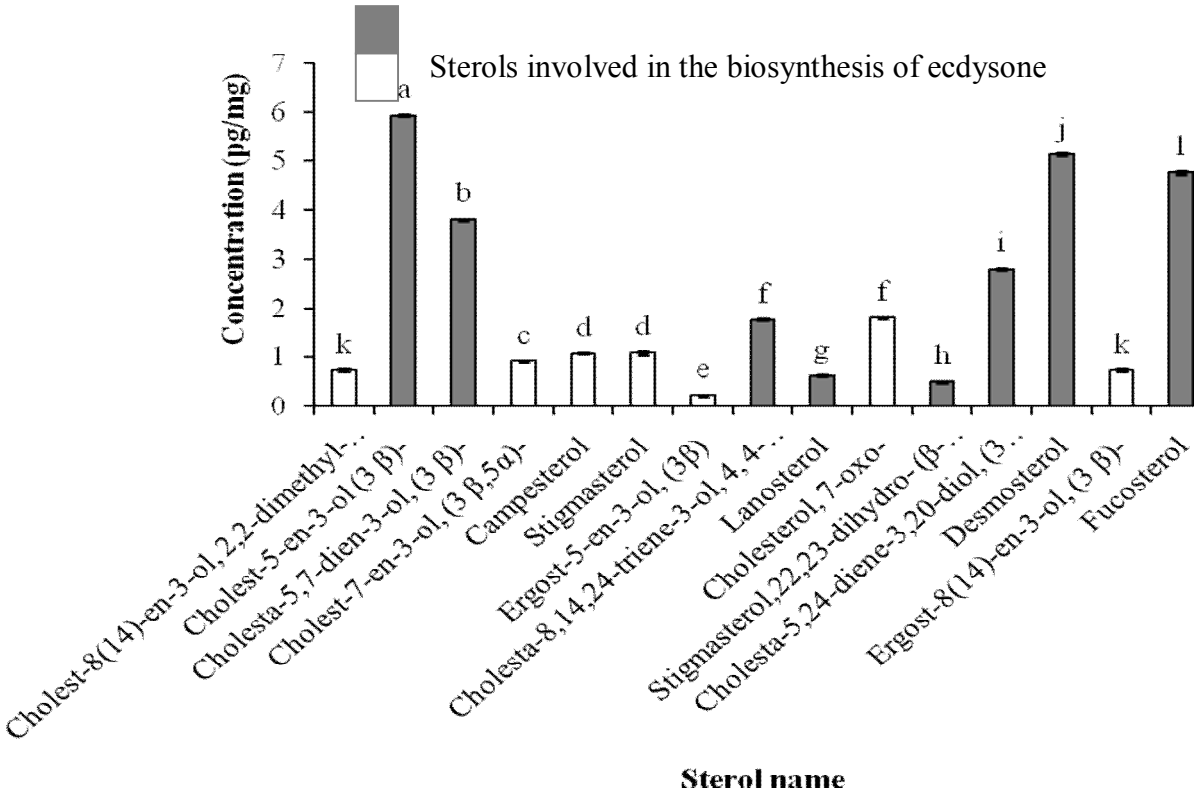


Figure 13.1: Mean concentration of sterols from midgut of 5th instars desert locust. Bars bearing the same letters are not significantly different (P≤0.05, Tukey's, HSD test).

3.4.3 Results for the hindgut extracts from the 5th instars desert locust fed on wheat seedling and wheat bran

Retention time comparison of cholesterol standard aided in identification of cholesterol in the sample while TMS derivatives were used as confirmatory indicators in identification of other sterols. Literature comparison of mass spectra

was used in the identification process. The following sterols shown in Table 7, Total ion chromatogram Figure 14 and mean concentration Figure 14.1 show sterol profiles for the hindgut extracts from the 5^{th} instars desert locust. A total of ten sterols were identified a drop from fifteen which were found in the fore- and midgut, an indication that five sterols were selectively absorbed in the midgut.

From the results, there was a significant ($P \leq 0.001$) difference in the mean amounts of sterols in the hindgut, with the highest (5.74 ± 0.04 pg/mg) being campesterol and the lowest (0.10 ± 0.02 pg/mg) being ergost-8(14)-en-3-ol, (3β). There were no significant differences in the amounts of sterols; cholest-5-en-3-ol (3β) & stigmasterol & ergost-5-en-3-ol, (3β), cholest-7-en-3-ol, (3 β,5α) & lanosterol.

From the results, 7.47 pg/mg of the total sterol found in the foregut contain double bond at position 7 in the tetracyclic nucleus (B-ring) and/or position 22 ($\Delta^{7,\,22}$) and 4.62 pg/mg of the total sterol found in the hindgut contain double bond at position 5 and or position 24 ($\Delta^{5,\,24}$).

Table 7: Sterol profile for hindgut extracts from the 5th instars desert locust fed on wheat plant and wheat bran.

	Peak no.	Sterols identified	RT (Min)	Mean Conc.(pg/mg)	Δ position
[b]	1	Cholest-8(14)-en-3-ol,2,2-dimethyl-, (3 β,5 α)-	34.008	0.60 ± 0.02	$\Delta^{8(14)}$
[a]	2	Cholest-5-en-3-ol (3 β)-	35.553	0.89 ± 0.01	Δ^{5}
[b]	3	Cholest-7-en-3-ol, (3 β,5α)-	36.034	0.74± 0.02	Δ^{7}
[b]	4	Campesterol	36.507	5.74 ± 0.02	Δ^{5},24-methyl
[b]	5	Stigmasterol	37.001	0.89 ± 0.01	$\Delta^{5,22}$,24-ethy
[b]	6	Ergost-5-en-3-ol,(3β)	38.279	0.87 ± 0.01	$\Delta^{5,7,22}$,24-methyl
[b]	7	lanosterol	39.503	0.77 ± 0.01	$\Delta^{8,24}$
[b]	8	Cholesterol, 7-oxo-	39.999	0.99 ± 0.01	Δ^{7}
[b]	9	Stigmasterol,22,23-dihydro-(β-Sitosterol)	41.887	0.42 ± 0.02	Δ^{5},24-ethyl
[b]	10	Ergost-8(14)-en-3-ol, (3 β)-	46.009	0.10 ± 0.01	$\Delta^{8(14)}$

[a] Identified by comparison with authentic sample, [b] Identified by comparison with literature spectra, concentration values expressed as means ± SE

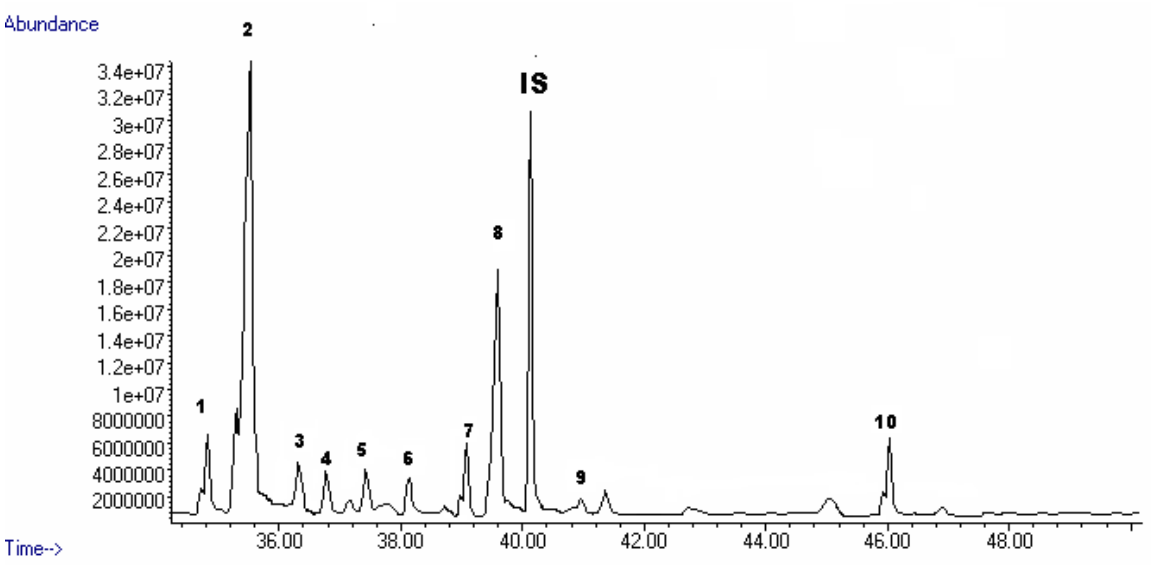


Figure 14: Total ion chromatogram showing sterol profile for the hindgut extracts of 5th instars desert locust fed on wheat seedling and wheat bran

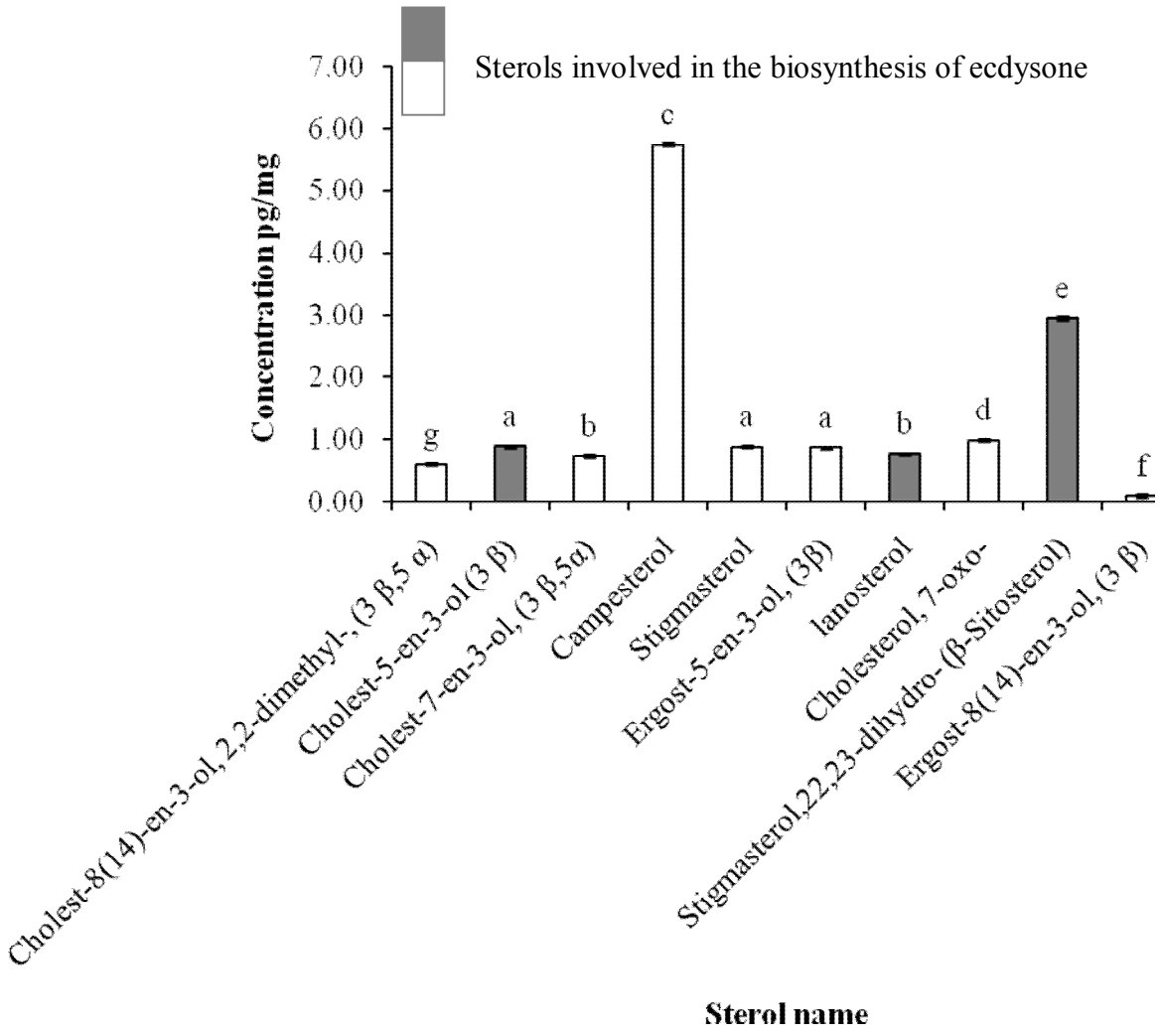


Figure 14.1: Mean concentration of sterols from hindgut of 5th instars desert locust fed on wheat seedling and wheat bran. Bars bearing the same letters are not significantly different (P≤0.05, Tukey's, HSD test).

3.4.2.1 The sterol profile of foregut, midgut and hindgut of the 5th instar desert locust after incubation with cholesterol-[4-^{13}C].

Retention time comparison of cholesterol-[4-^{13}C] and normal cholesterol standard aided in its identification in the sample. Literature comparison of mass spectra was used in the identification. From this experiment cholesterol-[4-^{13}C] and normal cholesterol were significantly transformed to new metabolites in the foregut and midgut but cholesterol remained unchanged in the hindgut. The following sterols shown in Table 8, tic Figure 15 show sterol profile for the foregut and midgut while Figure 16 shows sterol profile for the hindgut after incubation with the labeled cholesterol.

Table 8: Sterol profile for foregut and midgut extracts after incubation with cholesterol-[4-^{13}C] and normal cholesterol.

Peak No.	Compound name	RT (min)	MW (Normal cholesterol)	MW Cholesterol-[4-^{13}C]
1	Cholest-5-en-3-ol (3β)-	35.446	386	387
2	Cholesta-5,7-dien-3-ol, (3β)-	35.760	384	385
3	Cholest-7-en-3-ol, (3 β.5α)	35.939	386	387
4	Cholesta-3,5-dien-7-one	36.633	382	383
5	Cholest-4-en-3-one	37.395	384	385
6	Cholesta-4,6-dien-3-one	37.977	382	383
7	Cholestane-3,6-dione, (5α.,17α., 20S)-	40.866	400	401
8	Cholest-4-ene-3,6-dione	41.113	398	399
9	Cholesterol, 7-oxo-	41.314	400	401

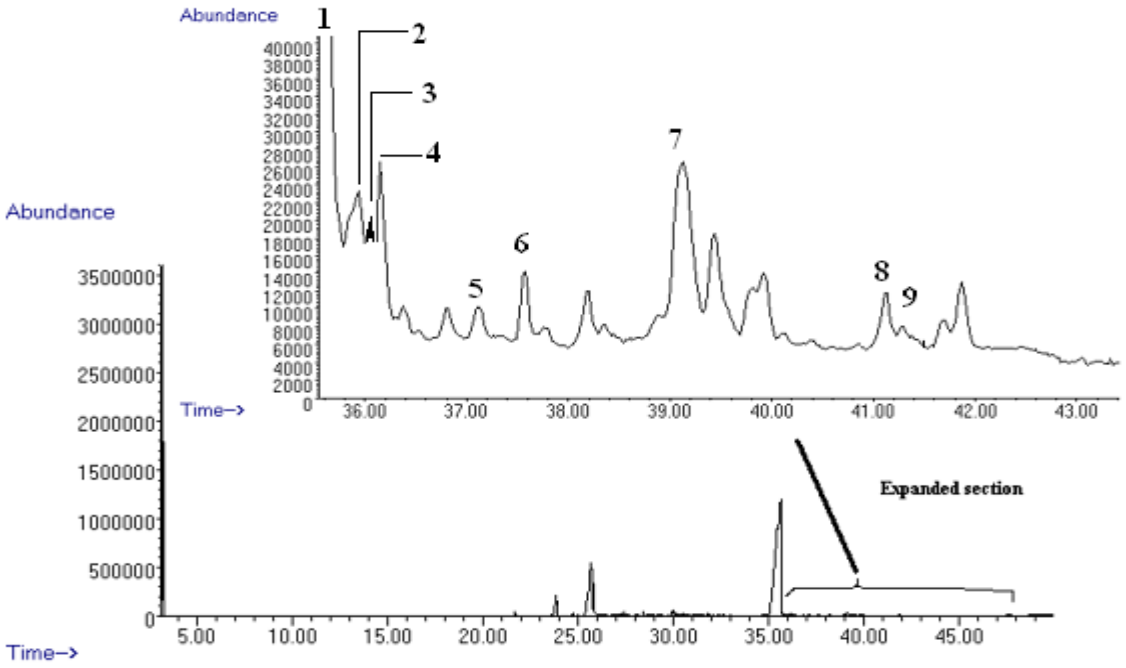


Figure 15: Representative total ion chromatogram showing sterols from foregut and midgut extracts after incubation with cholesterol-[4-^{13}C]

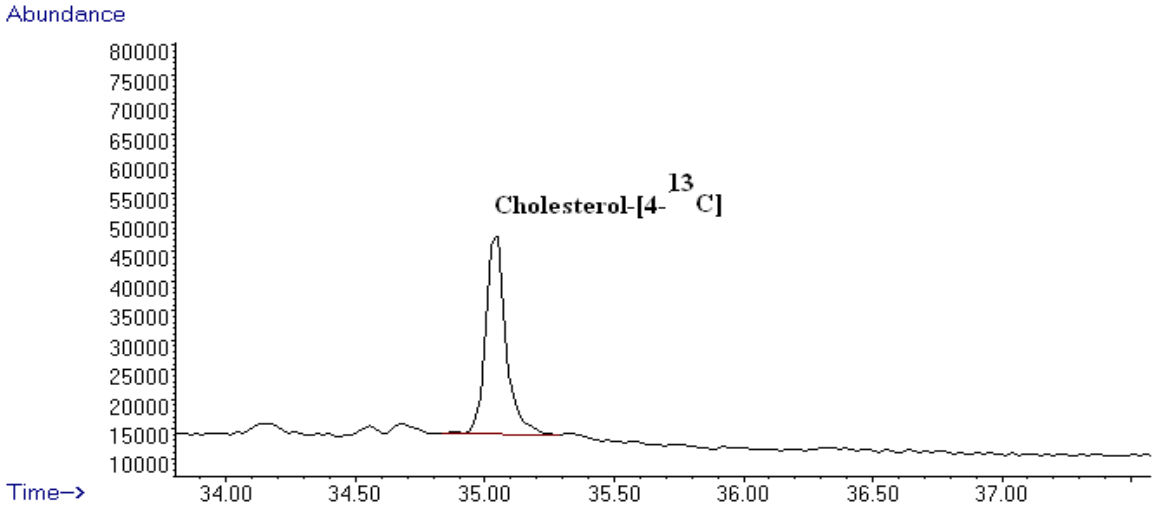


Figure 16: Representative total ion chromatogram showing sterols from hindgut after incubation with cholesterol-[4-^{13}C]

3.5. Results for the Frass extracts from 5th instars desert locust fed on wheat seedlings and wheat bran

Retention time comparison of cholesterol standarded aid in identification of cholesterol in the sample while TMS derivatives were used as confirmatory indicators in identification of other sterols. The following sterols shown in Table 9, tic Figure 17 and mean concentration 17.1 show sterol profiles for the frass extracts from the 5th instars desert locust fed on wheat seedlings and wheat bran. A total of ten sterols were identified similar to the ones found in the hindgut.

From the results, there was a significant ($P \leq 0.001$) difference in the mean amounts of sterols in the frass, with the highest (4.79 ± 0.03 pg/mg) being campesterol and the lowest (0.06 ± 0.01 pg/mg) being ergost-8(14)-en-3-ol,(3β). There were no significant differences in the amounts of sterols; cholest-5-en-3-ol (3β) & stigmasterol & ergost-5-en-3-ol, (3β), cholest-7-en-3-ol, (3 β,5α) & lanosterol.

From the results 6.22 pg/mg of the total sterol found in the frass contain double bond at position 7 in the tetracyclic nucleus (B-ring) and/or position 22 ($\Delta^{7,\ 22}$) and 3.86 pg/mg of the total sterol found in the frass contain double bond at position 5 and or position 24 ($\Delta^{5,\ 24}$).

Table 9: Sterol profile of frass from 5th instars desert locust fed on wheat seedlings and wheat bran.

	Peak no.	Sterols identified	RT (Min)	Mean Conc. (pg/mg)	Δ position
[b]	1	Cholest-8(14)-en-3-ol, 2,2-dimethyl-, (3 β,5 α)	34.008	0.51 ± 0.02	$\Delta^{8(14)}$
[a]	2	Cholest-5-en-3-ol (3 β)	35.553	0.74 ± 0.01	Δ^{5}
[b]	3	Cholest-7-en-3-ol, (3 β,5α)	36.034	0.61 ± 0.00	Δ^{7}
[b]	4	Campesterol	36.507	4.79 ± 0.02	Δ^{5},24-methyl
[b]	5	Stigmasterol	37.001	0.74 ± 0.01	$\Delta^{5,22}$,24-ethy
[b]	6	Ergost-5-en-3-ol, (3β)	38.279	0.74± 0.01	$\Delta^{5,7,22}$,24-methyl
[b]	7	lanosterol	39.503	0.63± 0.01	$\Delta^{8,24}$
[b]	8	Cholesterol, 7-oxo-	39.999	0.82 ± 0.01	Δ^{7}
[b]	9	Stigmasterol,22,23-dihydro-(β-Sitosterol)	41.887	0.42 ± 0.02	Δ^{5},24-ethyl
[b]	10	Ergost-8(14)-en-3-ol, (3 β)-	46.009	0.06 ± 0.01	$\Delta^{8(14)}$

[a] Identified by comparison with authentic sample, [b] Identified by comparison with literature spectra, concentration values expressed as means ± SE

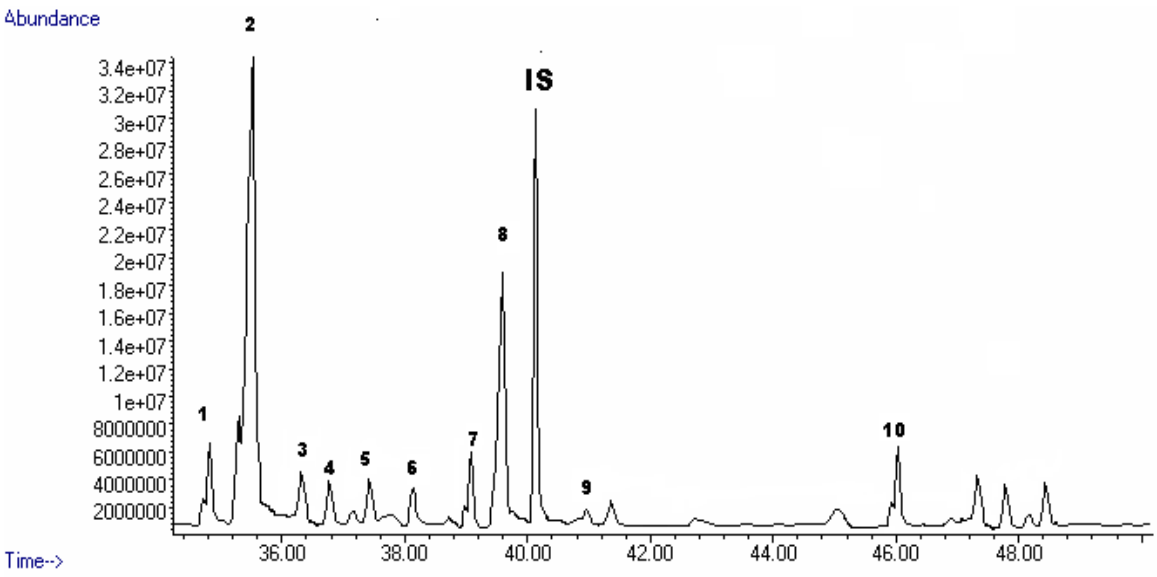


Figure 17: Total ion chromatogram showing sterol profile for the frass extracts from 5th instars desert locust fed on wheat seedlings and wheat bran

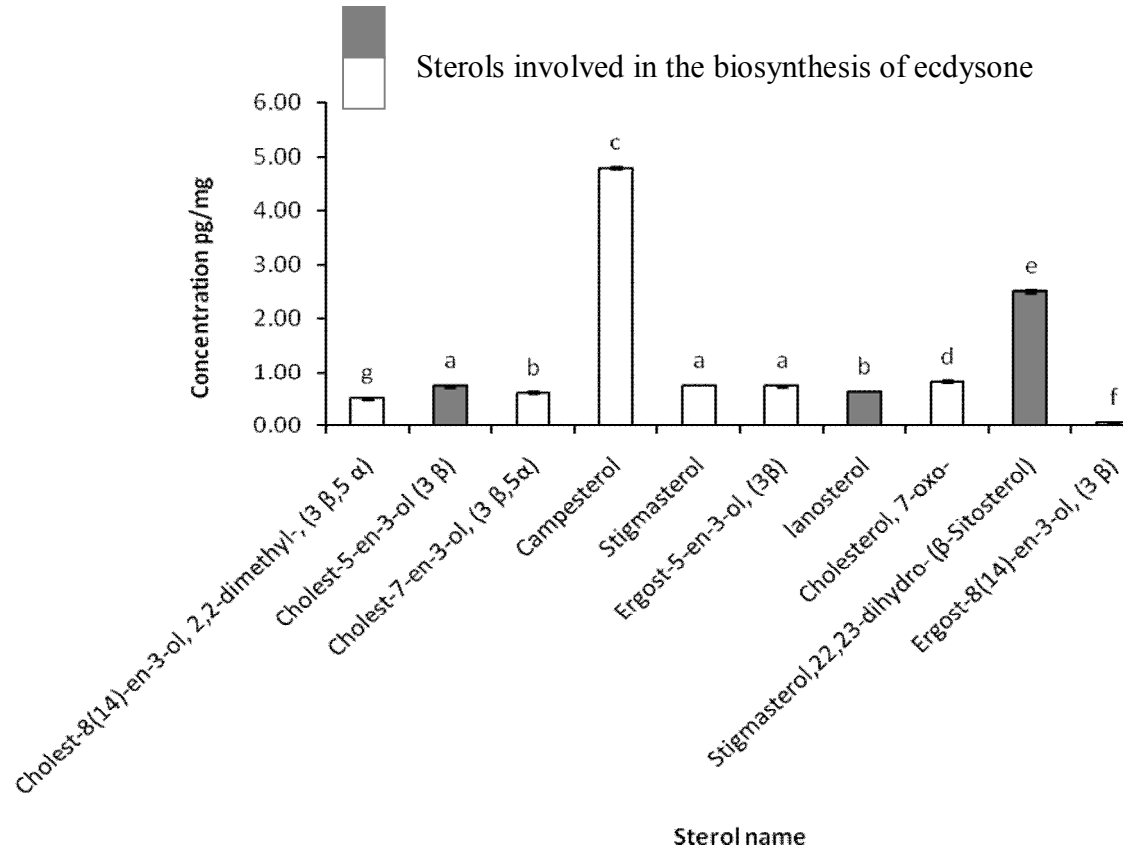


Figure 17.1: Mean concentration of sterols from frass of 5th instars desert locust. Bars bearing the same letters are not significantly different (P≤0.05, Tukey's, HSD test).

