Sterols in Mangrove sediments of the Cochin Estuary

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Dedicated with salutations unto

H.H Swami Chinmayananda,

who initiated me into the World of

Learning

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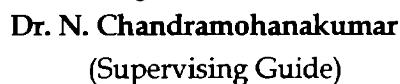
CERTIFICATE

This is to certify that the thesis titled "sterols in Mangrove Sediments of the Cochin Estuary" is an authentic record of the research work carried out by T.Narayanan under my supervision and guidance in the Department of Chemical Oceanography, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfilment of the requirements for Ph.D degree of Cochin University of Science and Technology and no part of this has been presented before for any degree in any university.



Kochi – 16
June, 2006

Dr. N. Chandramohanakumar
(Supervising Guide)

DECLARATION

I hereby declare that this thesis entitled "Sterols in Mangrove Sediments of the Cochin Estuary" is an authentic record of the research work carried out by me under the guidance and supervision of Dr. N. Chandramohanakumar, Professor and Head, Department of Chemical Oceanography, School of Marine Sciences, Cochin University of Science and Technology, and no part of this has previously formed the basis of the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Preface

Mangroves are the tidal forests of coastal wetlands, existing in the intertidal zones of sheltered shores, estuaries, tidal creeks, backwaters, lagoons, marshes and mud-flats of the tropical and sub tropical regions of the world. Mangroves have assumed great economic significance both in terms of direct resource of forest and fishery as well as their indirect value in protecting coastlands. The Mangrove ecosystems are considered to be the natural hatcheries of the crustaceans like shrimps, prawns and many fish varieties. They provide an excellent supply of organic detrital matter in the early food chain of coastal and insular habitats.

Sterols are considered to be excellent biomarkers for tracing diagenetic transformations in both recent and ancient sediments. The study of sterol distribution in sediments gives an idea about the contributing biota or dietary modifications brought about by the flora and fauna present in the environment. The sterols are