3.6 Results for the haemolymph extract from the 5th instars desert locust fed on wheat plant

Retention time comparison of cholesterol standard aided in identification of cholesterol in the sample while TMS derivatives were used as a confirmatory indicator in identification of other sterols. The following sterols shown in Table 10

tic Figure 18 and mean concentration Figure 18.1 show sterol profile for the hemolymph extract from 5^{th} instars desert locust fed on wheat seedlings and wheat bran. A total of eight sterols were identified, this sterols are the same as the ones which were absorbed in the midgut.

From the results, there was a significant ($P \leq 0.001$) difference in the mean amounts of sterols in the hemolymph, with the highest (1.50 ± 0.01 pg/µl) being cholest-5-en-3-ol (3β) and the lowest (0.32 ± 0.01 pg/µl) being cholesta-5,24-diene-3,20-diol, (3β,20R). There were no significant differences in the amounts of sterols; fucosterol & lanosterol.

From the result, 5.62 pg/µl of the total sterol found in the hemolymph contain double bond at position 5 and or position 24 ($\Delta^{5,24}$).

Table 10: Sterol profile for hemolymph extract from the 5th instars desert locust fed on wheat seedlings and wheat bran

	Peak no.	Sterols identified	RT (Min)	Mean Conc. (pg/µl)	Δ position
[a]	1	Cholest-5-en-3-ol (3 β)-	35.553	1.50 ± 0.01	Δ^5
[b]	2	Cholesta-5,7-dien-3-ol, (3 β)-	35.853	0.86 ± 0.02	$\Delta^{5,7}$
[b]	3	Cholesta-8,14,24-diene-3-ol, 4, 4-dimethyl-, (3 β, 5.α)	38.999	0.36 ± 0.01	$\Delta^{8,14,24}$
[b]	4	Lanosterol	39.503	0.52 ± 0.01	$\Delta^{8,24}$
[b]	5	Stigmasterol, 22,23-dihydro-(β-Sitosterol)	41.887	0.32 ± 0.00	Δ^5,24-ethyl
[b]	6	Cholesta-5,24-diene-3,20-diol, (3 β,20R)	42.303	0.75 ± 0.01	$\Delta^{5,24}$
[b]	7	Desmosterol	44.203	0.78 ± 0.01	$\Delta^{5,24}$
[b]	8	Fucosterol	48.003	0.52 ± 0.02	Δ^5,24-ethyl-1-ene

[a] Identified by comparison with authentic sample, [b] Identified by comparison with literature spectra, concentration values expressed as means ± SE

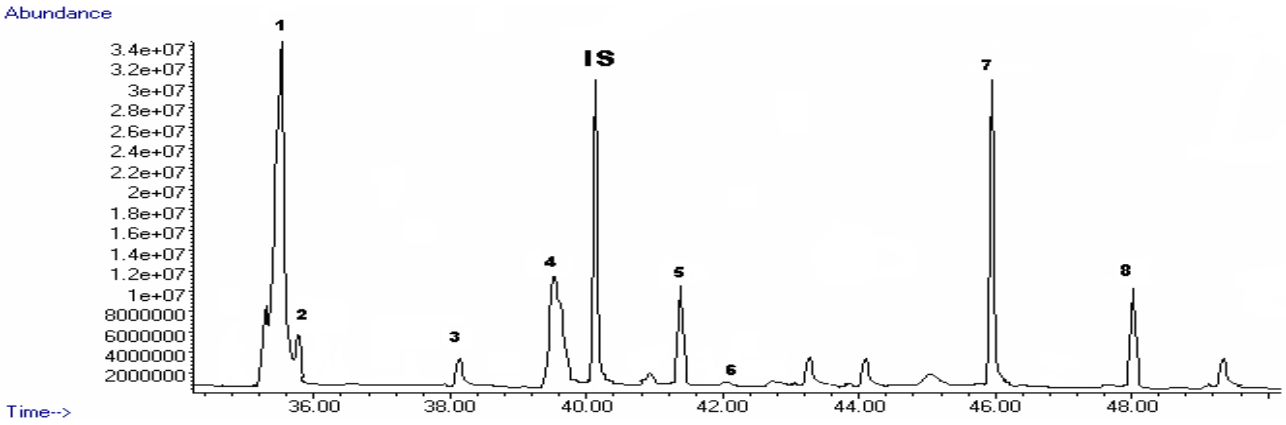


Figure 18: Total ion chromatogram showing sterol profile for hemolymph extracts from the 5th instars desert locust fed on wheat seedlings and wheat bran

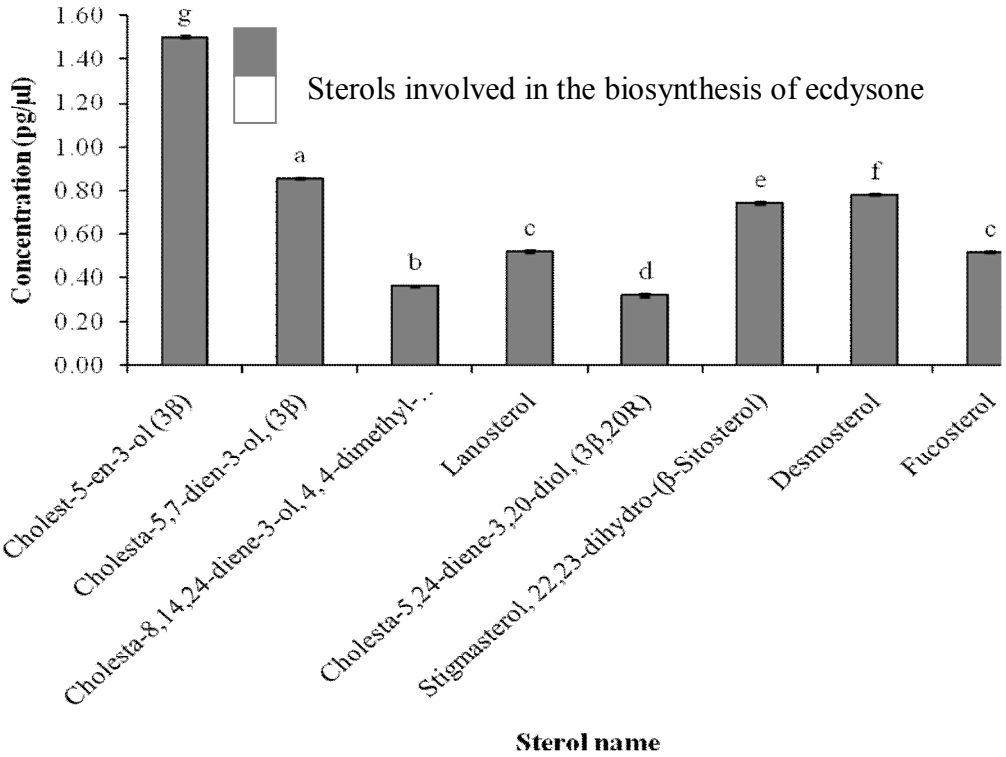


Figure 18.1: Mean concentration of sterols from hemolymph of 5th instars desert locust. Bars bearing the same letters are not significantly different (P≤0.05, Tukey's, HSD test).

3.7 Results for the fat bodies extract from the 5th instars desert locust fed on wheat seedling and wheat bran

Retention time comparison of cholesterol standard aided in identification of cholesterol in the sample while TMS derivatives were used as confirmatory indicators in identification of other sterols. The following sterols shown in Table 11, Total ion chromatogram Figure 19 and mean concentration Figure 19.1 show sterol profile of the fat bodies extracts from 5th instars desert locust fed on wheat seedlings and wheat bran.

From the results, there was a significant ($P \leq 0.001$) difference in the mean amounts of sterols in the fat bodies, with the highest (1.88 ± 0.003 pg/mg) being cholest-5-en-3-ol (3β) and the lowest (0.06± 0.002 pg/mg) being cholesta-5,24-diene-3,20-diol, (3β,20R). There were no significant differences in the amounts of sterols; lanosterol & desmosterol.

Based on the amounts found the following can be deduced; 5.12 pg/mg of the total sterol found in the fat bodies contain double bond at position 5 and or position 24 ($\Delta^{5,24}$).

Table 11: Sterol profile of fat bodies extract from the 5th instars desert locust fed on wheat seedling and wheat bran.

	Peak no.	Sterols identified	RT (Min)	Mean Conc. (pg/mg)	Δ position
[a]	1	Cholest-5-en-3-ol (3 β)-	35.553	1.88 ± 0.003	Δ^5
[b]	2	Cholesta-5,7-dien-3-ol, (3 β)-	35.853	0.92 ± 0.004	$\Delta^{5,7}$
[b]	3	Cholesta-8,14,24-diene-3-ol, 4, 4-dimethyl-, (3β,5α)	38.999	0.10 ± 0.004	$\Delta^{8,14,24}$
[b]	4	Lanosterol	39.503	0.23 ± 0.003	$\Delta^{8,24}$
[b]	5	Stigmasterol, 22,23-dihydro-(β-Sitosterol)	41.887	0.06 ± 0.002	Δ^5,24-ethyl
[b]	6	Cholesta-5,24-diene-3,20-diol, (3 β,20R)-	42.303	1.25 ± 0.003	$\Delta^{5,24}$
[b]	7	Desmosterol	44.203	0.24 ± 0.002	$\Delta^{5,24}$
[b]	8	Fucosterol	48.003	0.44± 0.003	Δ^5,24-ethyl-1-ene

[a] Identified by comparison with authentic sample, [b] Identified by comparison with literature spectra, concentration values expressed as means ± SE

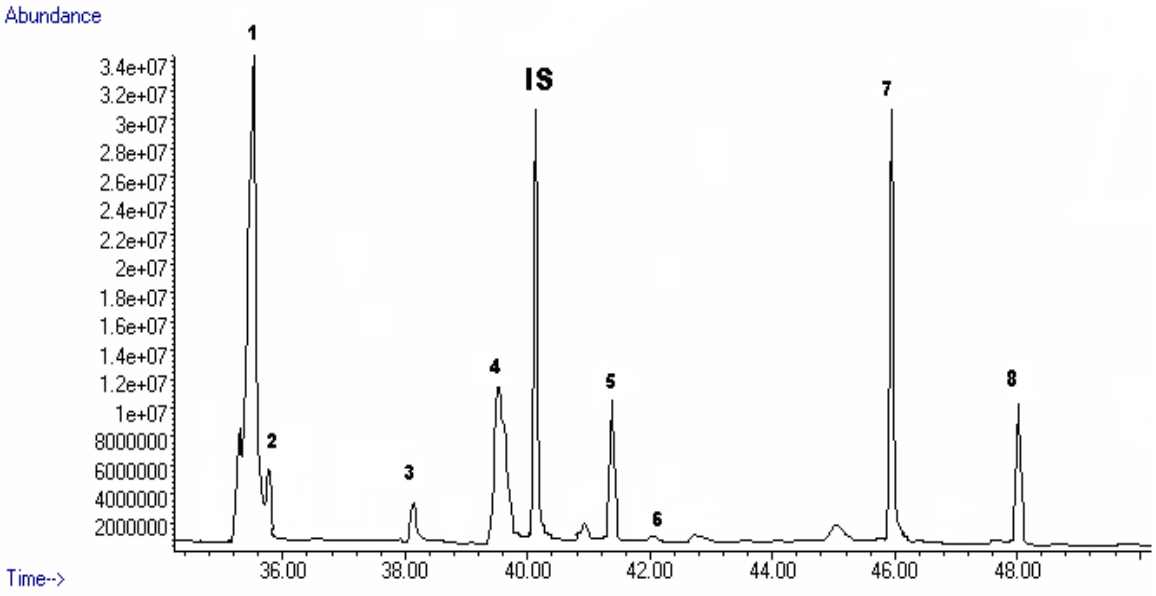


Figure 19: Total ion chromatogram showing sterol profile for fat bodies extracts from the 5th instars desert locust fed on wheat plant and wheat bran

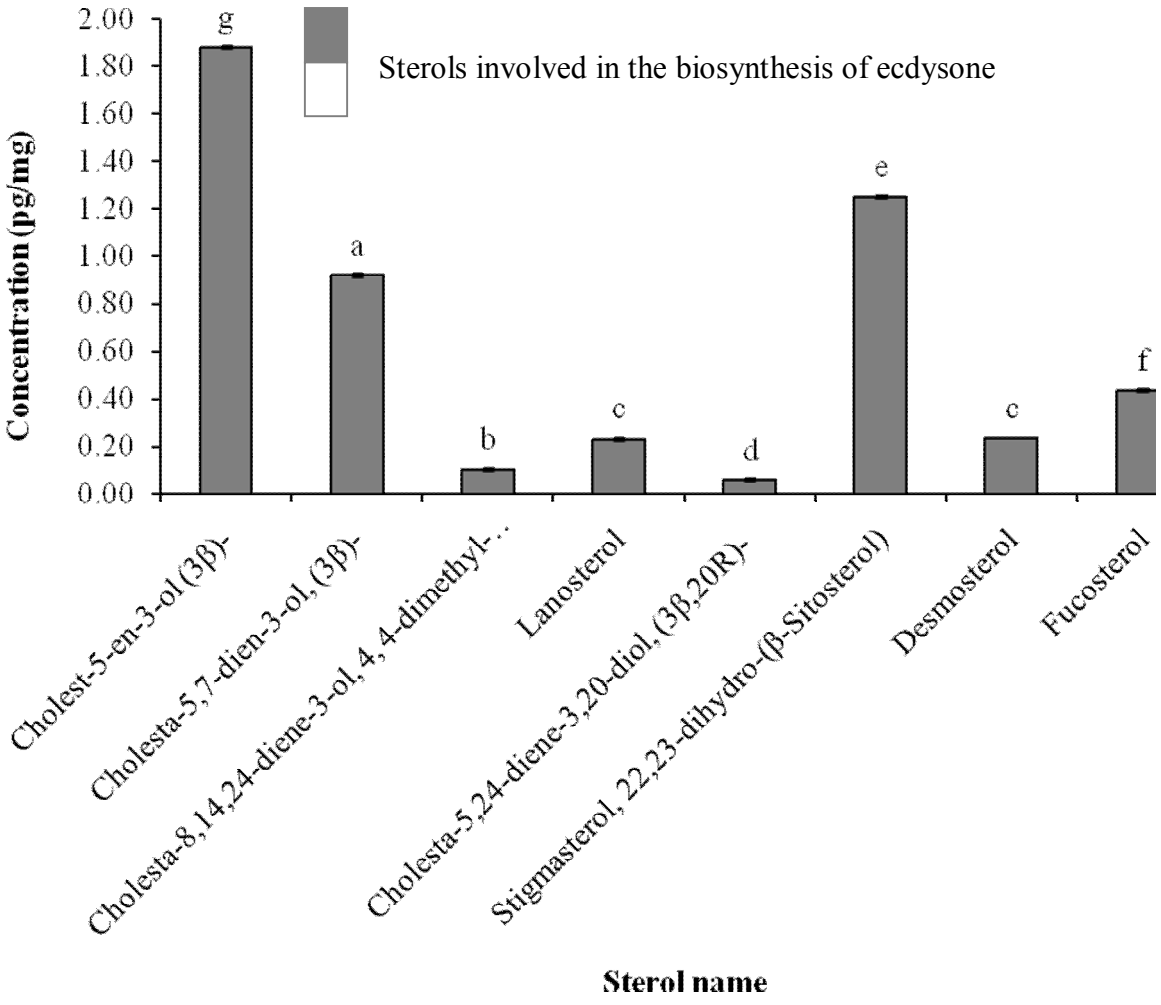


Figure 19.1: Mean concentration of sterols from fat bodies of 5th instars desert locust. Bars bearing the same letters are not significantly different (P≤0.05, Tukey's, HSD test).

3.8 Results for the prothoracic glands from the 5th instar desert locust fed on wheat seedlings and wheat bran.

Retention time comparison of cholesterol standard aided in identification of cholesterol in the sample while TMS derivatives were used as confirmatory indicators in identification of other sterols. The following sterols shown in Table 12, tic Figure 20 and mean concentration Figure 20.1 show sterol profile from pg extracts of 5th instars desert locust fed on wheat seedlings and wheat bran. A total of four sterols were identified.

From the results, there was a significant ($P \leq 0.001$) difference in the mean amounts of sterols in the PG, with the highest (0.699± 0.003 pg/mg) being cholest-5-en-3-ol (3β) and the lowest (0.132± 0.001 pg/mg) being Cholesta-5,7-dien-3-ol, (3β.).

Based on the amounts found the following can be deduced; 0.81 pg/mg of the total sterol found in the PG contain double bond at position 5 and or position 24 ($\Delta^{5,24}$).

Table 12: Sterol profile for the prothoracic glands from 5th instar desert locust fed on wheat seedlings and wheat bran

	Peak no.	Sterols identified	RT (Min)	Mean conc. (pg/mg)	Δ position
[a]	1	Cholest-5-en-3-ol (3 β)-	35.553	0.699 ± 0.003	Δ^5
[b]	2	Cholesta-5,7-dien-3-ol, (3β)	35.853	0.132 ± 0.002	$\Delta^{5,7}$
[b]	3	Cholesta-7-en-6-one-3,14-diol (3β)	47.008	0.169± 0.001	Δ^7
[b]	4	Cholesta-7-en-6-one-3,14,25-triol (3β)	49.876	0.421 ± 0.002	Δ^7

[a] Identified by comparison with authentic sample, [b] Identified by comparison with literature spectra, concentration values expressed as means ± SE

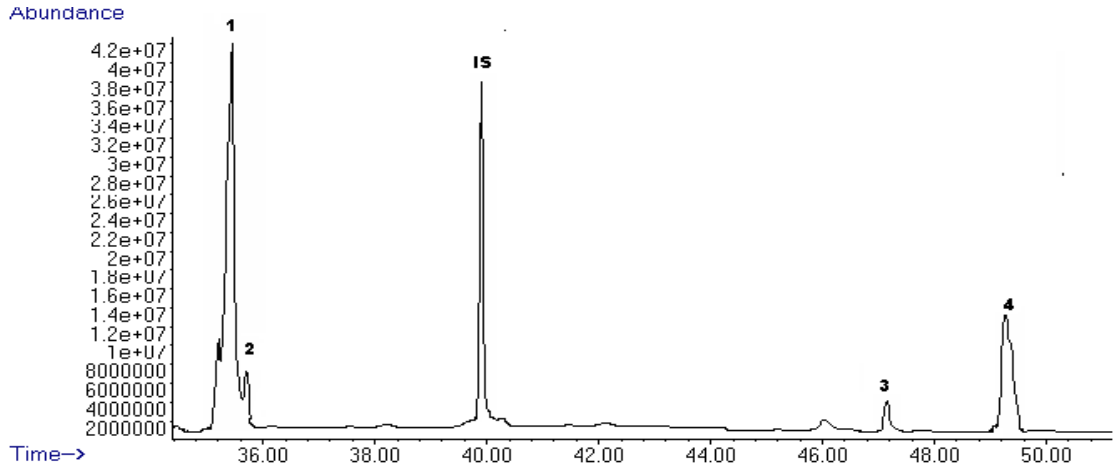


Figure 20: Total ion chromatogram showing sterol profile for the prothoracic glands from the 5th instar desert locust fed on wheat seedlings and wheat bran

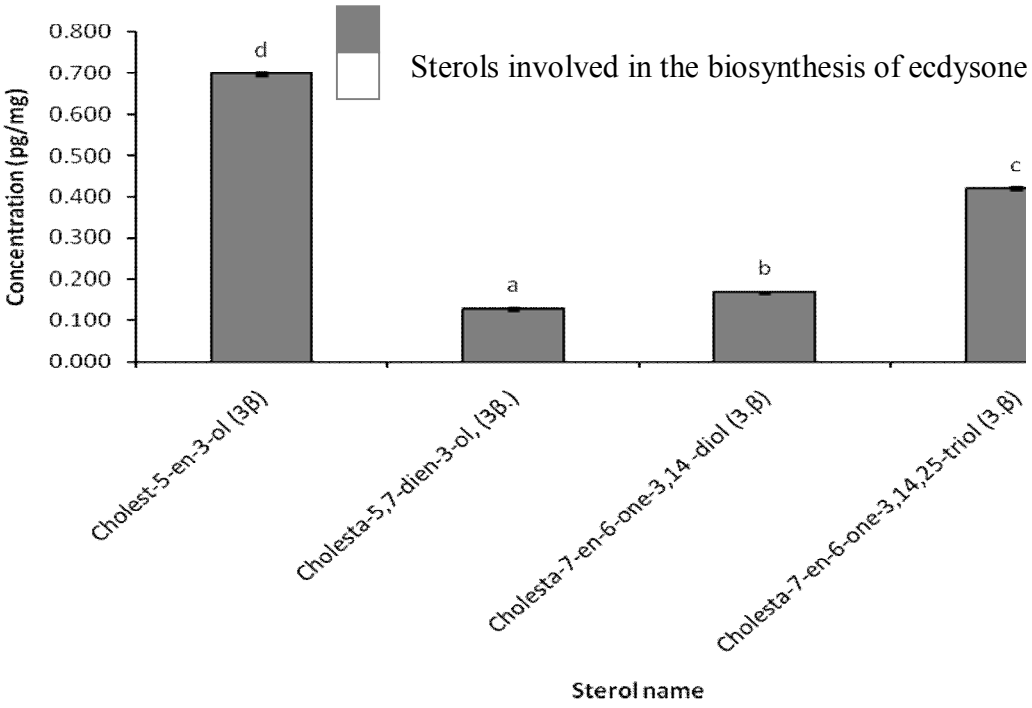


Figure 20.1: Mean concentration of sterols from prothoracic gland of 5th instars desert locust. Bars bearing the same letters are not significantly different (P≤0.05, Tukey's, HSD test).

3.9 Mass spectra Data analysis for sterols of interest

Mass spectra and structures of the sterols of interest found in the various stages of analysis including incubation experiments which incooperated the use of cholesterol-[4-^{13}C] and normal cholesterol are discussed in this section. The order is as follows: lanosterol; cholesta-8,14,24-triene-3-ol, 4,4-dimethyl, (3β,5α); cholesta-5,24-diene-3, 20-diol,(3β,20R); β-sitosterol; fucosterol; desmosterol; cholest-5-en-3-ol (3β); cholesta-5, 7-dien-3-ol, (3β); 5 β–ketodiol; cholesta-7-ene-6-one-3, 14, 25-triol (3β).

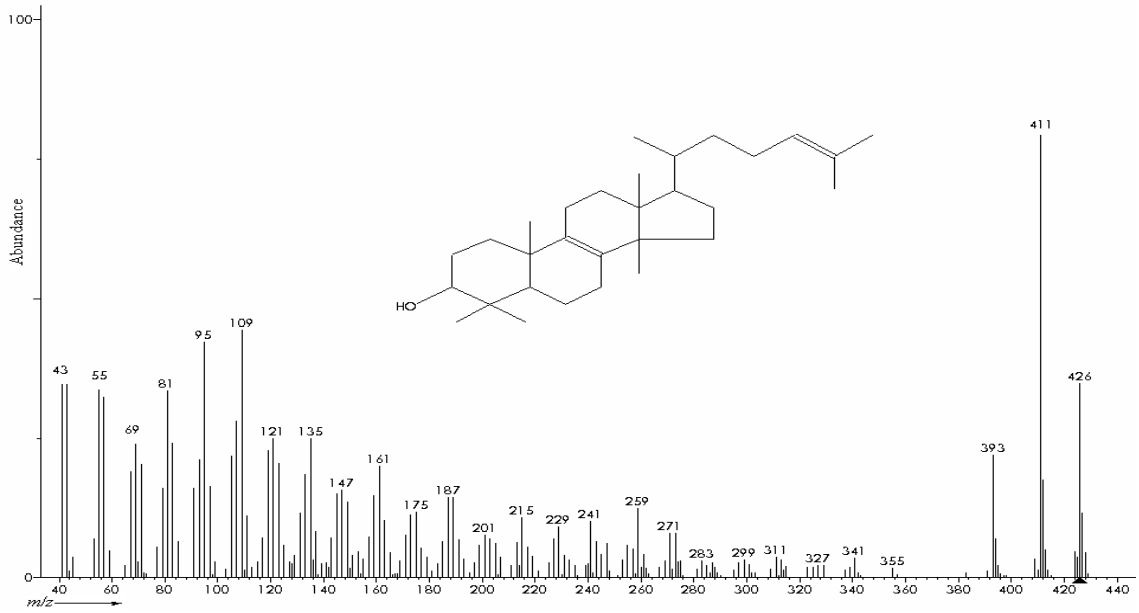
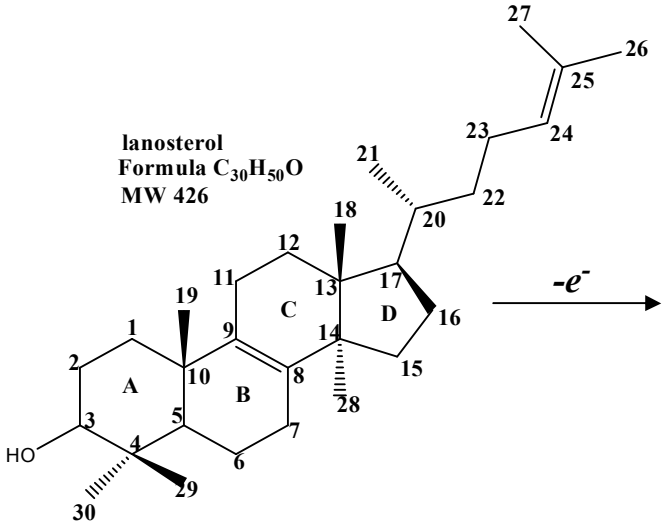
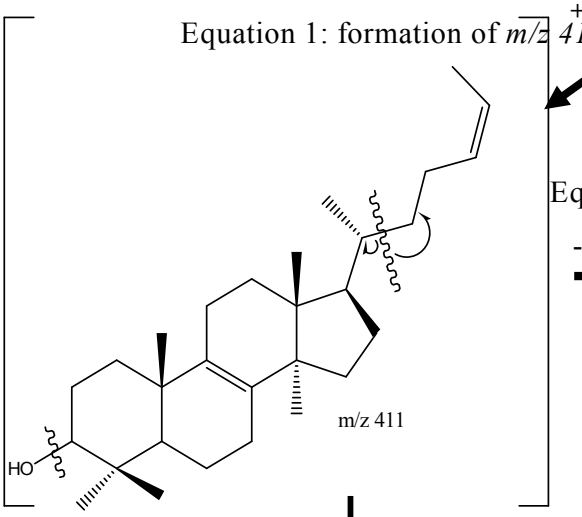


Figure 21: MS and structure for lanosterol

Lanosterol has a molecular weight of 426. The MS (Fig 21) displayed a molecular ion peak at *m/z* 426 [34%, M^+]. The base ion peak occurred at *m/z* 411 [90%, M-15]. Other prominent peaks appeared at *m/z,* 393 [22%, M-33]$^+$, 135 [25%, M-291]$^+$, 109 [44%, M-317]$^+$, 95 [42%, M-331]$^+$, 81 [33%, M-345]$^+$, 57 [32%, M-369]$^+$, 55 [64%, M-371]$^+$, 43 [91%, M-383]$^+$, 41 [64%,M-385]$^+$.

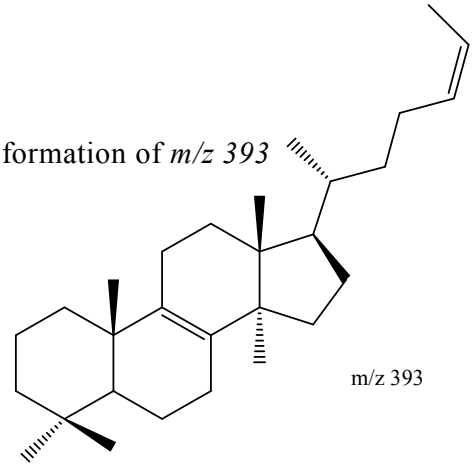


Equation 1: formation of *m/z 411*



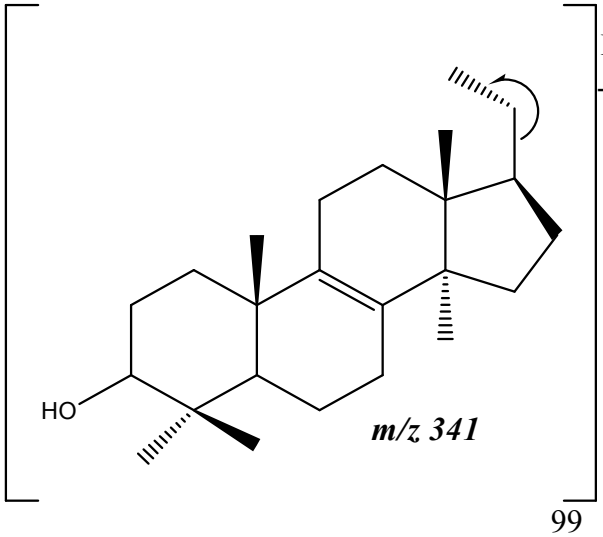
Equation 2: formation of *m/z 393*

- H_2O



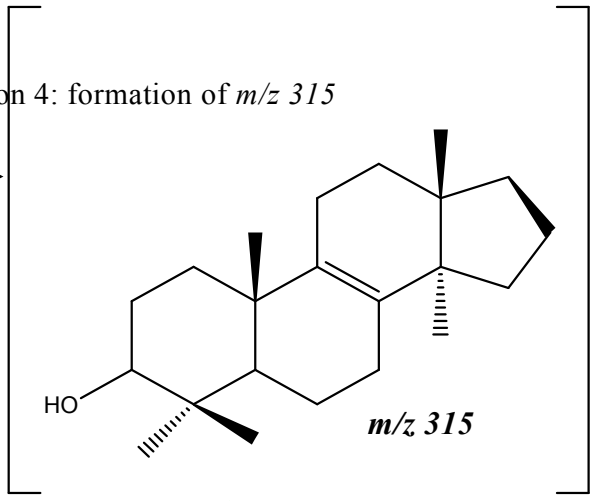
-

Equation3: formation of *m/z 341*



Equation 4: formation of *m/z 315*

- C_2H_5



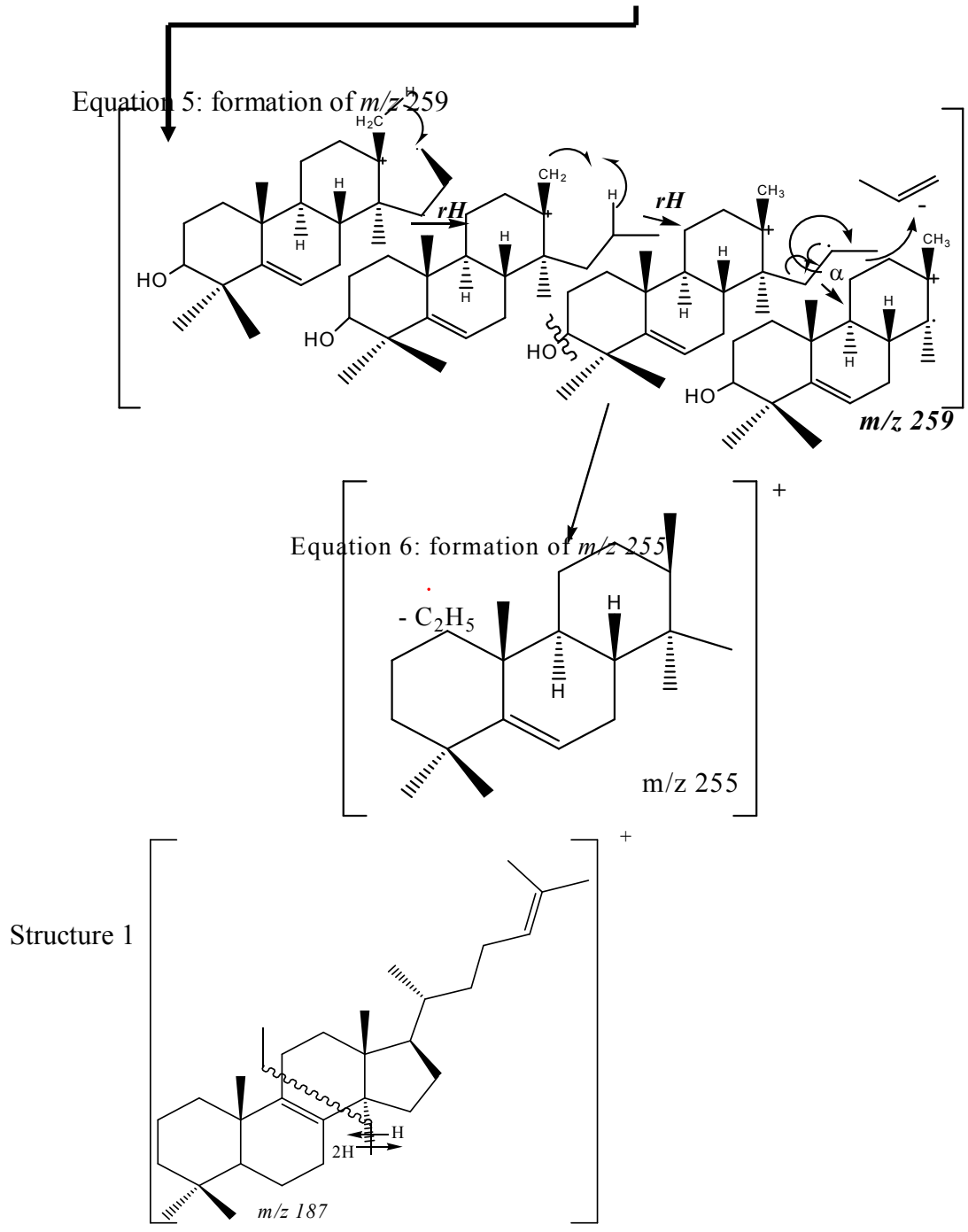


Figure 21.1: Possible fragmentation patterns for formation of m/z 109,135,393,411 and 95 in lanosterol

In the mass spectrum of lanosterol (Figure 21.1) , the $[M - CH_3]^+$, (equation 1) arises from the loss of methyl group at any of the following positions; C 30 (a), C 29 (b), C 19 (c), C 18 (d) , C 21 (e), C 26 (f), C 27 (g), C 28 (h) giving rise to *m/z* 411. The [M $–CH_3–H_2O$]$^+$, *m/z* 393 (equation 2) arise from the loss of water through a metastable reaction occurring at the source that is dehydration occurs before ionization producing spurious peak due to hot metallic inlet system. Loss of C_5H_{10} (equation 3) by the exocyclic fragmentation of the 20-22 bond explains the presence of *m/z* 341 while the loss of ethyl radical at cleavage of 17-20 bond (equation 4) yields *m/z* 315. The 13-17 cleavage of the D ring produces a stable tertiary carbonium ion. Radical site of this species can proceed either through reciprocal hydrogen transfer and 14-15 cleavage (equation 5) to yield the characteristic m/z 259 or through loss of ethyl radical and hydroxyl group (equation 6) giving rise to peak with m/z 255. Finally the m/z 187 ion, structure 1 is composed mainly of rings A and B but is formed in a complex mechanism operating in conjunction with a triple hydrogen transfer under the influence of stereochemistry of A/B rings (Seifert *et al*. 1972).

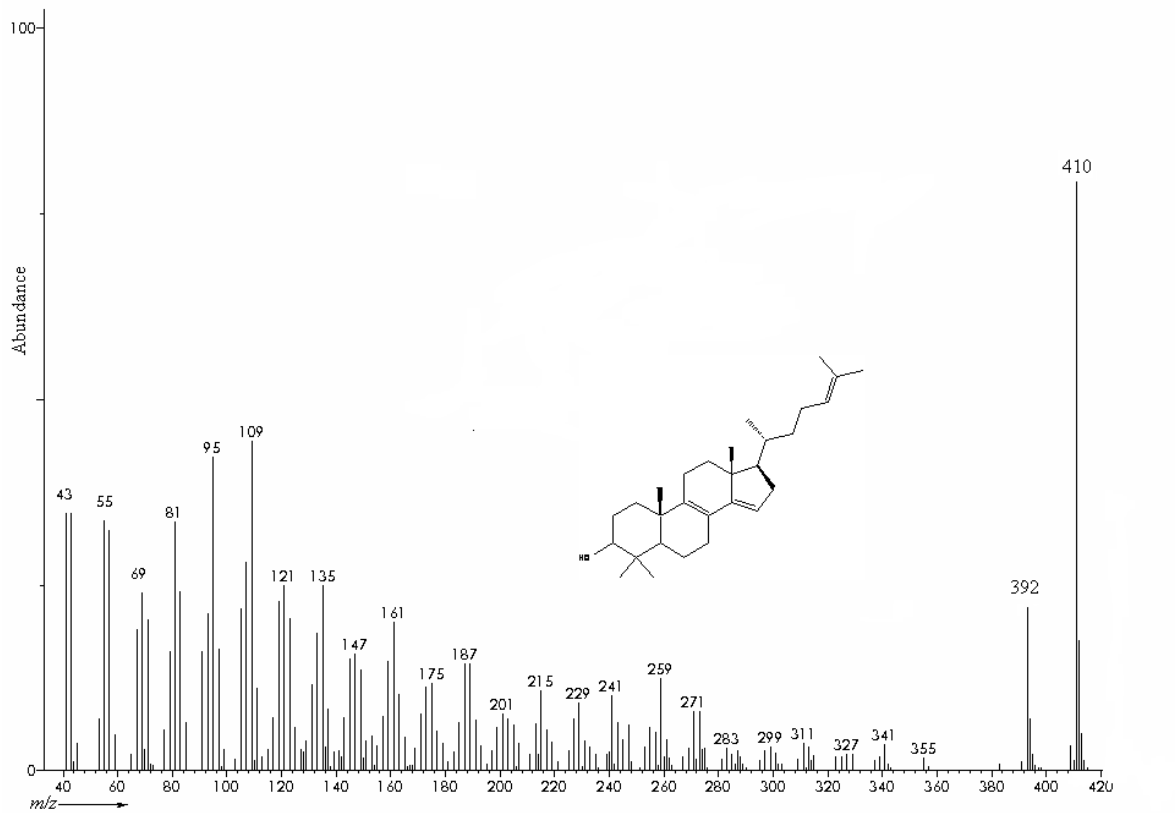
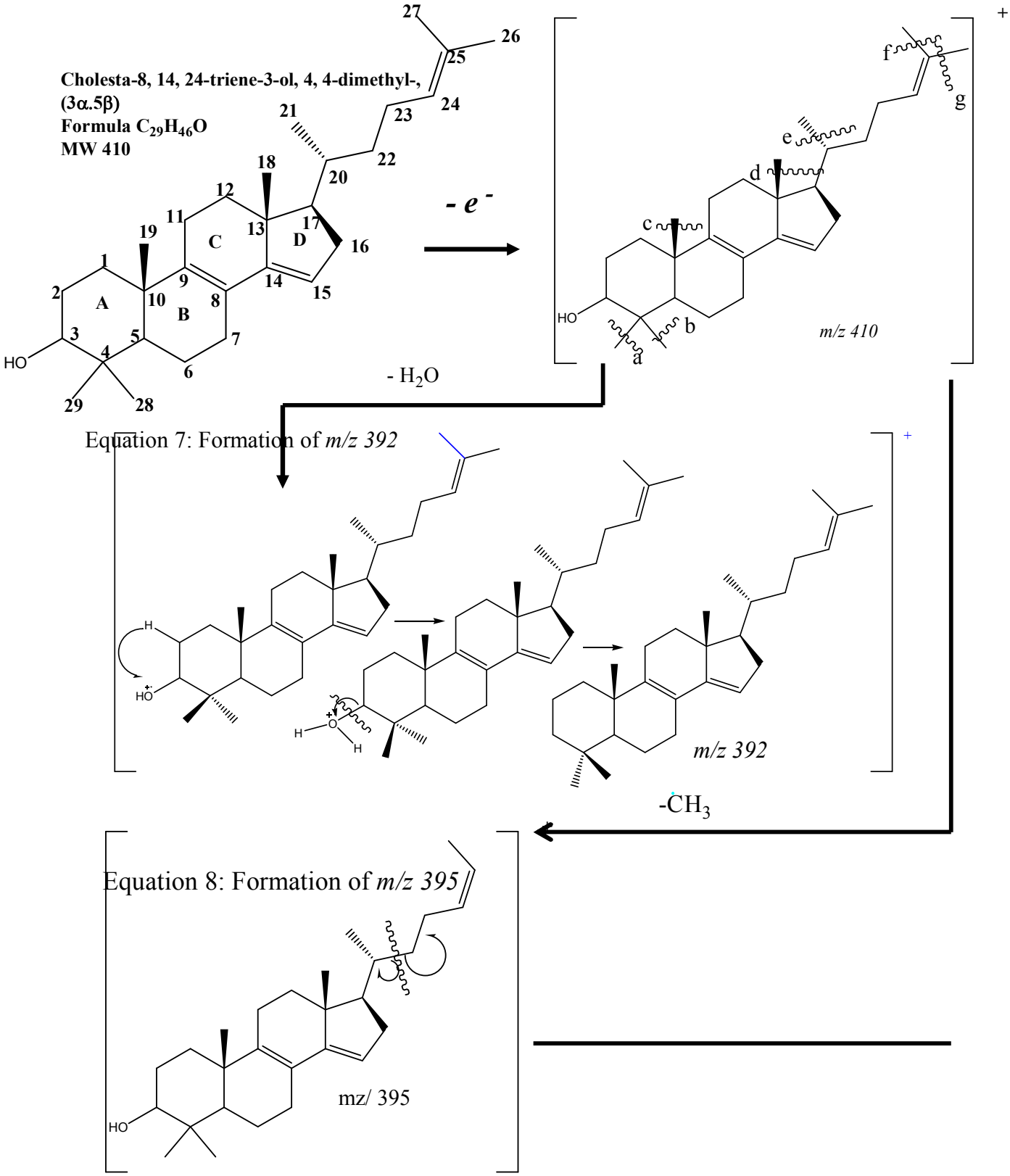


Figure 22: MS and structure for cholesta-8, 14, 24-triene-3-ol, 4, 4-dimethyl,(3β, 5α)

Cholesta-8,14,24 -triene-3-ol, 4,4-dimethyl-,(3β,5α) - has a molecular weight of 410. The MS (Fig 22) displayed a molecular ion peak at 410 [80%, M^+]. The base ion peak occurred at *m/z* 410 [99%, M]. Other prominent peaks appeared at *m/z* 392 [20%, M-18] $^+$, 395 [5%, M-15] $^+$, 325 [10%, M-85] $^+$, 299 [10%, M-111] $^+$.



Equation 7: Formation of *m/z 392*

Equation 8: Formation of *m/z 395*

Equation 9: Formation of *m/z 325*

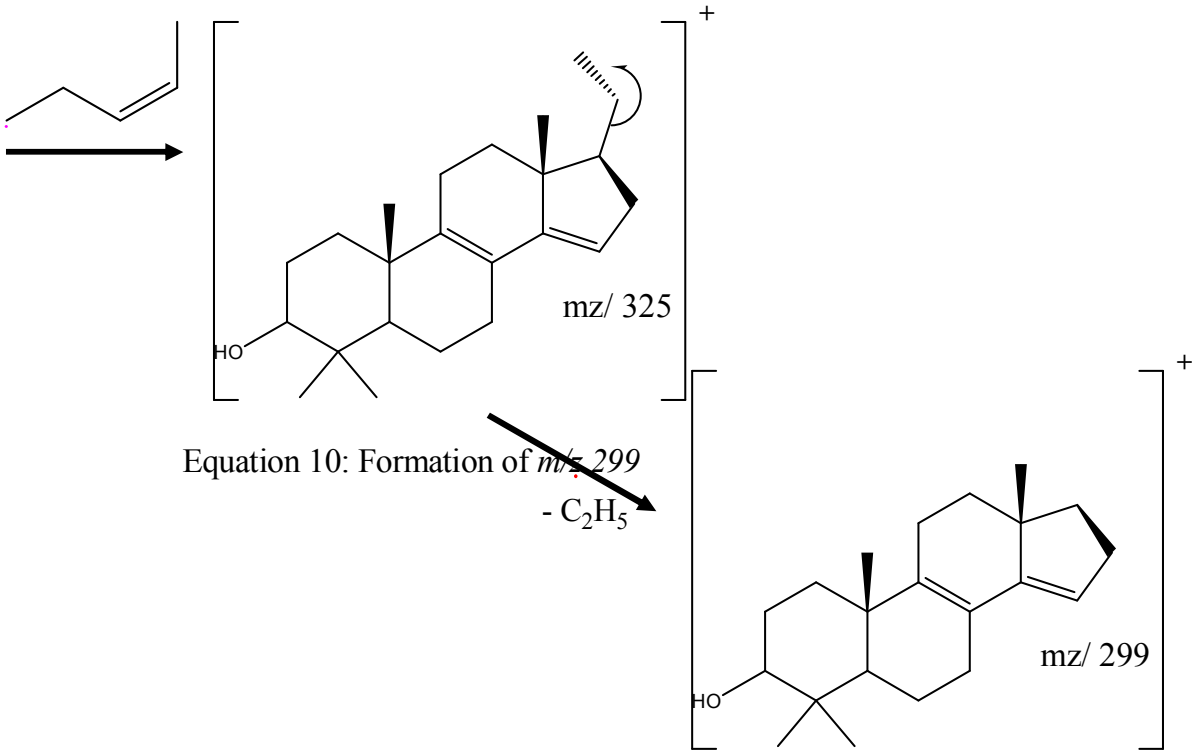


Figure 22.1: Possible fragmentation patterns for formation of m/z 410, 395, 392, 325,299 in cholesta-8, 14, 24-triene-3-ol, 4, 4-dimethyl-, (3 β,5α)

In the mass spectrum of cholesta-8,14,24-triene-3-ol,4,4-dimethyl,(3β,5α) (Fig 22.1), the $[M–H_2O]^+$, *m/z* 392 (equation 7) arise from the loss of water. The $[M–CH_3]^+$, (equation 8) arises from the loss of methyl group at any of the following positions; C 29 (a), C 28 (b), C 19 (c), C 18 (d), C 21 (e), C 26 (f), C 27 (g) giving rise to *m/z* 395. Loss of C_5H_{10} (equation 9) by the exocyclic fragmentation of the 20-22 bond explains the presence of *m/z* 325 while the loss of ethyl radical from cleavage of 17-20 bond (equation 10) yields *m/z* 299.

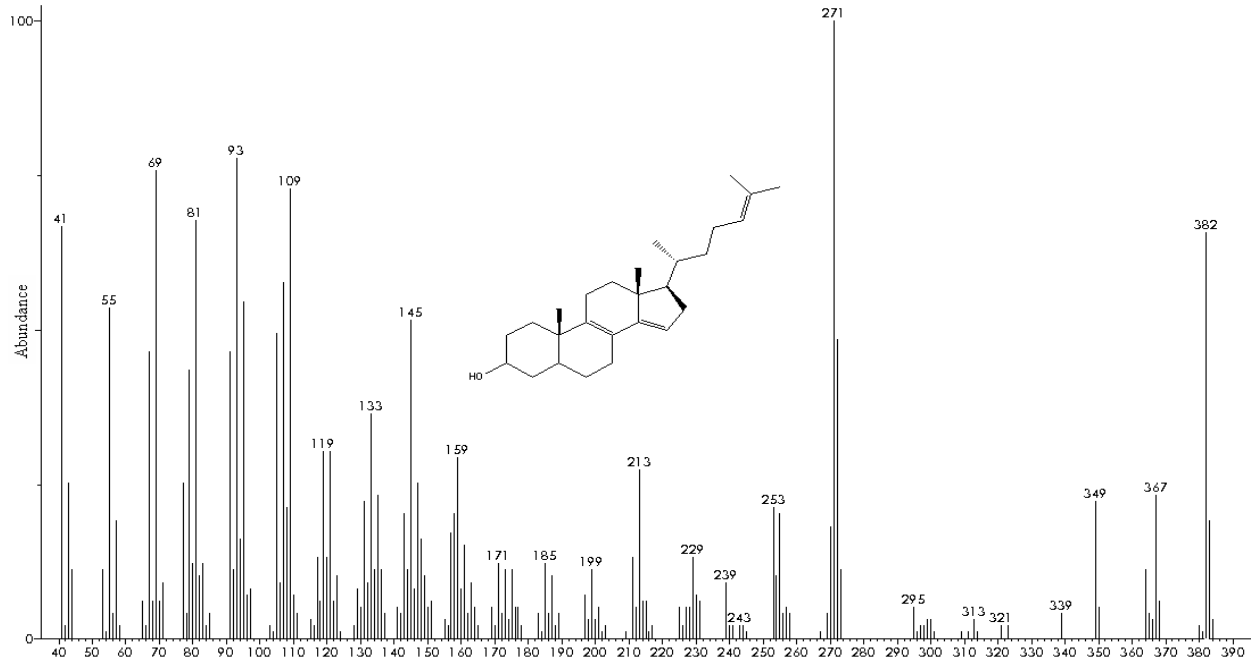
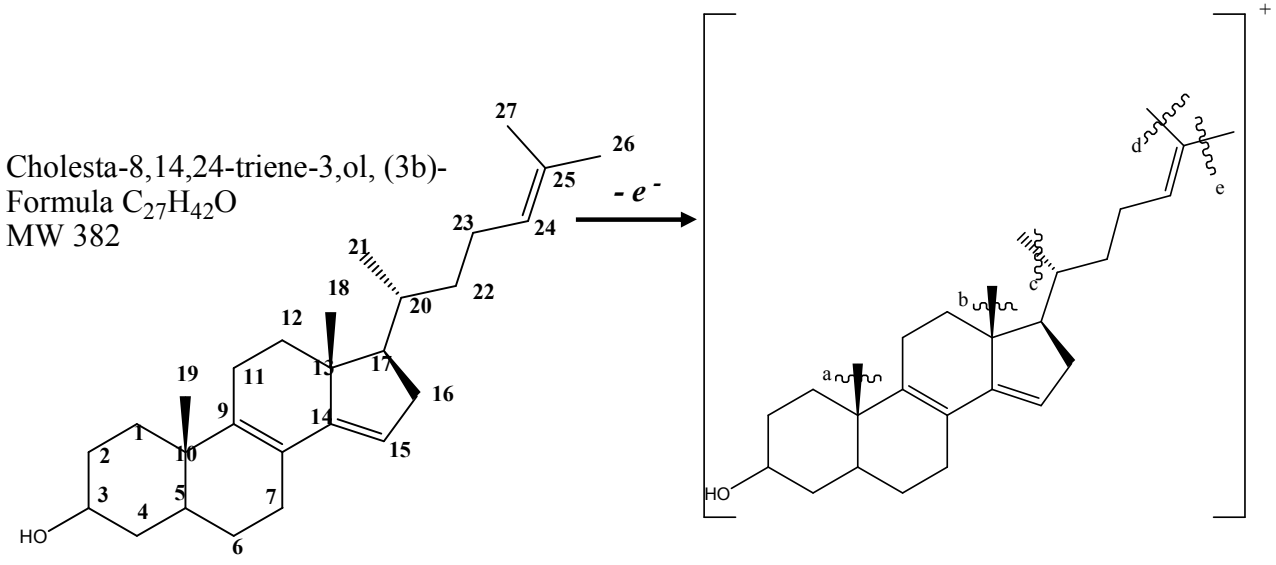


Figure 23: MS and cholesta-8,14,24-triene-3,ol, (3β)-

Cholesta-8,14,24-triene-3,ol, (3 β)- has a molecular weight of 382. The MS (Fig 23) showed the base ion peak occurred at m/z 271 [99%, M-111] $^{+}$. Other peaks appeared at m/z 364 [24%, M-18] $^{+}$, 367 [35%, M-15] $^{+}$, 349 [35%, M-33] $^{+}$, 299.



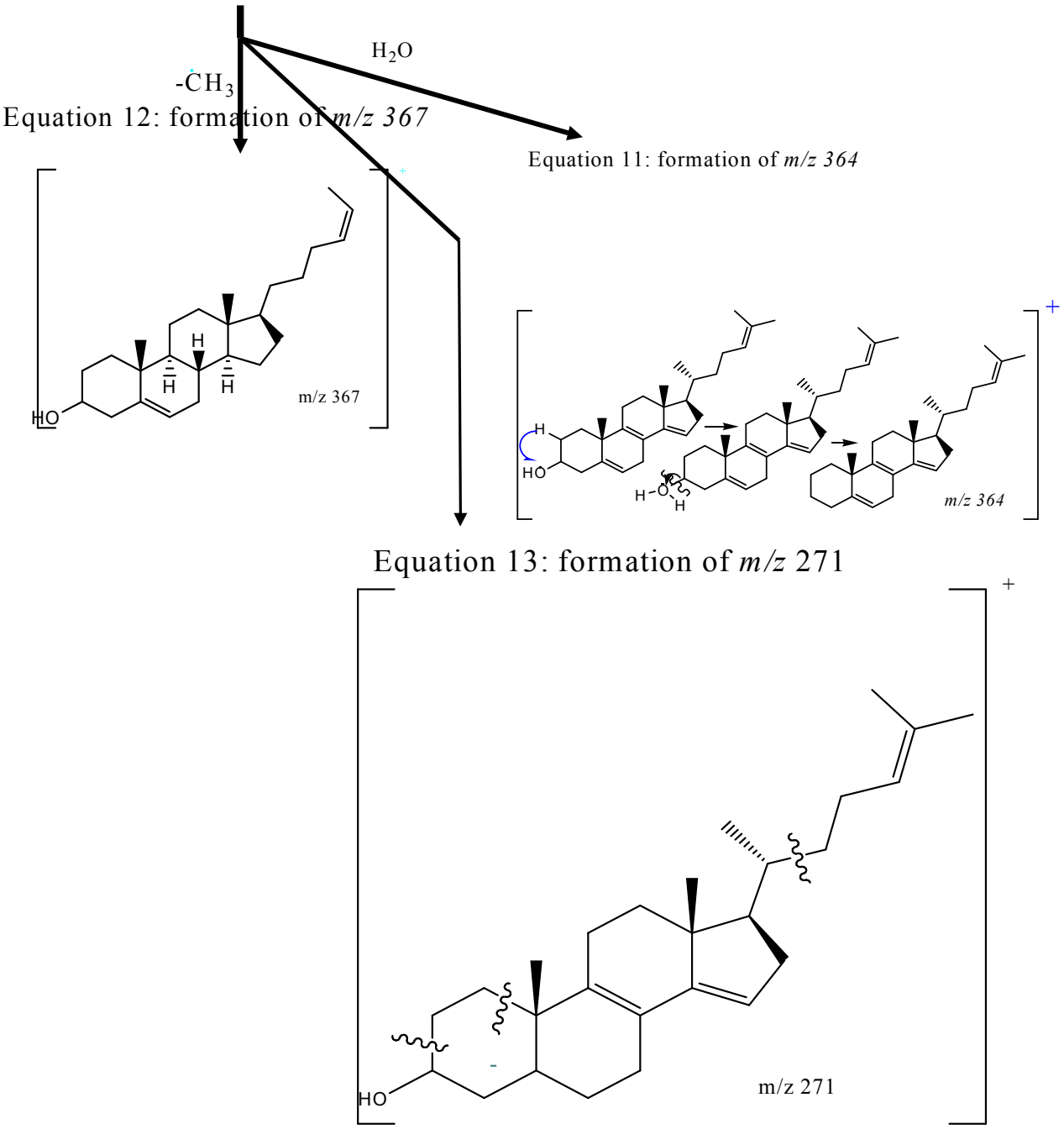


Figure 23.1: Possible fragmentation patterns for formation of m/z 271, 364, and 367 in cholesta-8,14,24-triene-3,ol,(3β)

In the mass spectrum of cholesta-8,14,24-triene-3,ol, (3β)- (Fig 23.1) , the $[M - H_2O]^+$, *m/z* 364 (equation 11) arise from the loss of water. The $[M - CH_3]^+$, (equation 12) arises from the loss of methyl group at any of the following positions; C 19 (a), C (b), C 21 (c), C 26 (d), C 27 (e) giving rise to *m/z* 385. When the following bonds beak C 2-3, C 1-10 and C 20-21 bond it yields *m/z* 271.

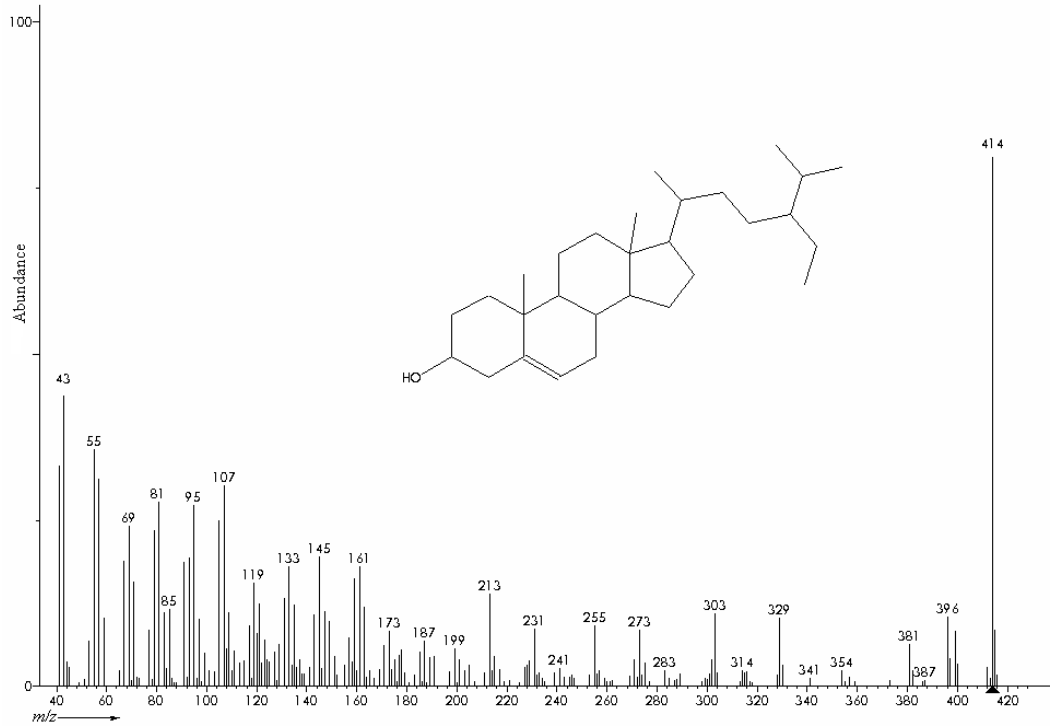
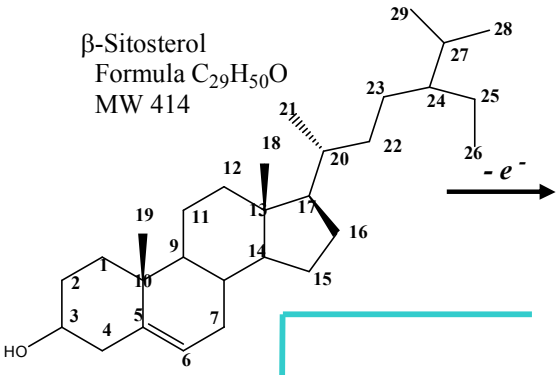


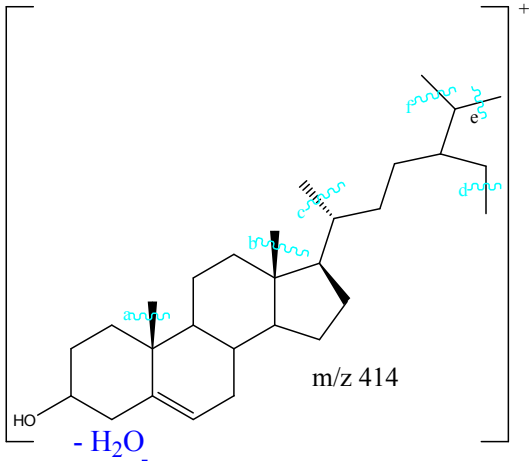
Figure 24: MS and structure for β-sitosterol

β-sitosterol has a molecular weight of 414. The MS (Figure 24) displayed a molecular ion peak at 414 [90%, M^+]. The base ion peak occurred at *m/z* 414 [90%, M]. Other prominent peaks appeared at *m/z* 396 [10%, M-18], 381[6%, M-33], 354 [2%, M-60], 329 [9%, M-85], 303 [11%, M-111], 314 [3%, M-100], 273 [8%, M-141].

β-Sitosterol
Formula $C_{29}H_{50}O$
MW 414

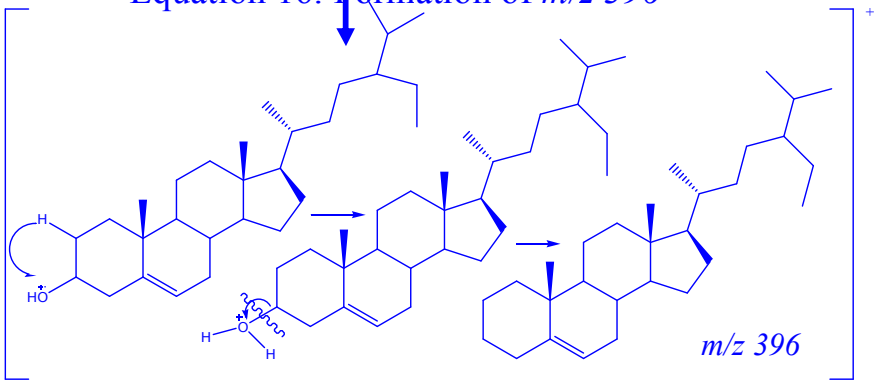


$- e^-$



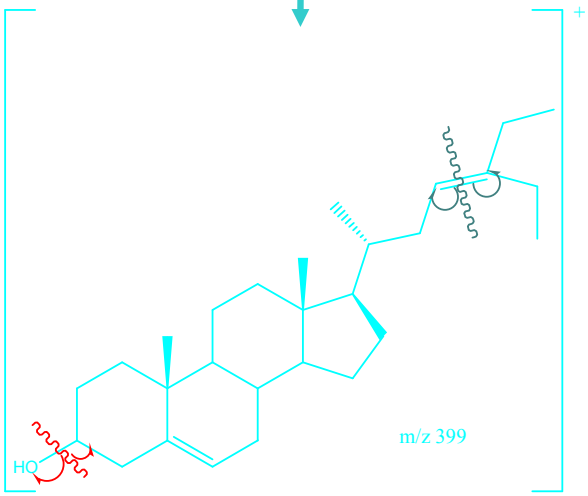
$- H_2O$

Equation 16: Formation of *m/z 396*



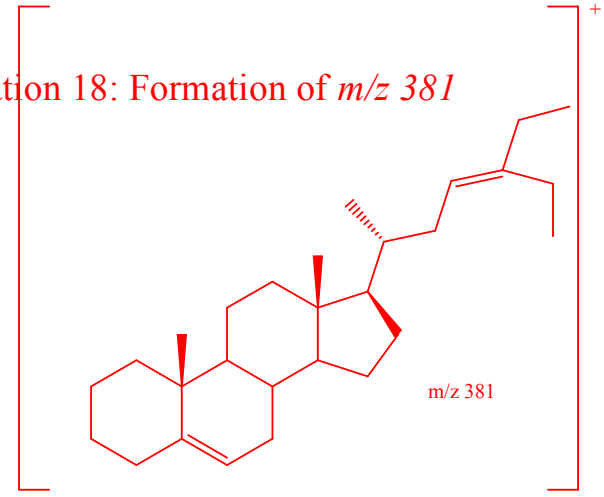
$-\dot{C}H_3$

Equation 17: Formation of *m/z 399*



Equation 18: Formation of *m/z 381*

$- H_2O$



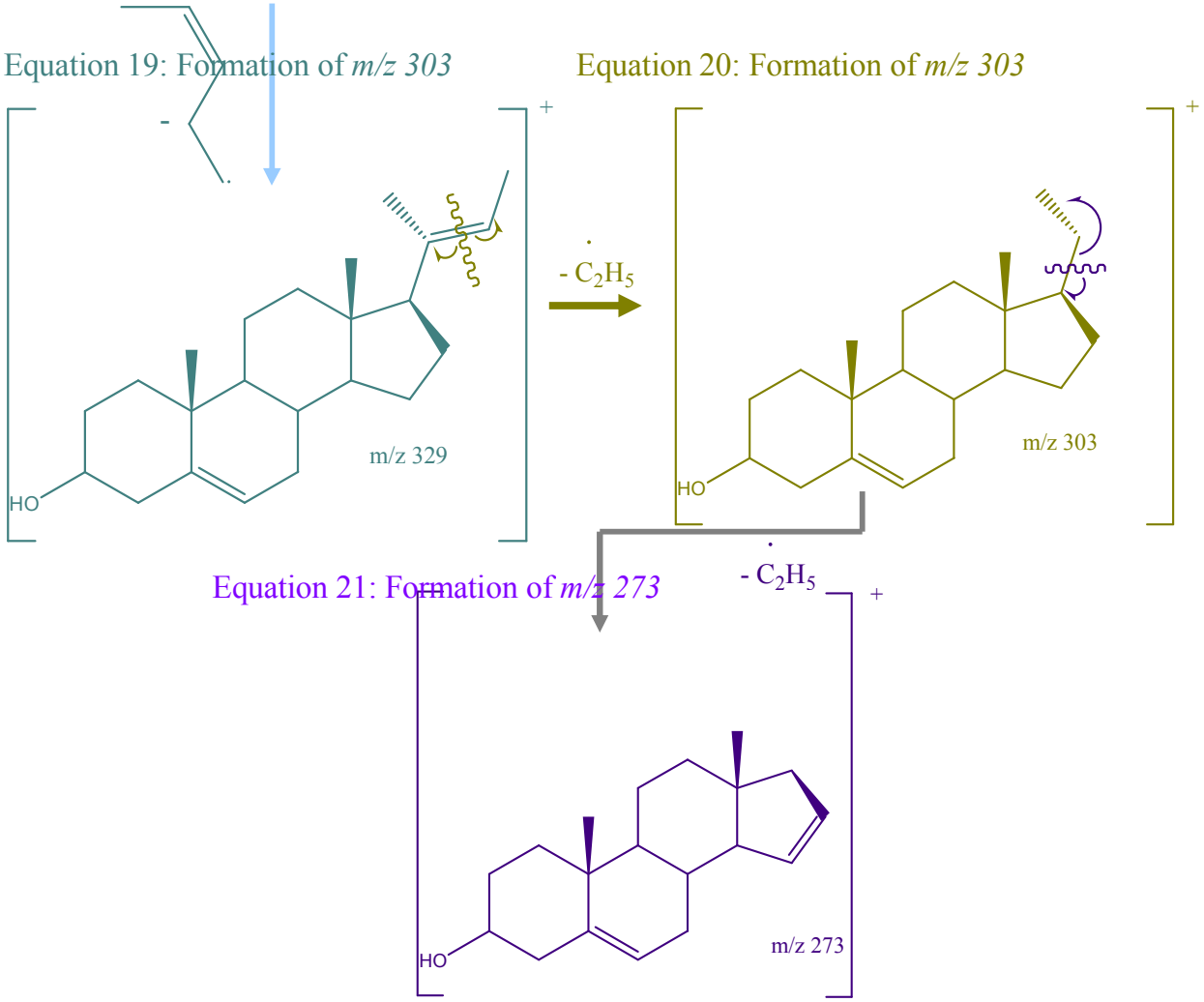


Figure 24.1: Possible fragmentation patterns for formation of m/z 381, 396 and 354 in β-sitosterol

In the mass spectrum of β-Sitosterol (Fig 24.1), the $[M - H_2O]^+$, *m/z* 396 (equation 19) arise from the loss of water. The $[M–CH_3]^+$, (equation 17) arises from the loss of methyl group at any of the following positions; C 19 (a), C 18 (b), C 21 (c), C 26 (d), C 28 (e), C 29 (f) giving rise to *m/z* 399. Loss of C_5H_{10} (equation 19) by the

exocyclic fragmentation of the 23-24 bond explains the presence of *m/z* 329 while the loss of methyl radical before losing water at C 3 (equation 18) gives rise to m/z 381. Ethyl radical cleavage from 20-22 (equation 20) explains the presence of *m/z* 303 while cleavage of 17-20 bond (equation 21) gives rise to *m/z 273*.

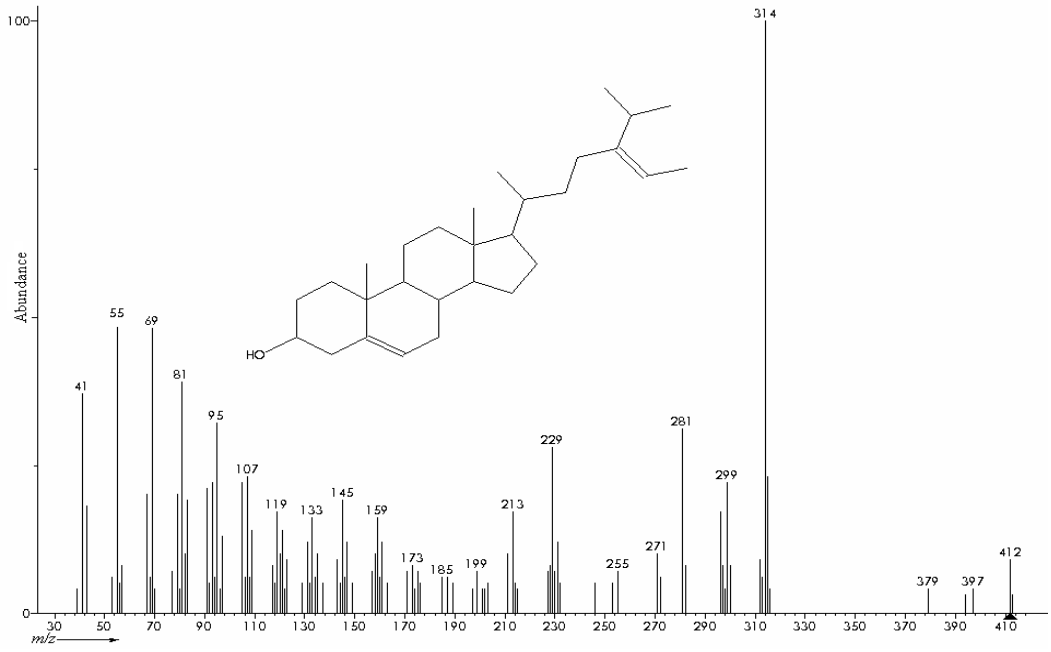
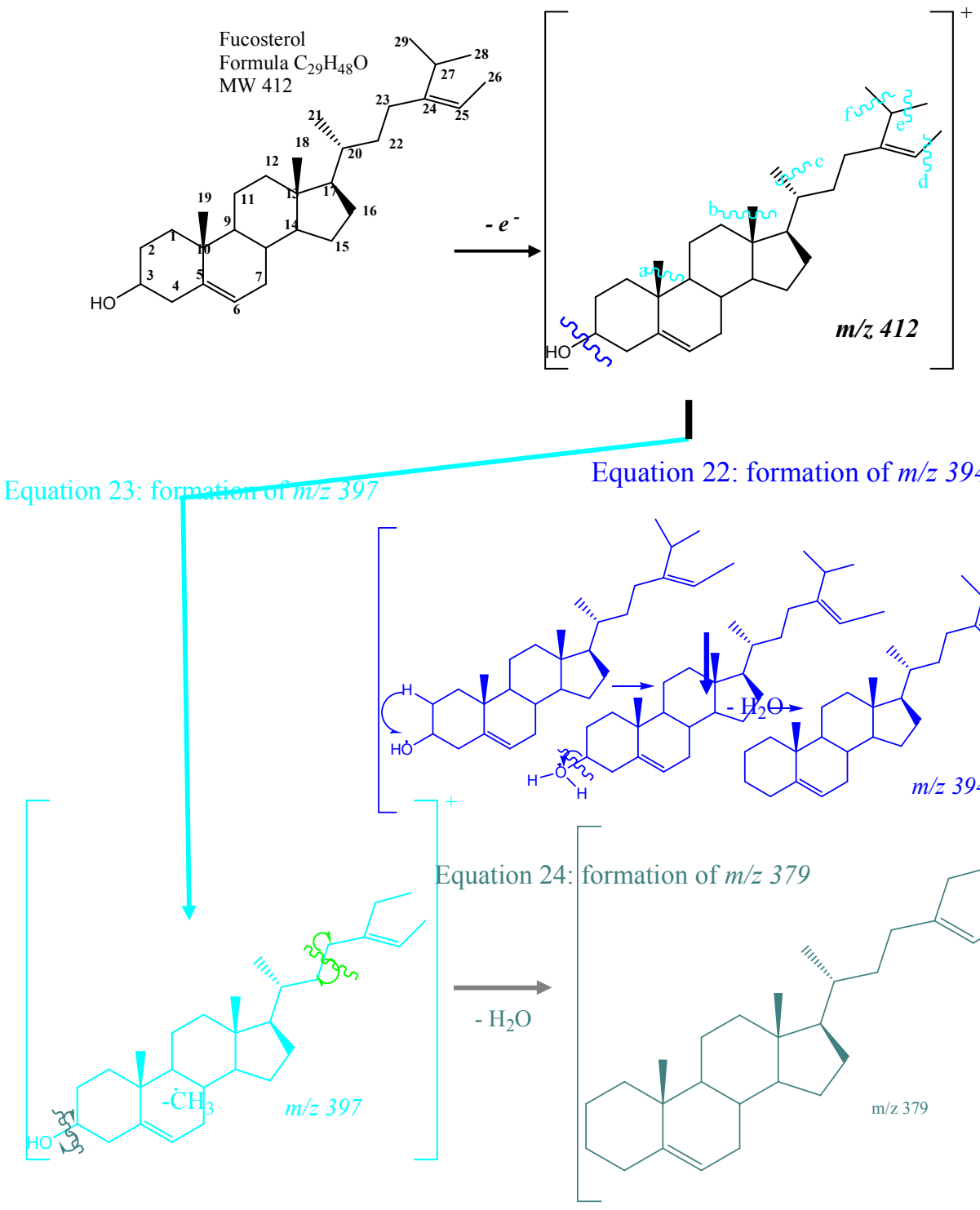


Figure 25: MS and structure for fucosterol

Fucosterol has a molecular weight of 412. The MS (Figure 25) displayed a molecular ion peak at 412 [9%, M^+]. The base ion peak occurred at *m/z* 314 [99%, M-98]. Other prominent peaks appeared at *m/z* 397 [40%, M-15), 394 [7%, M-18], 281 [31%, M-131], 229 [22%, M-183], 379 [40%, M-33], 159 [16%, M-253], 145 [19%, M-267], 107 [23%, M-305], 81 [39%, M-331], 55 [85%, M-357].


Fucosterol
Formula $C_{29}H_{48}O$
MW 412
- e -
m/z 412
Equation 22: formation of m/z 394
Equation 23: formation of m/z 397
- H_2O
m/z 394
Equation 24: formation of m/z 379
-$\dot{C}H_3$
m/z 397
- H_2O
m/z 379

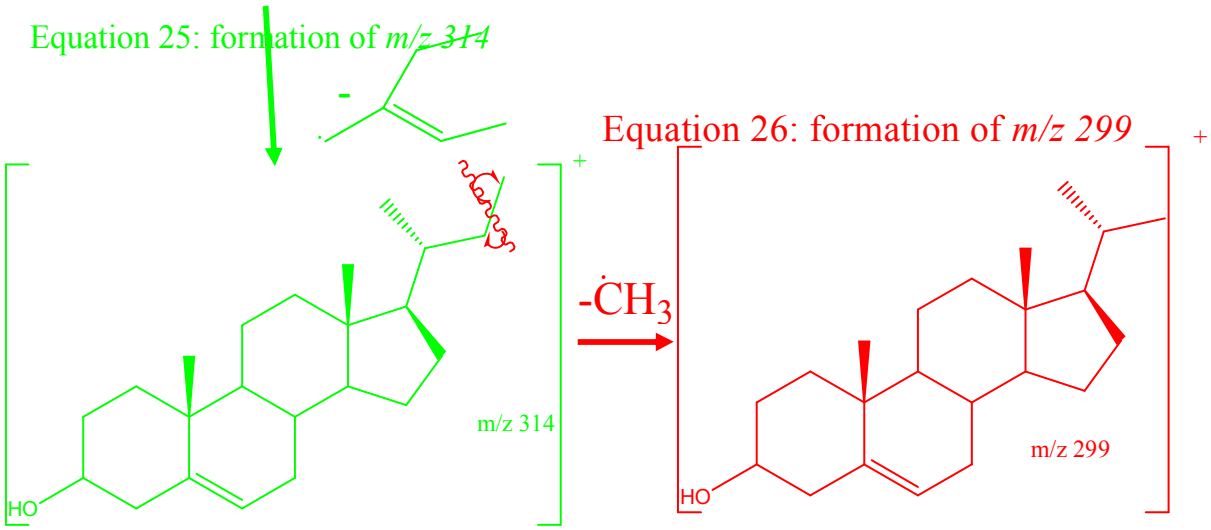


Figure 25.1: Possible fragmentation patterns for formation of m/z 394, 397, 394, 379, 314 and 299 in fucosterol

In the mass spectrum of fucosterol (Fig 25.1), the $[M - H_2O]^+$, *m/z* 394 (equation 22) arise from the loss of water .The $[M–CH_3]^+$, (equation 23) arises from the loss of methyl group at any of the following positions; C 19 (a), C 18 (b), C 21 (c), C 26 (d), C 28 (e), C 29 (f) giving rise to *m/z* 379. A special case arise whereby methyl radical is lost first then followed by the loss of water molecule this explains the presence of m/z 379 (equation 24). The base peak is formed from the loss of C_6H_{11} from m/z 397 through exocyclic fragmentation of 22-23 bond giving rise to m/z 314 (equation 25) while the loss of methyl radical at 21-22 explains the presence of m/z 299 (equation 26).

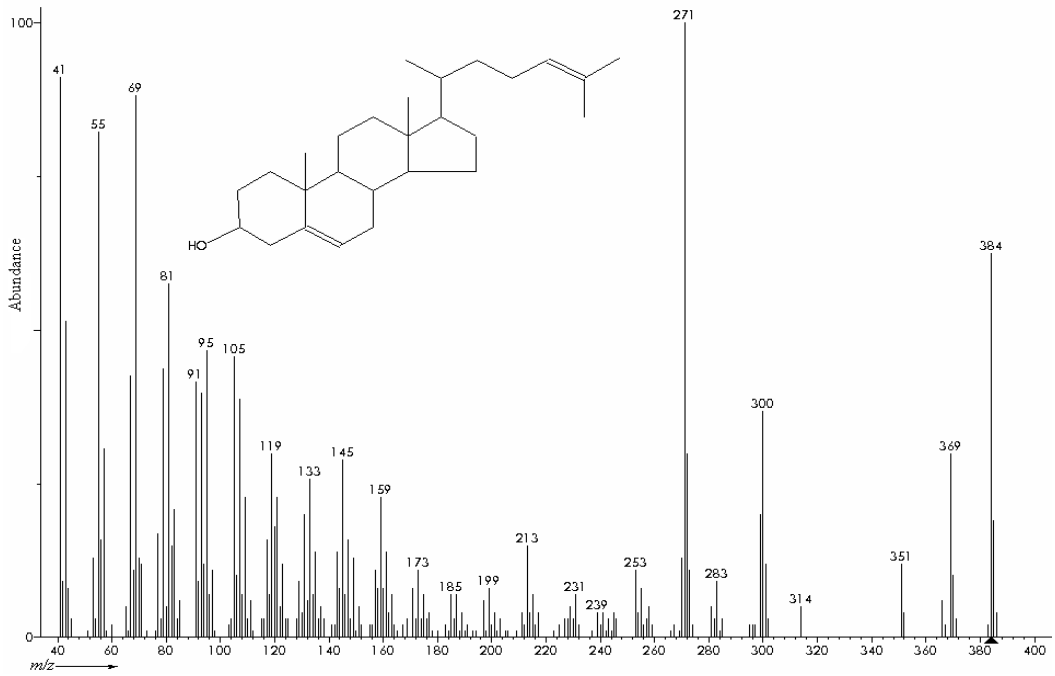


Figure 26: MS and structure for desmosterol

Desmosterol has a molecular weight of 384. The MS (Fig 26) displayed a molecular ion peak at *m/z* 384 [62%, M^+]. The base ion peak occurred at *m/z* 271 [100%, M-113]. Other prominent peaks appeared at *m/z* 369 [28%, M-15], 300 [37%, M-84], 95 [47%, M-289], 81 [58%, M-301], 79 [44%, M-305], 69 [88%, M-315], 55 [82%, M-329], 43 [51%, M-341], 41 [91%, M-343].

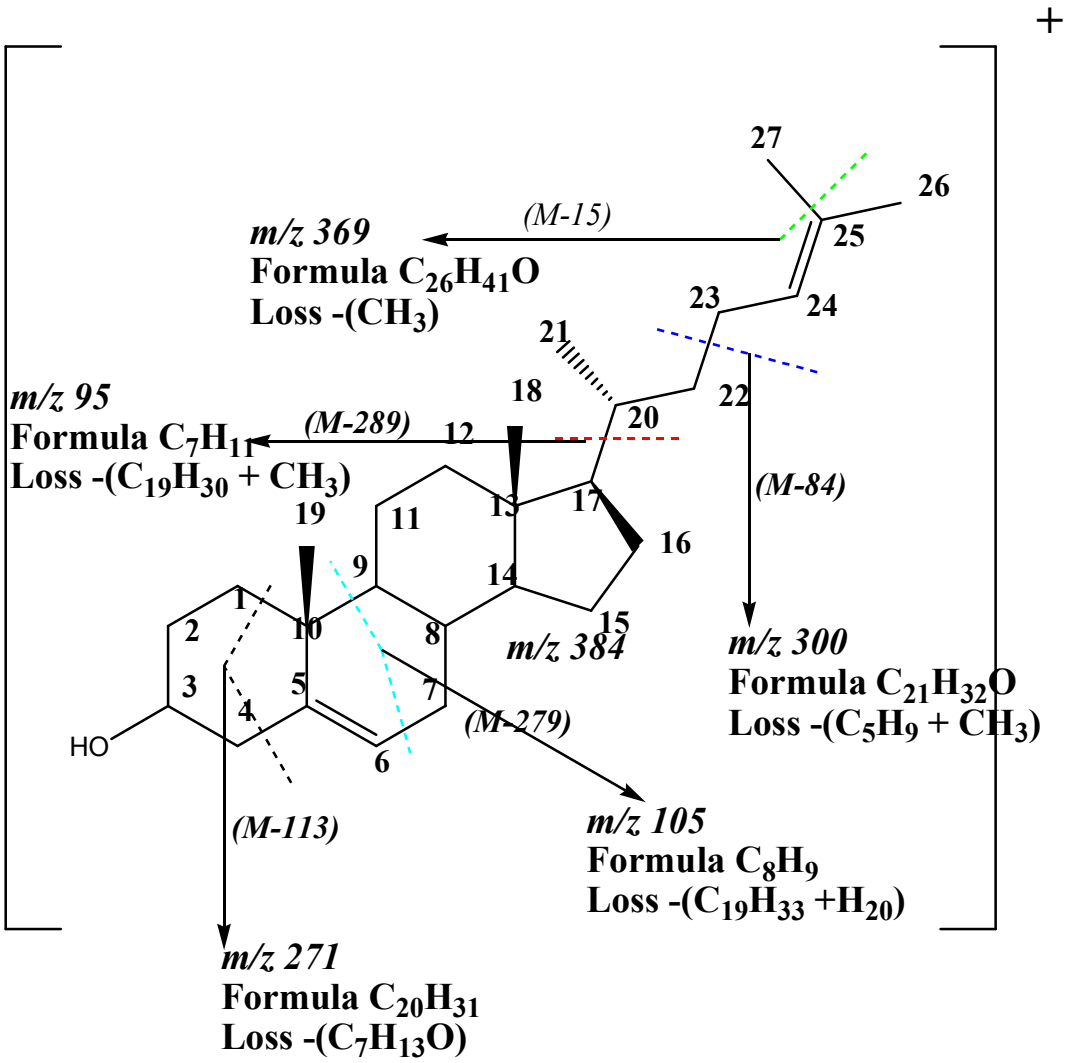


Figure 26.1: Possible fragmentation patterns for formation of m/z 369,271,300,105 and 95 in desmosterol

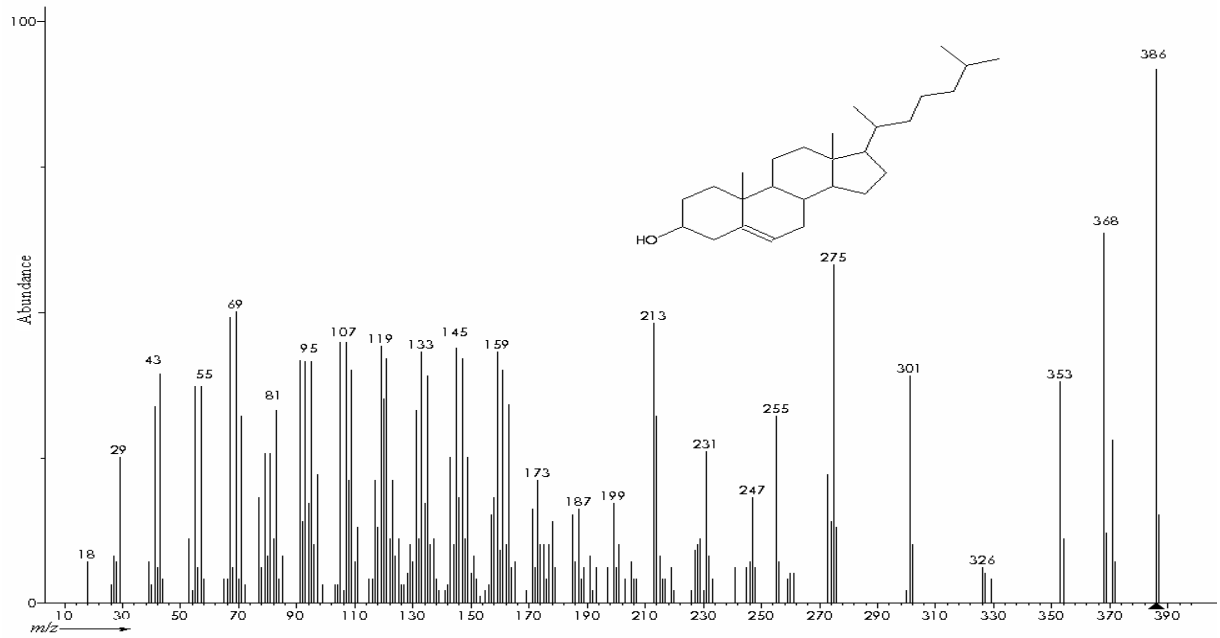
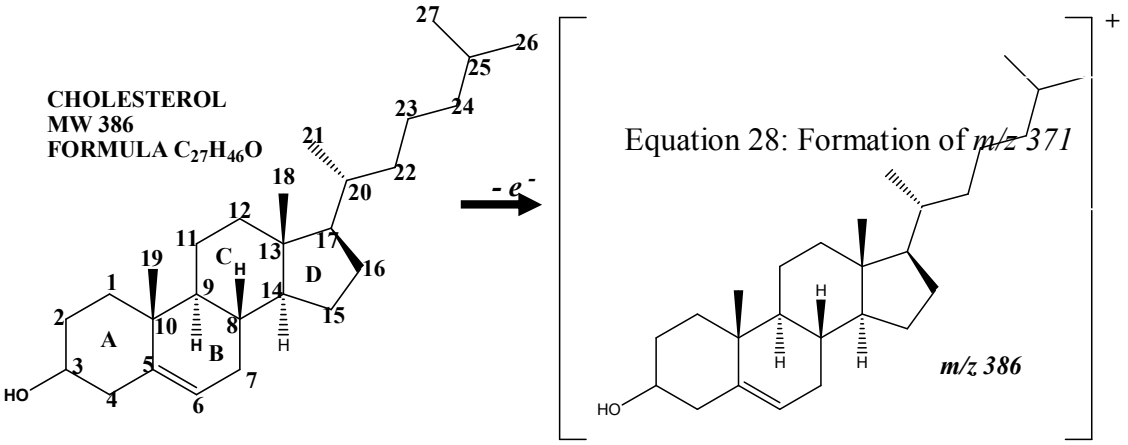
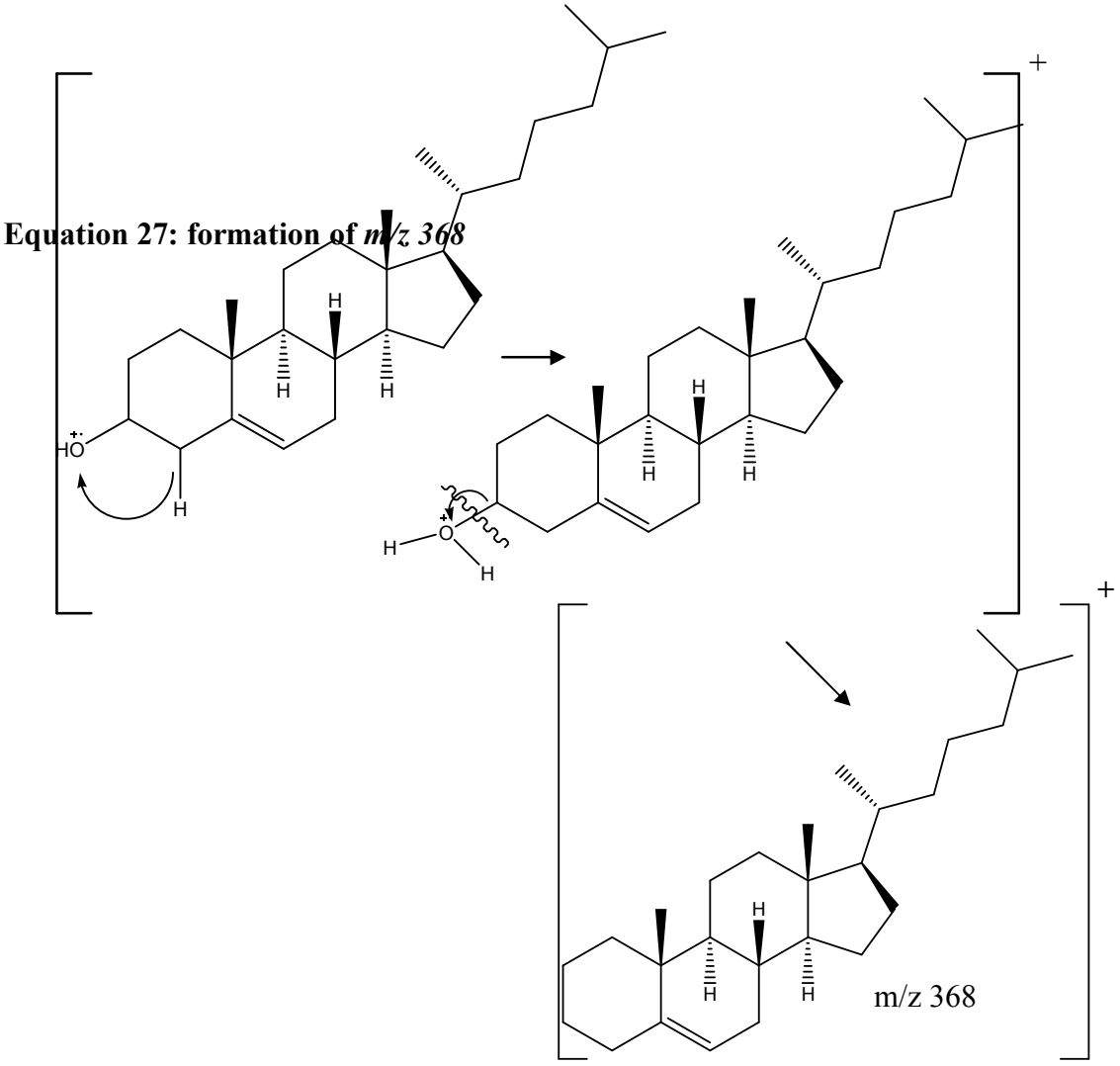


Figure 27: MS and structure for cholest-5-en-3-ol (3.β)

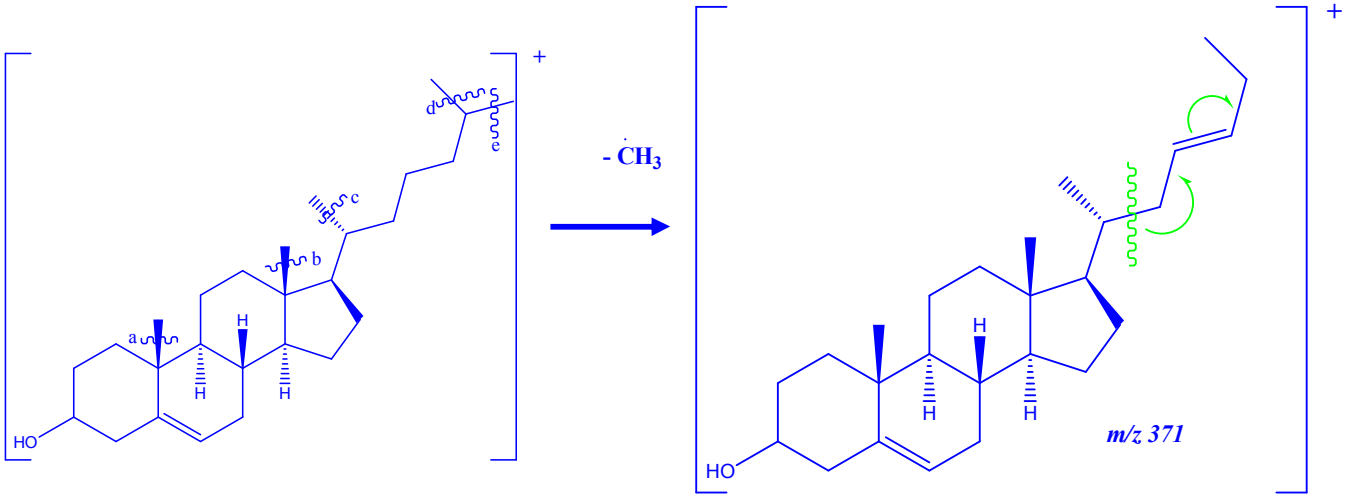
Cholest-5-en-3-ol (3β) - has a molecular weight of 386. The MS (Fig 27) displayed a molecular ion peak at *m/z* 386 [99%, M^+]. The base ion peak occurred at *m/z* 386[99%, m]. Other prominent peaks appeared at cholesterol m/z 371 [28%, M-15] 368 [37%, M-18], 301 [66%, M-85], 275 [82%], and 255 [50%, M-131], 145 [62%, M-241], 107 [63%, M-277], 105 [61%, M-281], 95 [57%, M-291],



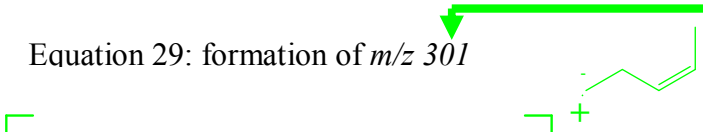
Equation 28: Formation of *m/z 371*



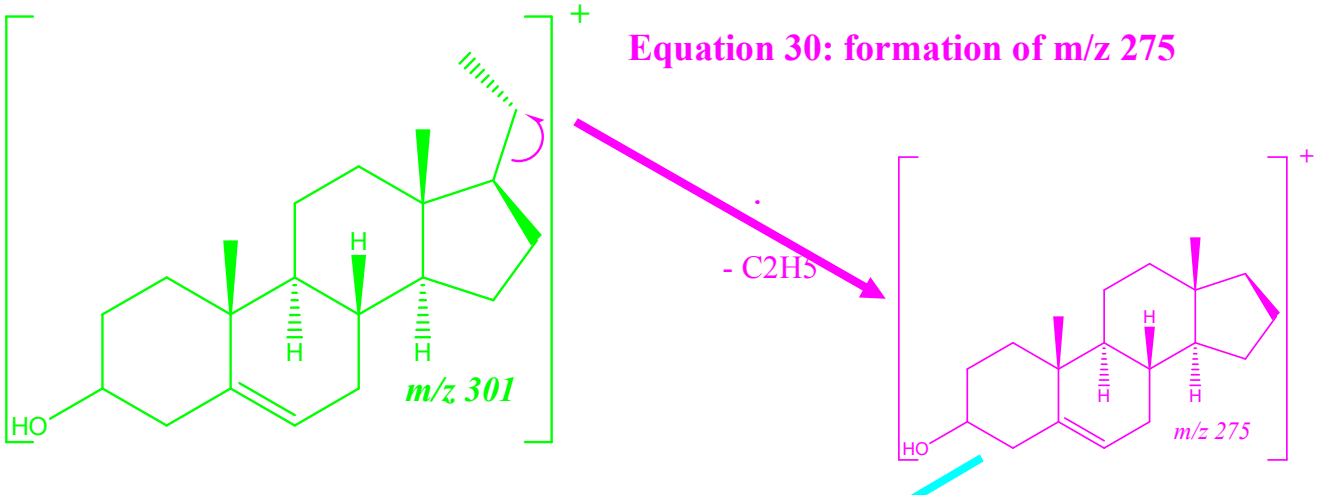
Equation 27: formation of *m/z 368*



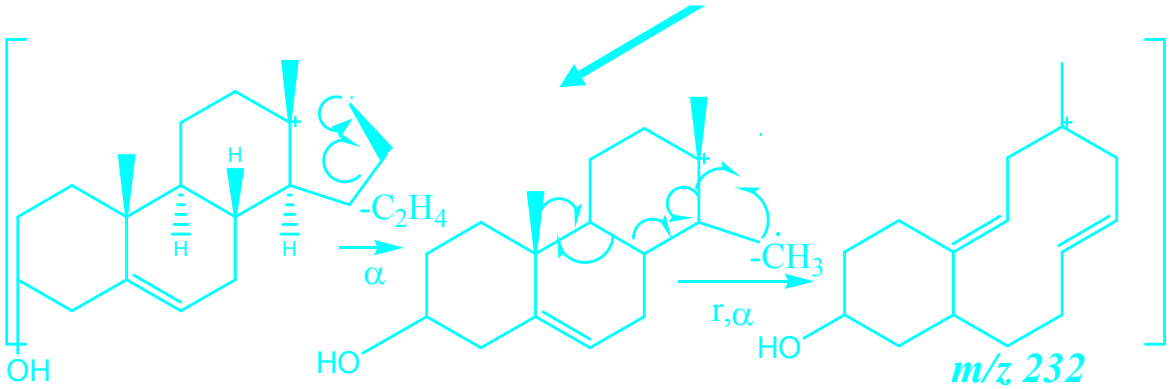
Equation 29: formation of *m/z 301*



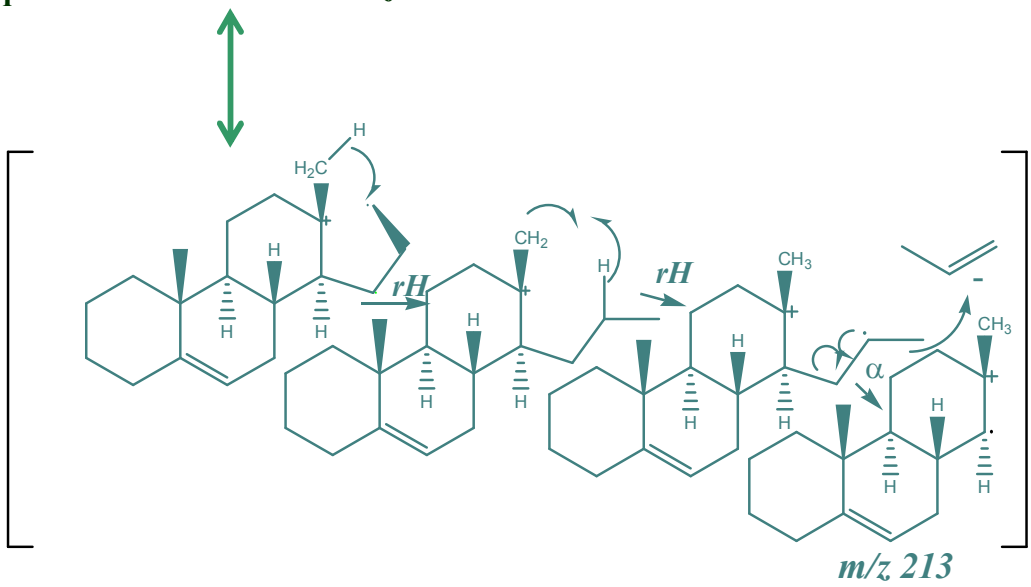
Equation 30: formation of m/z 275



Equation 31: formation of *m/z* 232



Equation 32: Formation of *m/z 213*



Structure 2

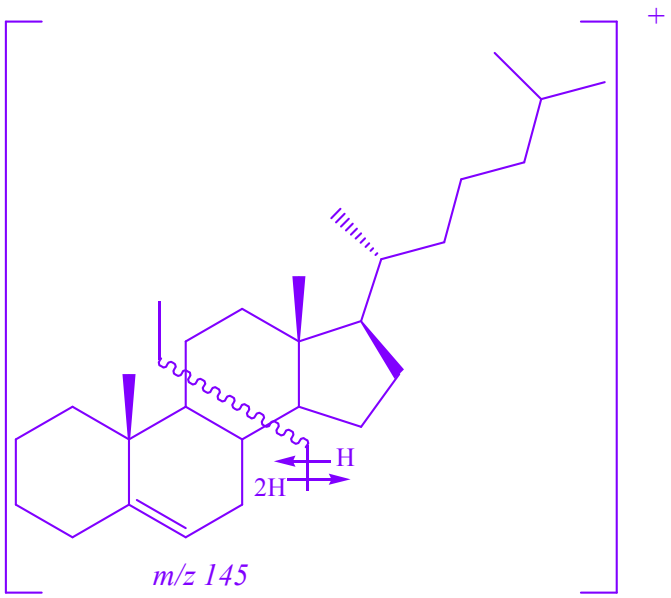


Figure 27.1: Possible fragmentation patterns for formation of m/z 386, 371, 368, 301, 275, 232, 213 and 145 in cholest-5-en-3-ol (3β)

In the mass spectrum of cholest-5-en-3-ol (3*β*) (Fig 27.1) the $[M - H_2O]^+$, *m/z* 368 (equation 27) arise from the loss of water .The $[M–CH_3]^+$, (equation 28) arises from the loss of methyl group at any of the following positions; C 19 (a), C 18 (b), C 21 (c), C 25 (d, e) giving rise to *m/z* 371. Loss of C_5H_{10} (equation 29) by the exocyclic fragmentation of the 20-21 bond explains the presence of *m/z* 301 while the loss of ethyl radical at cleavage of 17-20 bond (equation 30) yields *m/z* 275. The 13-17 cleavage of the D ring produces a stable tertiary carbenium ion. Radical site of this species can proceed either through reciprocal hydrogen transfer and 14-15 cleavage (equation 31) to yield the characteristic OE^+ m/z 213 or through electron donation to the neighboring bond, resulting in the m/z 232 peak 15-16 cleavage (equation 32). Finally the m/z 145 ion (structure 2) is composed mainly of rings A and B but is formed in a complex mechanism operating in conjunction with a triple hydrogen transfer under the influence of stereochemistry of A/B rings (Seifert *et al*. 1972).

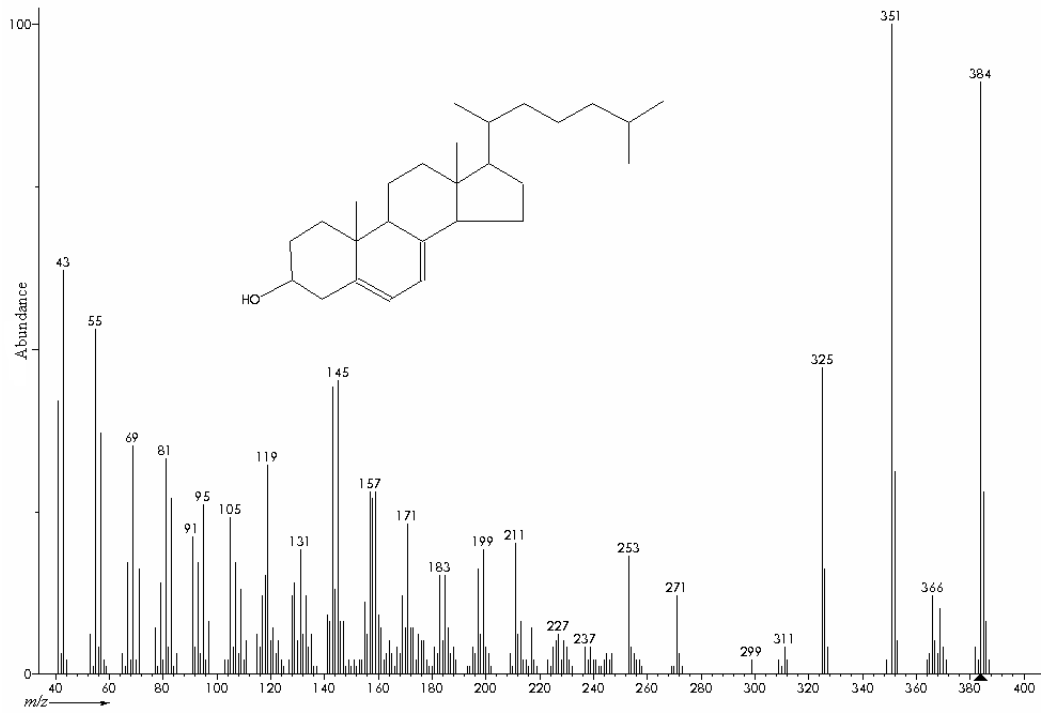
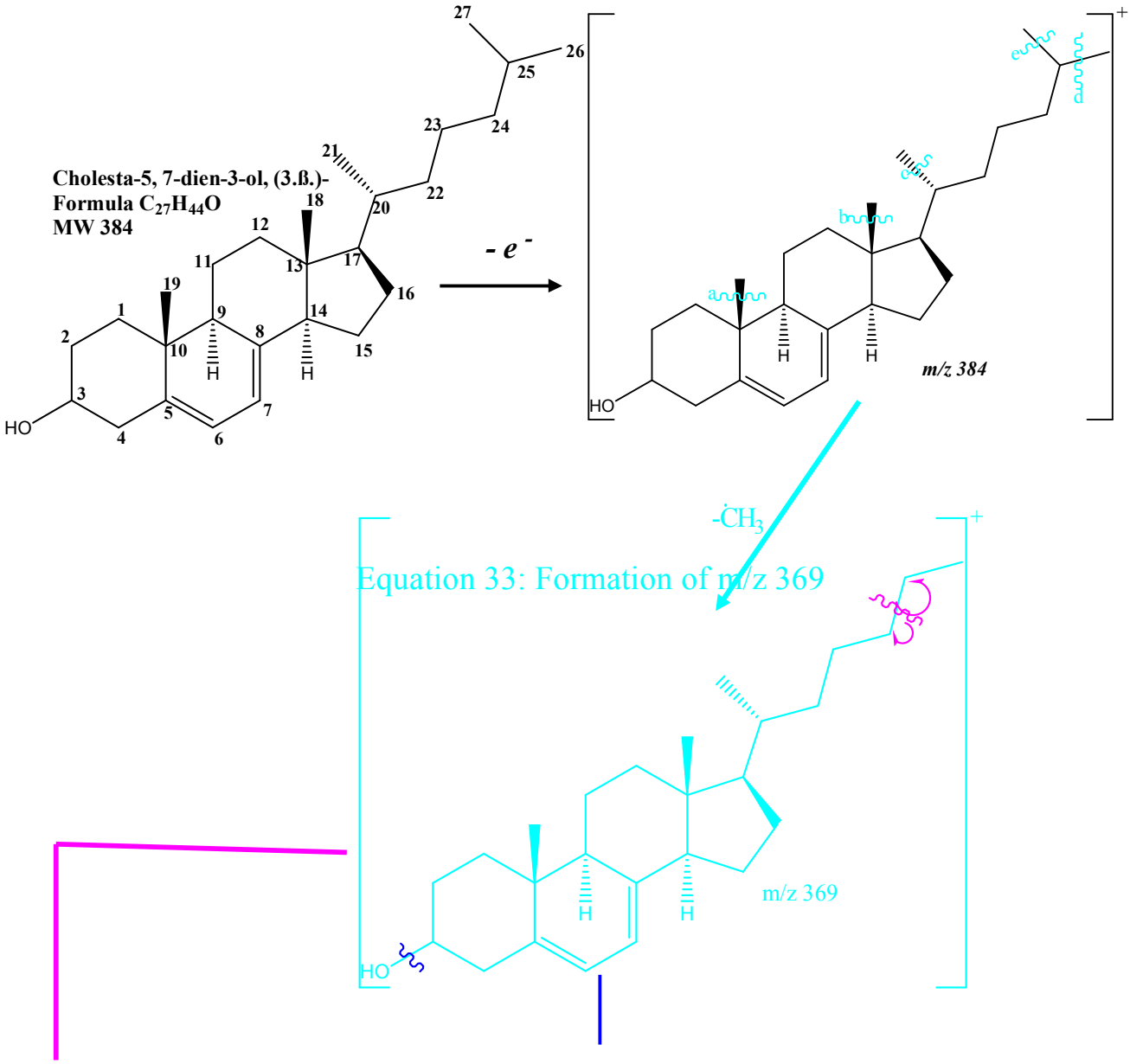


Figure 28: MS and structure for cholesta-5,7-dien-3-ol, (3β)

Cholesta-5,7-dien-3-ol,(3β)- has a molecular weight of 384. The MS (Fig 28) displayed a molecular ion peak at *m/z* 384 [91%, M^+]. The base ion peak occurred at *m/z* 351[100%, M-33]. Other prominent peaks appeared at *m/z* 366 [12%, M-18], 325 [47%, M- 59], 145 [45%, M-329], 143 [44%, M-241], 69 [35%, M-315], 57 [37%, M-327], 55 [63%, M-329], 43 [62%, M-342], 41 [42%, M-343].



Equation 33: Formation of m/z 369

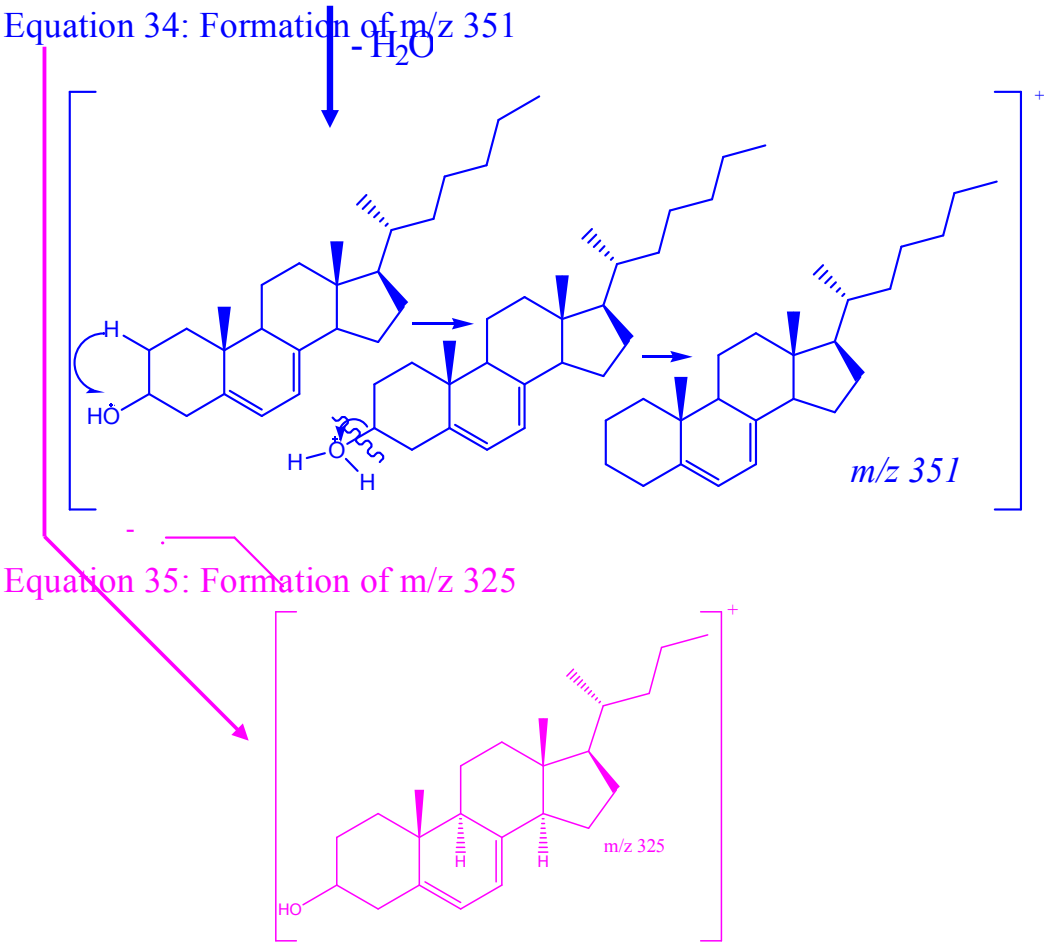


Figure 28.1: Possible fragmentation pattern for formation of m/z 351, 145, 366, 325 and 211 in cholesta-5, 7-dien-3-ol,(3β)

In the mass spectrum of cholesta-5,7-dien-3-ol, (3β)- (Fig 28.1), The $[M–CH_3]^+$, (equation 33) arises from the loss of methyl group at any of the following positions; C 19 (a), C 18 (b), C 21 (c), C 26 (d), C 28 (e), C 29 (f) giving rise to *m/z* 369, the $[M–H_2O]^+$, *m/z* 351 (equation 34), which is the base peak, arise from the loss of water. The exocyclic fragmentation of 24-25 bond gives rise to m/z 325 (equation 35).

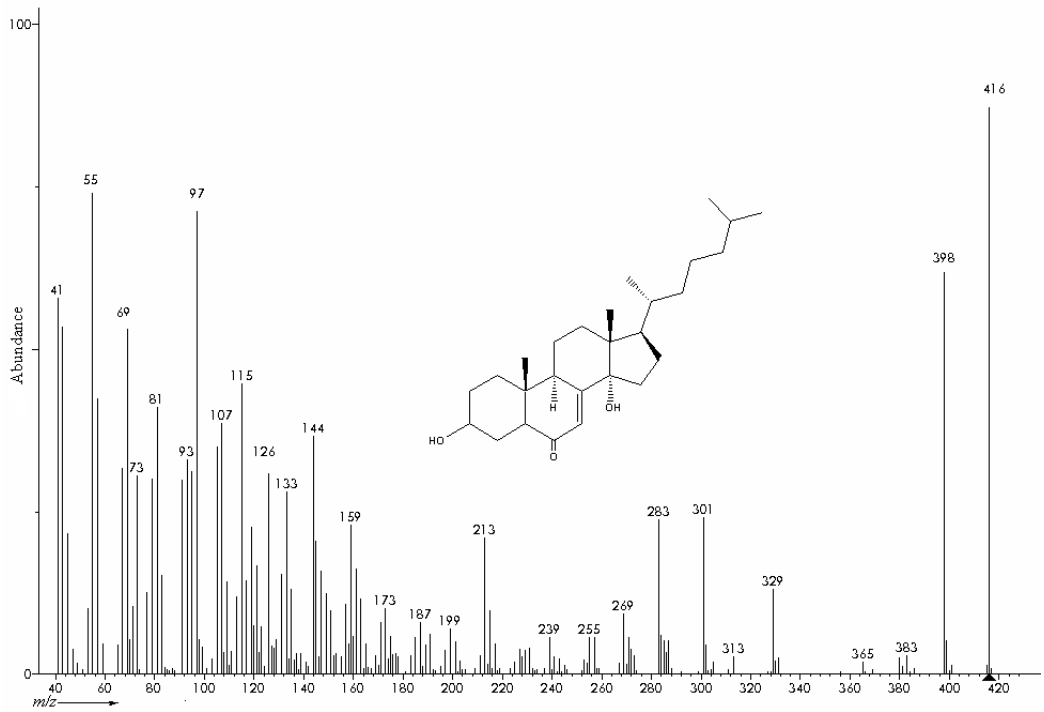


Figure 29: MS and structure for cholesta-7-ene-6-one-3,14-diol (3β) (2,22,25-Trideoxyecdysone (5β-ketodiol))

Cholesta-7-ene-6-one-3,14-diol (3β) has a molecular weight of 416. The MS (Fig 29) displayed a molecular ion peak at *m/z* 418 [47%, M^+]. The base ion peak occurred at *m/z* 400 [99%, M-18]. Other prominent peaks appeared at *m/z* 287 [15%, M-131], 382 [14%, M-36], 367 [9%, M-51], 343 [10%, M-75], 287 [10%, M-131], 121 [30%, M-297], 107 [30%, M-311], 95 [54%, M-323].

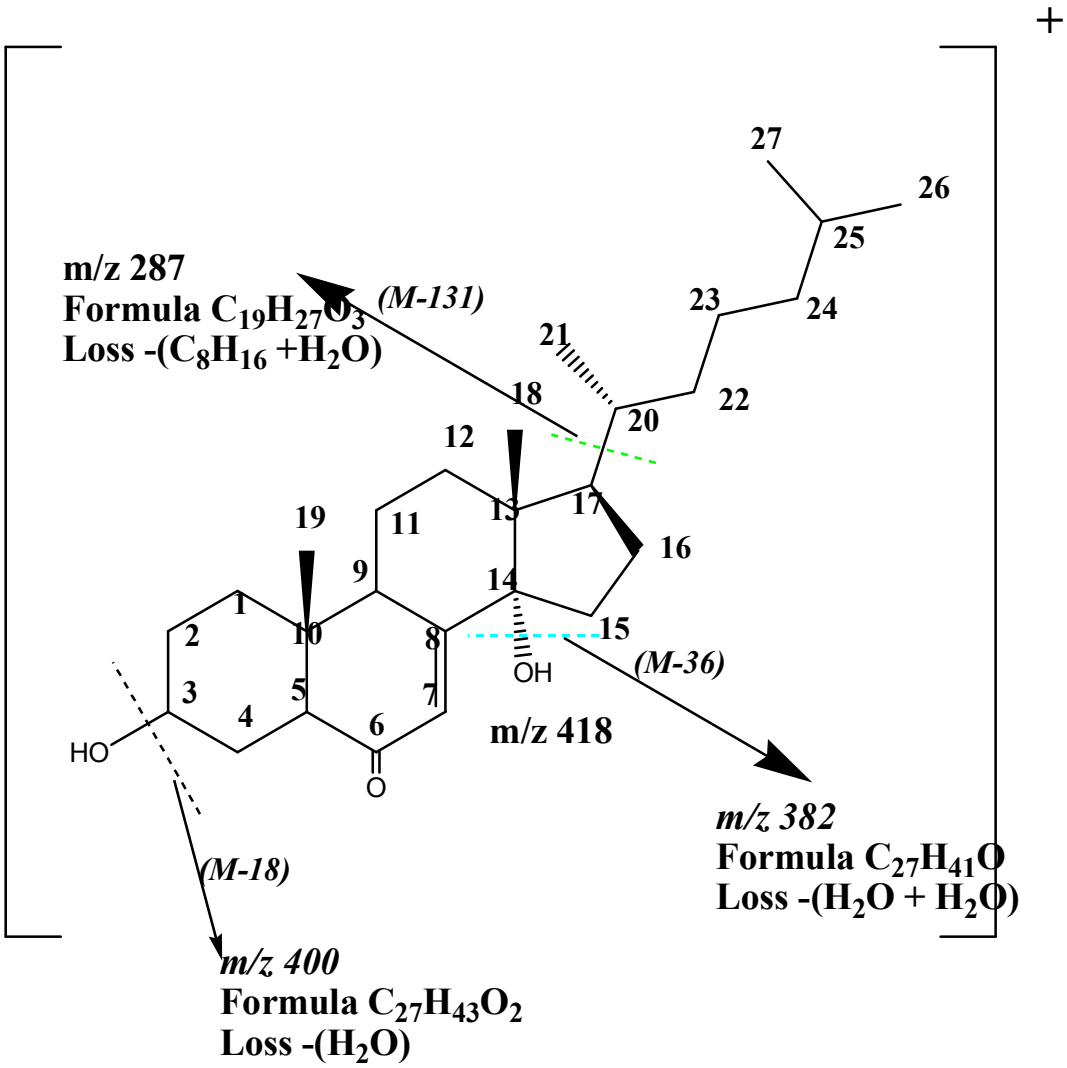


Figure 29.1: Possible fragmentation pattern for formation of m/z 287,400 and 382 in cholesta-7-ene-6-one-3, 14-diol (3β)

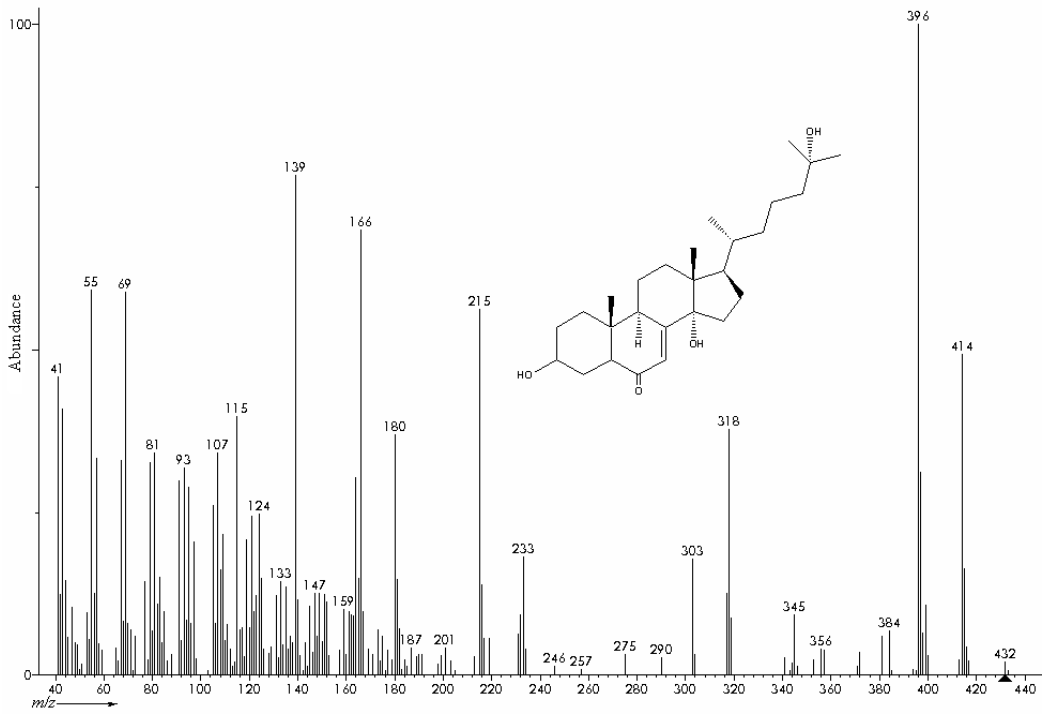


Figure 30: MS and structure for cholesta-7-ene-6-one-3, 14, 25-triol (3β)

Cholesta-7-ene-6-one-3, 14, 25-triol (3β) has a molecular weight of 432. The MS (Fig 30) displayed a molecular ion peak at *m/z* 432 [17%, M^+]. The base ion peak occurred at *m/z* 414 [90%, M-18]. Other prominent peaks appeared at *m/z*396 [45%, M-36], 404 [60%, M-28], 381 [17%, M-51], 233 [40%, M-199], 283 [20%, M-149]

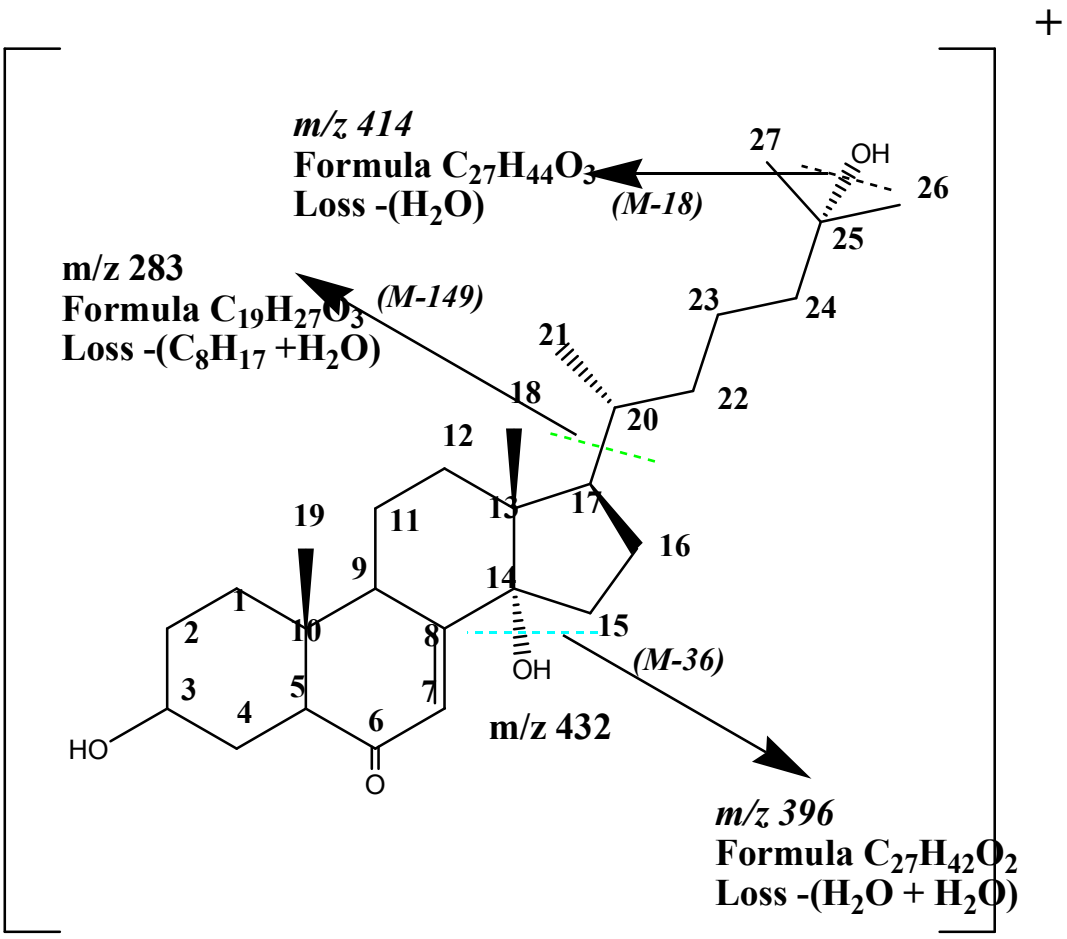


Figure 30.1: Possible fragmentation pattern for formation of m/z 414, 283, 396 in cholesta-7-ene-6-one-3, 14, 25-triol (3β)

3.9.1 MS of sterols after incubation cholesterol-[4-^{13}C] and normal cholesterol

This section presents the mass spectra of sterol metabolites after incubation of the foregut and midgut with normal cholesterol and cholesterol-[4-^{13}C].

(* indicates the position of the labeled carbon (carbon 4 in ring A) in the cholesterol moiety).

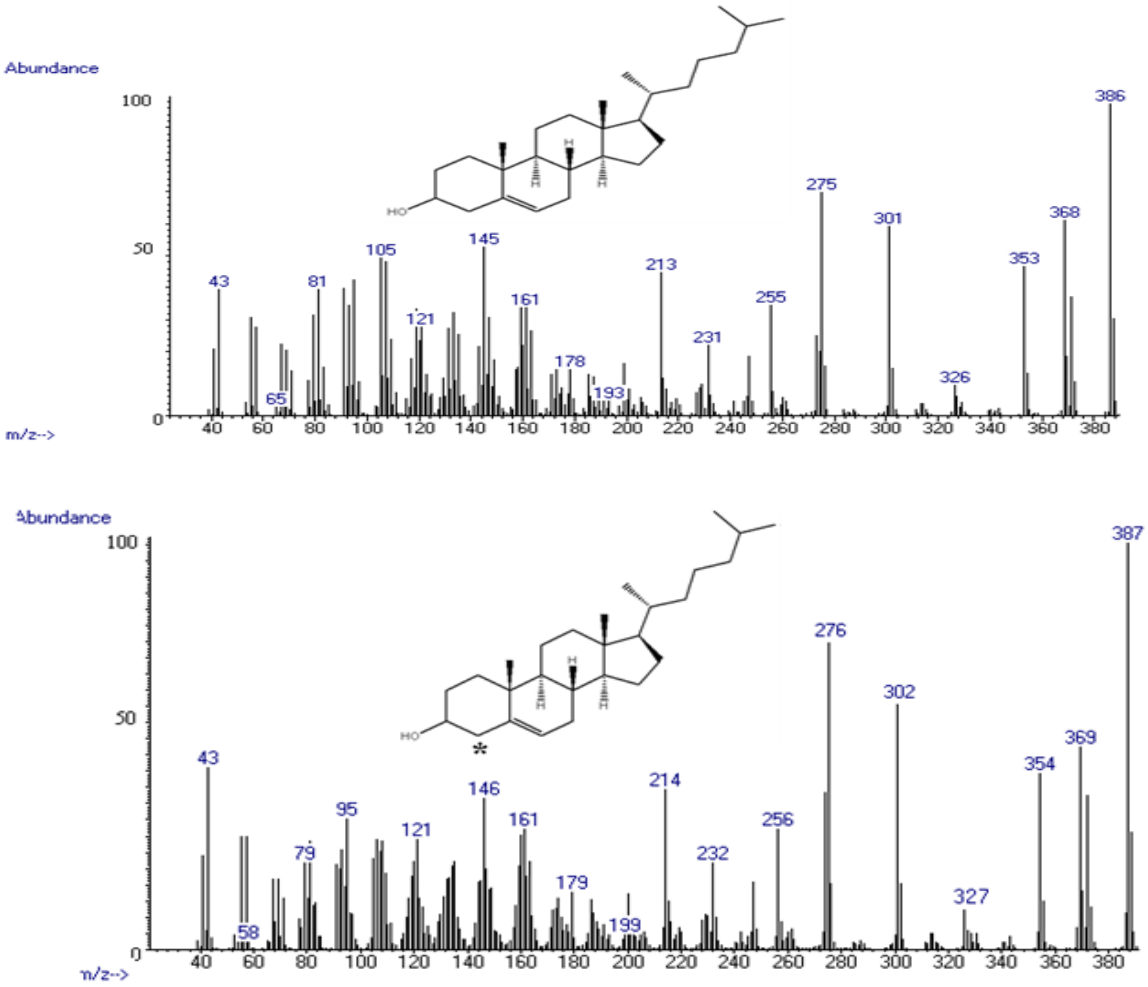


Figure 31: MS and structure for cholesterol & cholesterol-[4-^{13}C]

Cholesterol has a molecular weight of 386 (Fig 31), cholesterol-[4-^{13}C] being labeled at Carbon 4 with isotopic carbon with mass 13 has a molecular weight of 387 an increase of 1a.m.u. Other diagnostic ions are displayed at *m/z* 368, 353, 326, 301, 275 & 387, 354, 327, 302, 276 for normal cholesterol and cholesterol-[4-^{13}C] respectively.

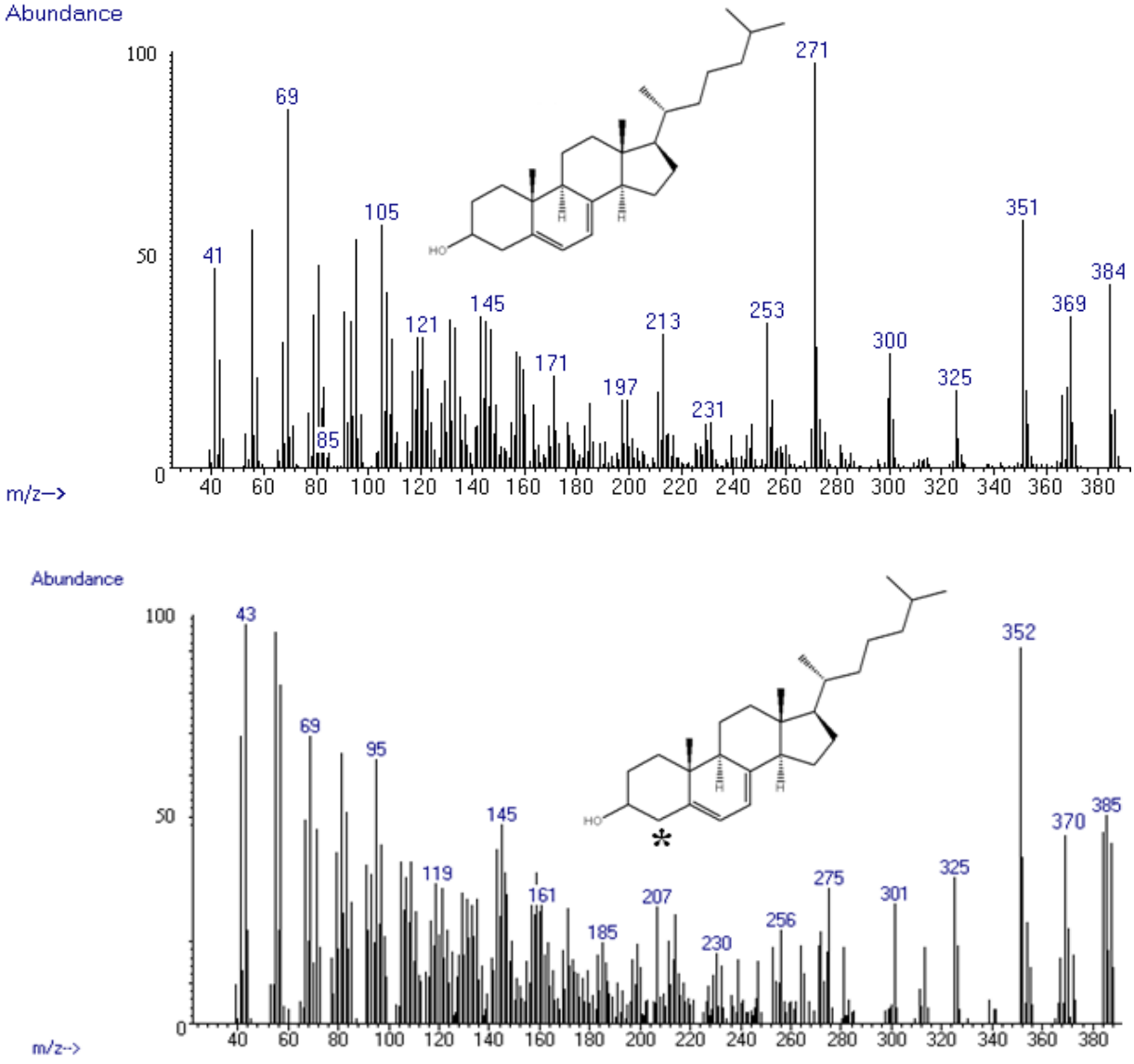


Figure 32: MS and structure for cholesta-5,7-dien-3-ol

Cholesta-5,7-dien-3-ol has a molecular weight of 384 (Fig 32) after incubation with the normal cholesterol and 385 after incubation with cholesterol-[4-^{13}C] an increase of 1a.m.u. Other diagnostic ions are displayed at *m/z* 384, 369, 351 & 385, 370, 352 for normal cholesterol and cholesterol-[4-^{13}C] respectively.

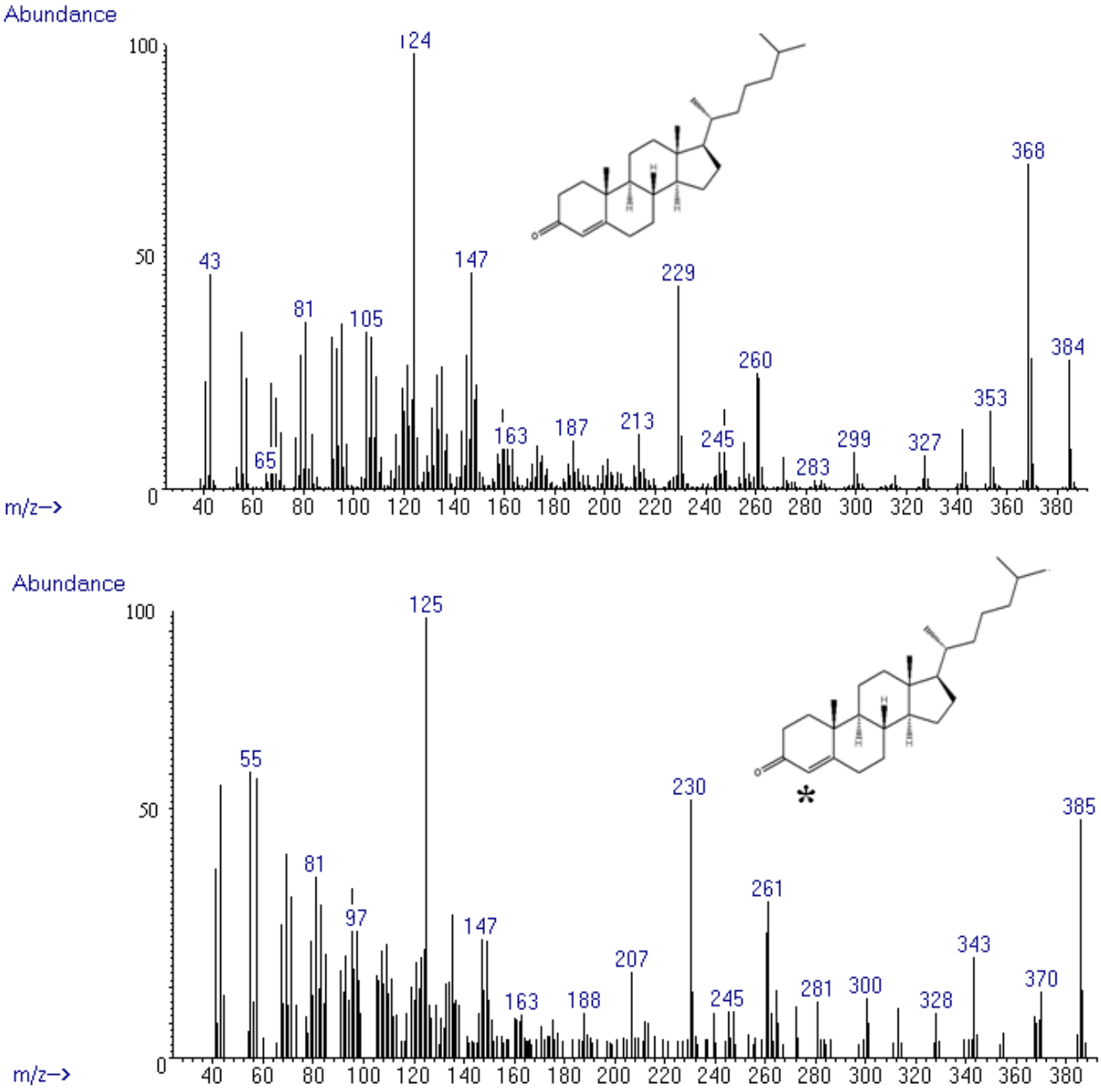


Figure 33: MS and structure for cholest-4-en-3-one

Cholest-4-en-3-one has a molecular weight of 384 (Fig 33) after incubation with the normal cholesterol and 385 after incubation with cholesterol-[4-^{13}C] an increase of 1a.m.u. Other diagnostic ions are displayed at *m/z* 369, 260, 229 & 385, 261, 230 for normal cholesterol and cholesterol-[4-^{13}C] respectively.

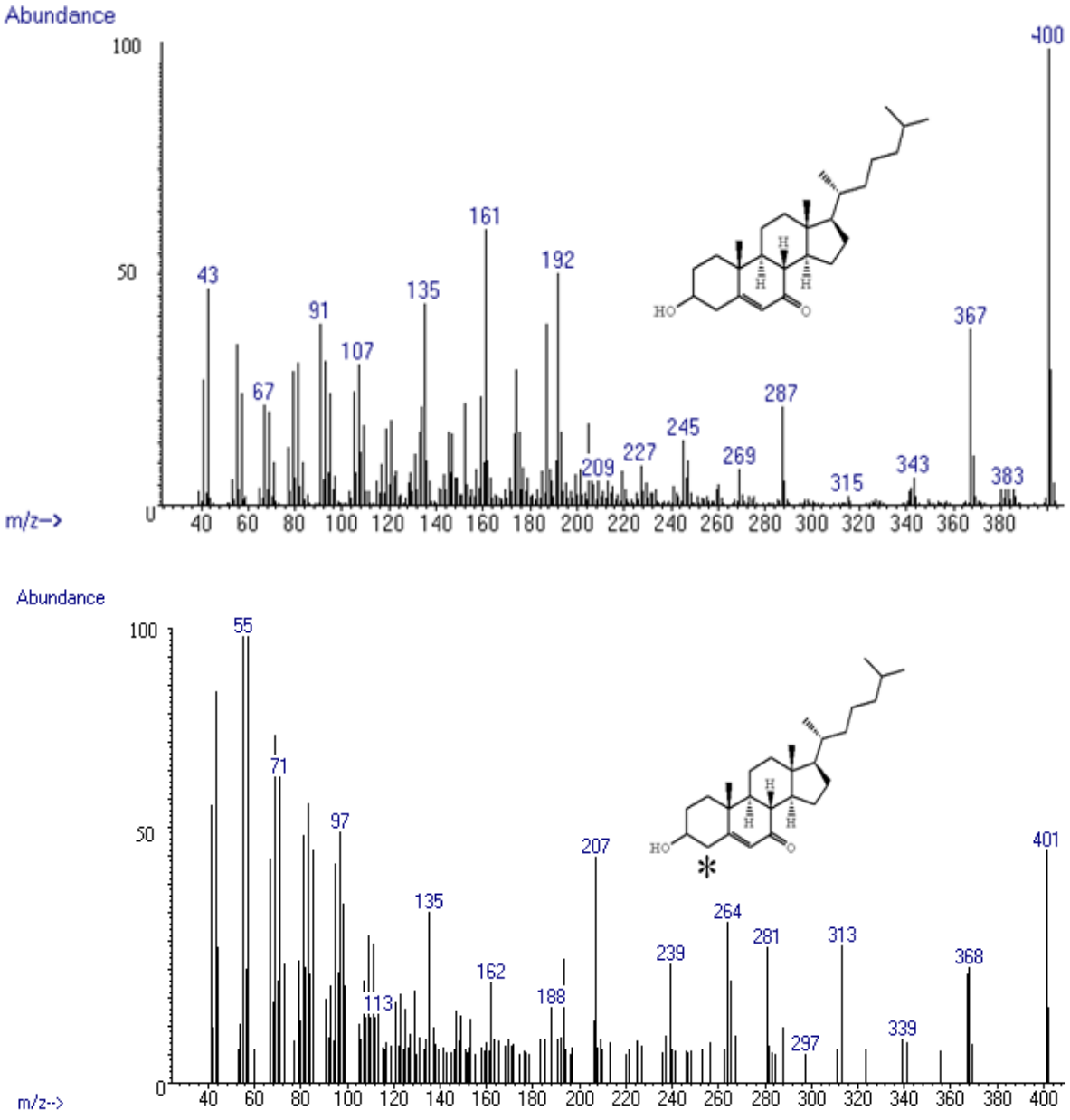


Figure 34: MS and structure for cholesterol 7-oxo

Cholesterol 7-oxo has a molecular weight of 400 (Fig 34) after incubation with the normal cholesterol and 401 after incubation with cholesterol-[4-^{13}C] an increase of 1a.m.u. Other diagnostic ions are displayed at *m/z* 367 & 368 for normal cholesterol and cholesterol-[4-^{13}C] respectively.

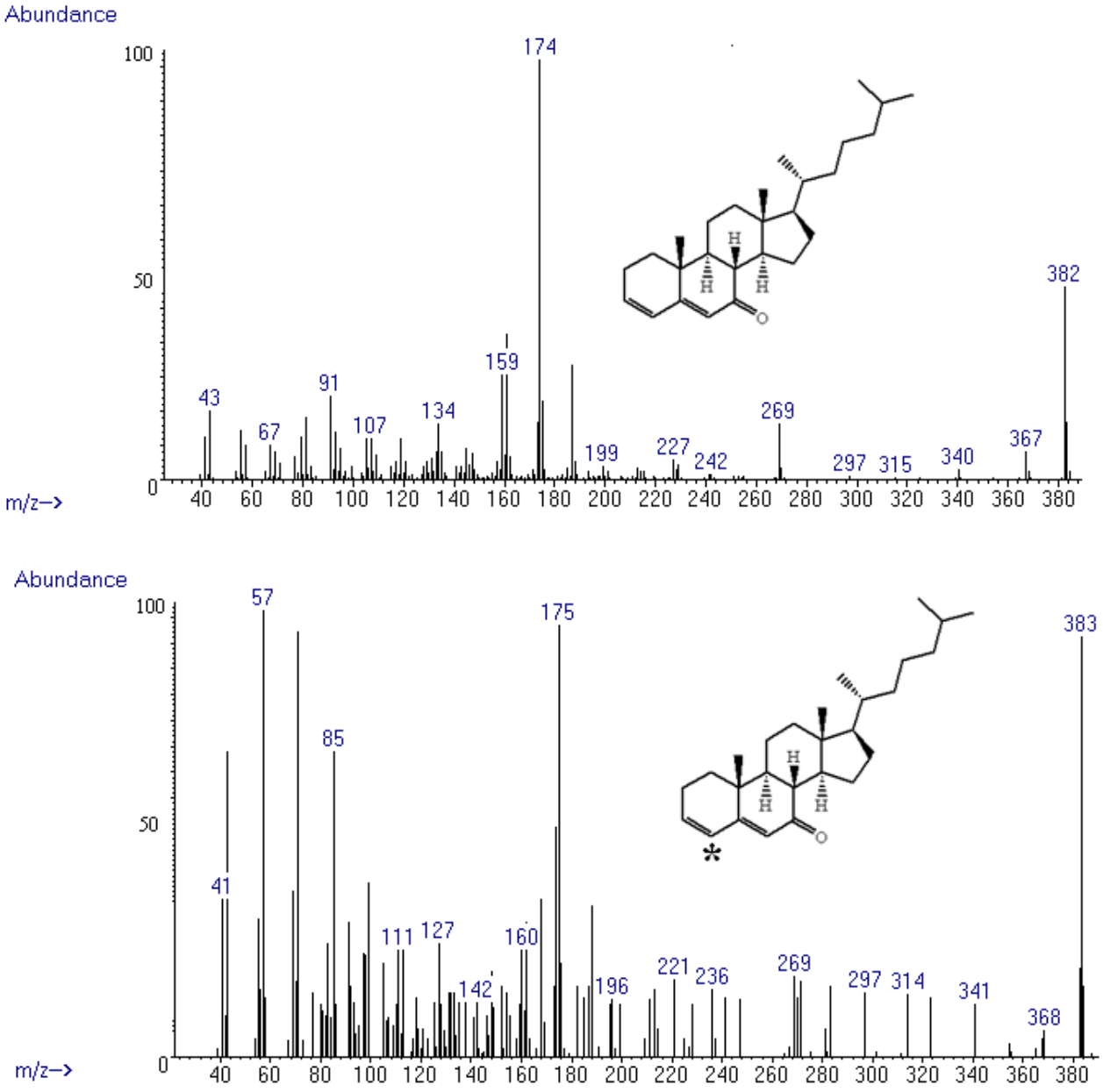


Figure 35: MS and structure for cholesta-3,5-dien-7-one

Cholesta-3,5-dien-7-one has a molecular weight of 382 (Fig 35) after incubation with the normal cholesterol and 383 after incubation with cholesterol-[4-^{13}C] an increase of 1a.m.u. Other diagnostic ions are displayed at *m/z* 367, 340, 174 & 368, 341, 175 for normal cholesterol and cholesterol-[4-^{13}C] respectively.

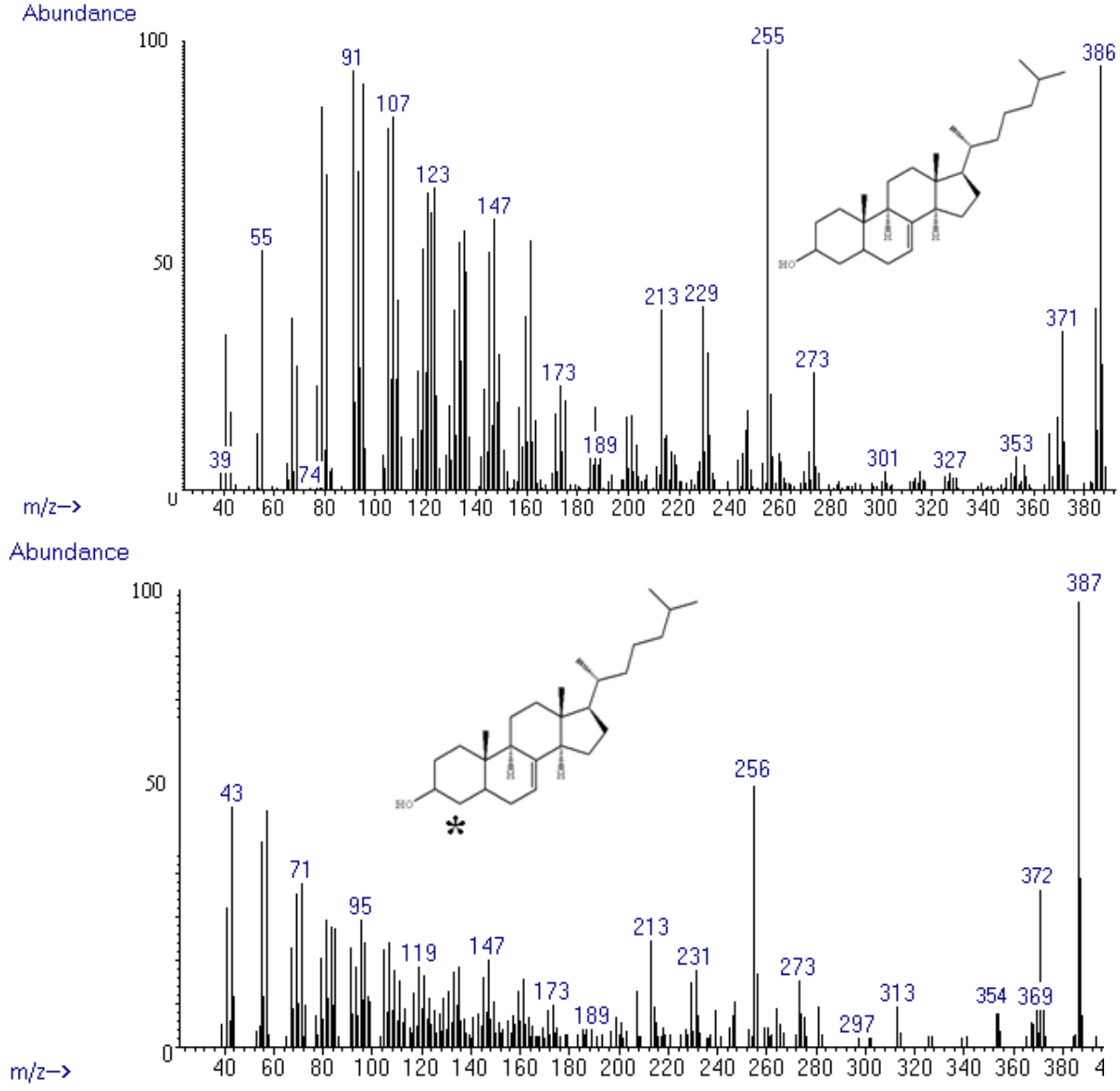


Figure 36: MS and structure for cholest-7-en-3-ol, (3 β,5α)

Cholest-7-en-3-ol, (3 β,5α) has a molecular weight of 386 (Fig 36) after incubation with the normal cholesterol and 387 after incubation with cholesterol-[4-^{13}C] an increase of 1a.m.u. Other diagnostic ions are displayed at *m/z* 371, 353, 255 & 387, 372, 256 for normal cholesterol and cholesterol-[4-^{13}C] respectively.

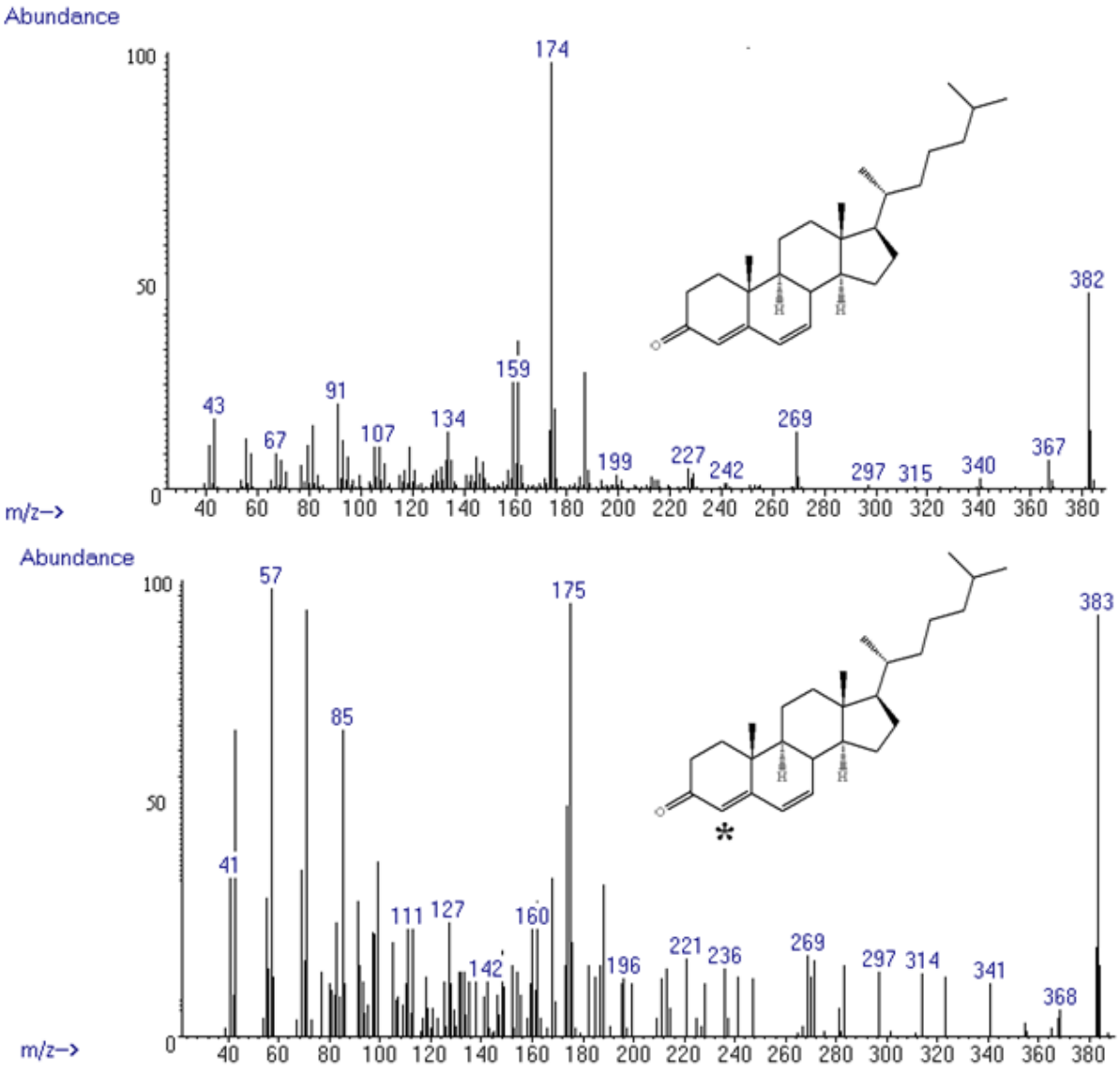


Figure 37: MS and structure for cholesta-4,6-dien-3-one

Cholesta-4,6-dien-3-one has a molecular weight of 382 (Fig 37) after incubation with the normal cholesterol and 383 after incubation with cholesterol-[4-^{13}C] an increase of 1a.m.u. Other diagnostic ions are displayed at *m/z* 367, 340, 174 & 368, 341, 175 for normal cholesterol and cholesterol-[4-^{13}C] respectively.

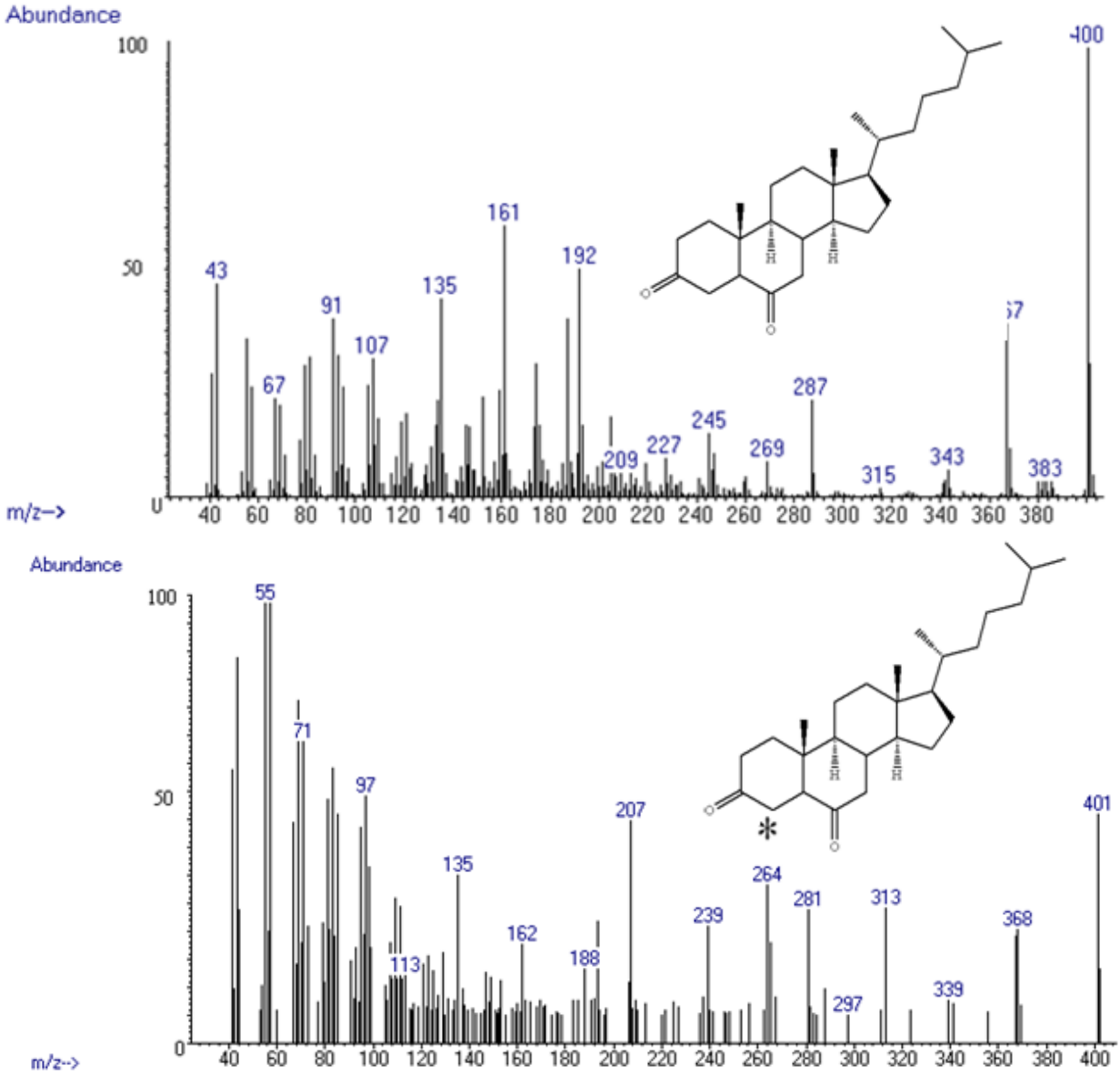


Figure 38: MS and structure for cholestane-3,6-dione, (5α,17α,20S)

Cholestane-3,6-dione, (5α,17α,20S)- has a molecular weight of 400 (Fig 38) after incubation with the normal cholesterol and 401 after incubation with cholesterol-[4-^{13}C] an increase of 1 a.m.u. Other diagnostic ions are displayed at *m/z* 367& 368 for normal cholesterol and cholesterol-[4-^{13}C] respectively.

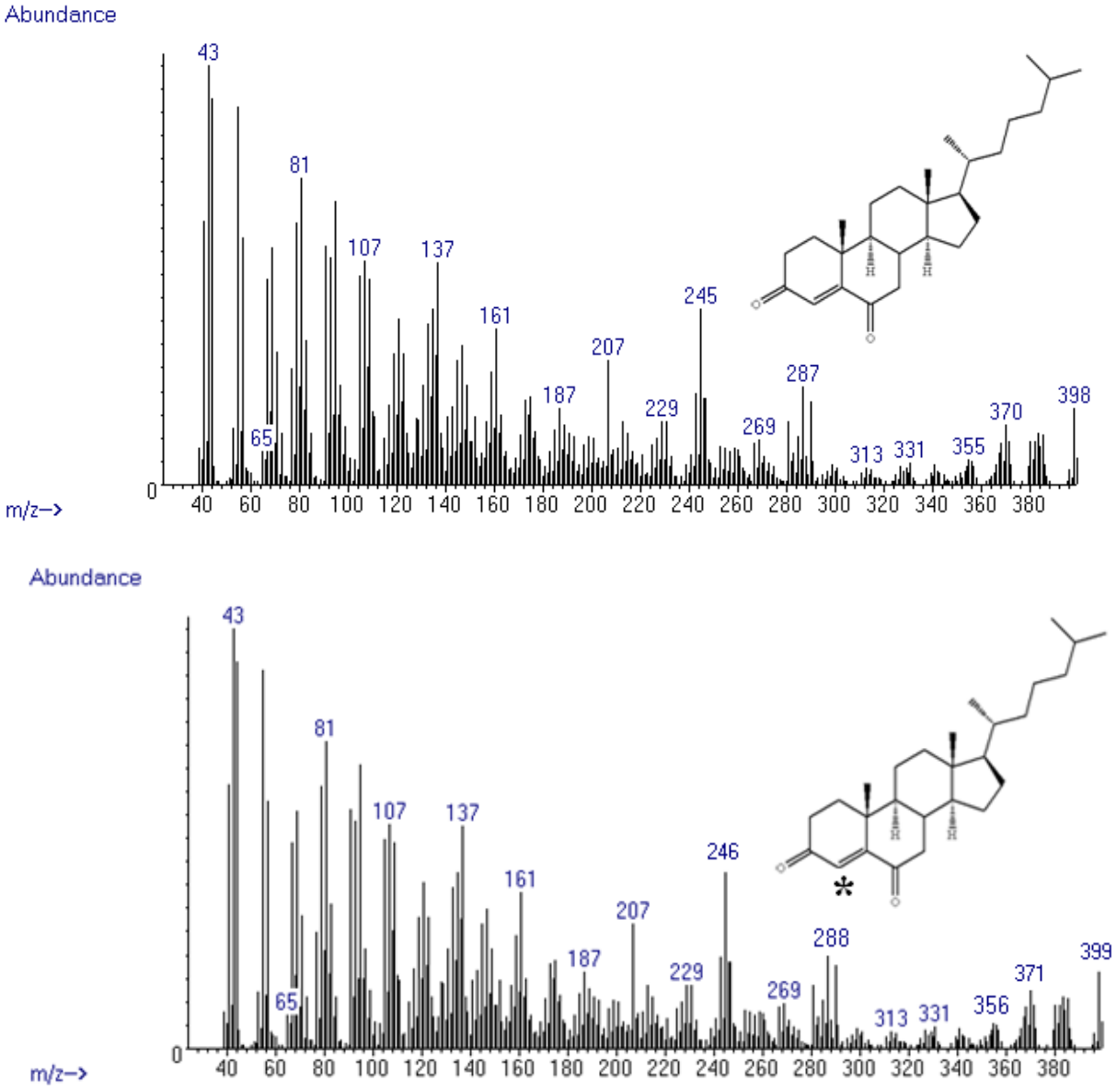


Figure 39: MS and structure for cholest-4-ene-3,6-dione

Cholest-4-ene-3,6-dione has a molecular weight of 398 (Fig 39) after incubation with the normal cholesterol and 399 after incubation with cholesterol-[4-^{13}C] an increase of 1a.m.u. Other diagnostic ions are displayed at *m/z* 370, 287, 245& 371, 288, 246 for normal cholesterol and cholesterol-[4-^{13}C] respectively.

CHAPTER FOUR

4.0 DISCUSSION,

Ten sterols were identified in wheat seedlings (Table 2, Fig 9); this to some extent, is in agreement with results obtained from other studies analyzing phytosterols from flowering plants where stigmasterol, β-sitosterol and campesterol were identified as common plant phytosterols sterols (Patterson 1994; Heywood 1993; Guo *et al.,* 1995), but differs in context by presence of cholesterol.

After feeding on wheat seedlings, fifteen sterols were identified in each of the different sections of the locust gut system including the oral secretion, foregut and midgut. Among these sterols included the ten sterols identified in the wheat seedlings. Analysis of the oral secretion from newly emerged unfed first instars desert locust (Fig 10, p 72) identified cholesterol as the only sterol hence confirming that wheat served as a dietary source of sterols for the locust. This finding suggests the locust gut system is capable of metabolizing new sterols. Interestingly, five new metabolites were identified in the gut including: (a) cholesta-8,14,24-triene-3-ol, 4,4-dimethyl-,(3β,5α) **14** (b) cholesta-8,14,24-triene-3,ol, (3β) **15** (c) fucosterol **19** (d) desmosterol **20** and (e) cholesta-5,7-dien-3-ol, (3 β),**22** (Scheme 8).

Compounds **14**, **15** and **20** are likely to be dealkylated products of lanosterol (compound **13,** Scheme 8); based on the following evidence; the mass spectrum of compounds; **13** (Fig 21, p 101), **14** (Fig 22, p 105) and **15** (Fig 23, p 108) molecular

ion peaks *m/z* 426 [M^+], *m/z* 410 [M^+], and *m/z* 382 [M^+] respectively. Compounds **13** and **14** differed in their molecular ion peaks by 16 mass units suggesting loss of a methyl group at carbon in position 28 and formation of $\Delta^{14,15,}$-double bond. Compounds **14** and **15** differed in their molecular ion peaks by 28 mass units suggesting loss of two methyl groups at carbon in positions 29, 30.

It is proposed that dealkylation of compound **13** to **14, 15** and **20** involves oxidative removal of a 4α-, 4ß- and 14α- methyl groups. 14α- methyl group is eliminated first and the replacement by hydrogen proceeds with retention of configuration. After oxidation to a 3-ketone, the 14α- methyl group is eliminated. The 4β-methyl group, after the loss of 14α- methyl group, adopts the α-orientation on equilibration of C-4, i.e. having a more stable equatorial substituent. The methyl groups are eliminated after oxidation to an aldehyde at 14α, and to a carboxyl group for the 14α- methyl, thus being eliminated respectively as formic acid and carbon dioxide. The oxidations proceed stepwise (methyl to primary alcohol to aldehyde to acid) and are catalyzed throughout by mixed function microsomal oxygenases (Svoboda *et al,* 1994). The decarboxylations are favoured by the presence of a neighboring ketone group at position β- to the point of attack. Thus C-3 is alternatively oxidized to carbonyl and reduced to alcohol during the demethylation process (Scheme 5, p 18).

Mass spectrum of compound **20** (Fig 26, pg 116) displayed a molecular ion peak at m/z 384 [M^+]; it is a reduced product of zymosterol (compound **16**) an intermediate not observed in the various sections of the desert locusts analyzed and it is believed

to involve Δ^{14}-double bond in which the 14α H is derived from NADPH and 15β-H from the medium to form zymosterol. The transfer of Δ^{8} to Δ^{5} double bond involves the isomerisation from Δ^{8} to Δ^{7} double bond. The Δ^{5}-double bond is introduced by dehydrogenation of the Δ^{7}-steroid to the $\Delta^{5,\,7}$-diene. In the final step the Δ^{7}-double bond is reduced by the two proteins enzyme to form desmosterol.

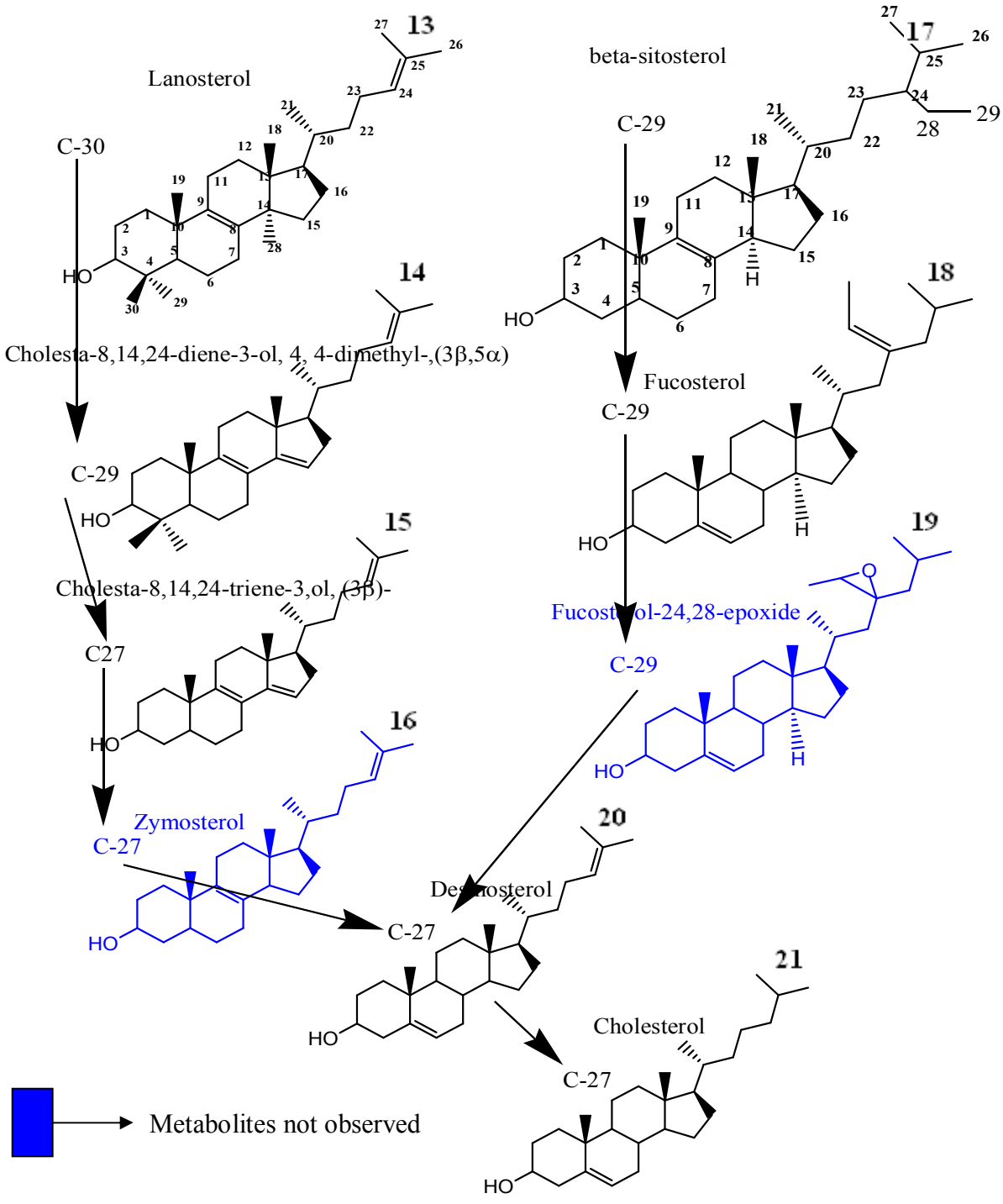
Compound **20** is a also a dealkylated product of sitosterol (compound **17**) and has been shown to follow through compound **18**; In the present study evidence for this pathway is described as follows: The mass spectrum of compounds; **17** (Fig 26, pg 116) and **18** (Fig 25, p 113); displayed molecular ion peaks at *m/z* 414 [M^{+}] and *m/z* 412 [M^{+}] respectively. The molecular ion peak of compound 2**0** differed from compound **17** by an mass unit which can solely be explained by loss of [CH_3^{+} & CH_2^{+}].

The presence of lanosterol metabolites; compounds **14**, **15** and **20** in the oral secretion, foregut and midgut strongly suggests that the desert locust is likely to contain enzymes that are able to convert lanosterol to cholesterol (Scheme 8). This conversion phenomenon has only been shown to occur in the rat liver Soo-Han and Young-Ki 1997, hence these results represent the first record of lanosterol dealkylation in the desert locust.

The presence of sitosterol metabolites; compounds **18** and **20** in the oral secretion, foregut and midgut also strongly suggests that enzymes are present in the gut that are able to convert β-sitosterol to cholesterol (Scheme 8). This reconfirms the fact that

the midgut of the desert locust is able to dealkylate sitosterol to cholesterol (Svoboda 1984).

Evidence exists that dealkylation of the predominant phytosterols; sitosterol, campesterol and stigmasterol occur via the analogous pathway shown in (Scheme 4, pg 14, Wolf 1994) in several insect species. Irrespective of the substrate, the initial step involves oxidation to produce $\Delta^{24\,(28)}$-bond, followed by an epoxidation reaction before dealkylation of the C_1 or C_2 fragment forming Δ^{24}. For stigmasterol dealkylation (Svoboda 1989) an extra step is required, reduction of the Δ^{22}-bond of 22E-cholesta-5,22,24-trien-3β-ol, to yield common terminal intermediate, desmosterol before being reduced to cholesterol. A summary of the dealkylation pathway for lanosterol and sitosterol to cholesterol together with their intermediates is given in Scheme 7 below:



Scheme 8: Dealkylation pathway of lanosterol and β-sitosterol to cholesterol

Cholesta-5,7-dien-3-ol (compound 2**2**, Scheme 8) is a metabolite of cholesterol (compound **21**) and a key intermediate in ecdysone formation, initially shown to be formed through a direct bioconversion reaction restricted in the prothoracic gland (Kappler *et al*., 1988; Grieneisen *et al*., 1993). The current study identified this metabolite (compound **22**) for the first time in the oral secretion, foregut and midgut. A further proof of this bioconversion was sorted through gut incubation with labeled cholesterol.

Incubation of cholesterol-[4-^{13}C] in the foregut, midgut and hindgut under aseptic conditions showed the substrate can be converted to eight other metabolites in the foregut and midgut but not in the hindgut. The metabolites were identified as: (a) Cholesta-5,7-dien-3-ol, (3β), **22** (b) Cholest-7-en-3-ol, (3 β.5α),**23** (c) Cholesterol,7-oxo **24** (d) Cholest-4-en-3-one **26** (e) Cholesta-4,6-dien-3-one **27** (f) Cholesta-3,5-dien-7-one **28** (g) Cholest-4-ene-3,6-dione **29** and (h) Cholestane-3,6-dione, (5α.,17α,20S), **30**.

The results revealed that conversion of cholesterol **(21)** to 7-dehydrocholesterol **(22)** starts early in the foregut and midgut of the desert locust and it involves other intermediates. At this point it can be deduced that 7,8-*dehydrogenase* found in the prothoracic gland or a similar enzyme may also be present in the foregut and midgut..

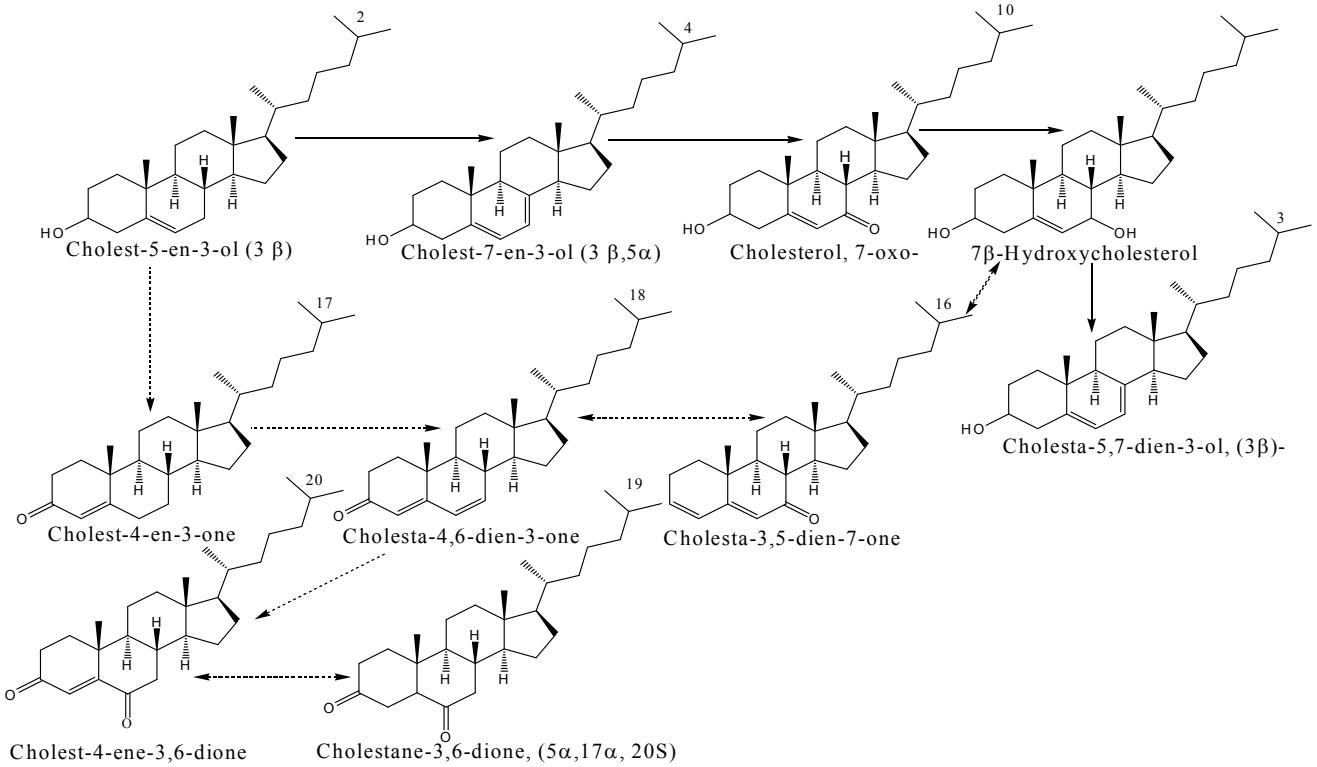
Cherbas, & Cherbas (1970) conducted studies with *D. pachea* and showed that it readily converted lathosterol (cholest-7-en-3-ol) **(23)**, to 7-dehydrocholesterol (Goodnight & Kircher 1971). Labeling experiments with a Δ^7-sterol, [3α,6α-2H2]- or

[3α,6β-2H2] lathosterol in *Ajuga* hairy roots, showed that the 6β-proton migrates to the 5β–position, whereas the 6α-H is eliminated (Ohyama *et al.,* 1999).

In the present study, the results indicated that both the foregut and midgut of the desert locust were able to convert cholesterol to the following metabolites lathosterol, cholesterol 7-oxo and 7-dehydrocholesterol. These data strongly suggest that cholesterol is indirectly converted to 7-dehydrocholesterol via either cholesterol 7-oxo or to first lathosterol before cholesterol 7-oxo, and eventually the later is converted to 7β-hydroxycholesterol and finally 7-dehydrocholesterol. This is the first study of its kind to demonstrate formation of 7-dehydrocholesterol in the gut of the desert locust and the intermediates involved.

Other metabolites synthesized form cholesterol in gut incubation were identified as; cholesta-3,5-dien-7-one, cholest-4-en-3-one, cholesta-4,6-dien-3-one, cholest-4-ene-3,6-dione, cholesterol,7-oxo and cholestane-3,6-dione, (5α,17α,20S), all these metabolites can be attributed to play an unknown important physiological a function in the desert locust.

M. vicina and *D. melanogaster* has been shown to utilize Δ^4-cholestenone to some extent but was inactive in the locusts, (Levinson *et al*,1957). The reason why the desert locust synthesis Δ^4-cholestenone despite it being proven to be a growth inhibiting compound in these species? The following biosynthetic pathway involved in early stages of ecdysone biosynthesis in the desert locust can be proposed (Scheme 8):



Scheme 9: Nine metabolites identified after incubation of the locust gut with cholesterol-[4-13C]. Solid lines indicate suggested biosynthesis pathway from the literature, and dashed lines pathway inferred from structural similarities

Analysis of hindgut and frass revealed ten sterols which were similar to the once found in the wheat seedlings, indicating that the five new metabolites which were present in the oral secretion, foregut and midgut were all selectively absorbed through the midgut of the desert locust, into the hemolymph and eventually stored in the fat body. This inference is corroborated in the hemolymph and fat body extracts of the insects by the detection of these five metabolites. The phytosterol substrates for these metabolites; cholesterol, lanosterol and sitosterol were also present but in minute concentrations in the hindgut and frass: cholesterol ~0.89 pg/mg, lanosterol ~0.77 pg/mg and sitosterol ~0.42 pg/mg as compared to ~9.76 pg/mg, ~1.00 pg/mg and ~0.83 pg/mg respectively for the three sterols in the fore and midgut. These results suggest that the desert locust requires specific amounts of sterols for its normal growth and development.

Following absorption into the midgut, sterols must be transported through the haemolymph to organs of utilization and storage. However, the hydrophobic nature of sterols requires that they are carried through the cytosol and haemolymph by a specialized carrier system. The specific mechanisms by which cholesterol and other sterols are delivered from the hemoceol through the midgut to different tissues and organs are poorly understood. Recently, however, a putative sterol carrier protein thought to be involved in intracellular transport of cholesterol has been identified in the yellow fever mosquito, *A. aegypti* (Krebs and Lan 2003). Likewise, high-density lipophorin (HDLp), a non-covalent mixture of lipid and protein, has been identified as the sole haemolymph carrier of cholesterol in M. sexta (Jouni *et al.,* 2002a).

Whether similar lipophorin molecules were involved in the transport of cholesterol in the desert locust is unknown. This would require further research.

Analysis of prothoracic glands revealed four sterols; Cholesterol, 7-dehydrocholesterol, Cholesta-7-ene-6-one-3,14-diol (3β) (ketodiol) and Cholesta-7-ene-6-one-3,14,25-triol(3β), (2,22-dideoxyecdysone) hence the following conclusion can be made: Path A (scheme 1, page 7) is the preferred pathway for ecdysone biosynthesis in the desert locust this is because majority of sterol metabolites: Cholesterol, 7-dehydrocholesterol, Cholesta-7-ene-6-one-3,14-diol (3β) (ketodiol) and Cholesta-7-ene-6-one-3,14,25-triol(3β), (2,22-dideoxyecdysone) were identified in the analyzed tissues.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The major discovery for the study is that it identified the following metabolites of cholesterol which are involved in the biosynthesis of ecdysone; cholesta-5,7-dien-3-ol, (3β), a metabolite of cholest-7-en-3-ol,(3β5α), and cholesterol, 7-oxo obtained after incubation of cholesterol-[4-^{13}C] in the, foregut and midgut; A literature search shows that cholesta-5,7-dien-3-ol, (3β) is synthesized in the prothoracic glands. However, the present study identified this intermediate in the gut system of the desert locust suggesting that the biosynthesis of ecdysone intermediates occurs early in the locust, in the gut system.

The study also showed for the first time the presence of the following metabolites of cholesterol in the foregut and midgut after cholesterol incubation; cholesta-3,5-dien-7-one, cholest-4-en-3-one, cholesta-4,6-dien-3-one, cholestane-3,6-dione, (5α,17α,20S) and cholest-4-ene-3,6-dione, further research would be required to ascertain their roles in the physiology of the locust.

This study has demonstrated how versatile GC-MS is as a simple method to identify triterpenes and useful tool for rapid chemical characterization of sterols and elucidation of their biosynthesis.

5.2 Recommendation

1. I wish to underline the absolute need for molecular biological approaches in this area in order to address the following basic questions:

a) Further work should be done to identify which desert locust gut system cells (all or specialized ones) does ecdysteroid biosynthesis start to take place.

b) A study should be under taken to identify if whole biosynthetic pathway take place in the same cells or does it involve some 'cooperation', as is often the case for animal steroids.

c) Further research should be carried to ascertain the viability of lanosterol as a dietary sterol for the desert locust.

2. Further studies should be done which in-cooperates wheat bran.

3. Further work should be carried out to unequivocally characterize and quantify cholesterol in wheat seedlings (or a close analogue)

4. Experiments to confirm identities of all preliminarily identified metabolites by direct chromatographic and spectral comparison with authentic samples.

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APPENDICES

APPENDIX I: Summary of Anova Table of sterols from wheat seedling and the tissues of the desert locust

Part analyzed	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Wheat seedlings	9	330208	36690	4132.9	< 0.001
Oral secretion	14	6.1398	1.86713	11333	< 0.001
foregut	14	406.01	29.0010	21859	< 0.001
Midgut	14	150.590	10.756	11131	< 0.001
Hindgut	9	76.205	8.4673	12916	< 0.001
Frass	9	53.489	5.9432	16249	< 0.001
Hemolymph	7	2.98393	0.42628	8682.4	< 0.001
Fat bodies	7	8.9698	1.28140	41671	< 0.001
Prothoracic gland	3	0.6221	0.207365	16997	< 0.001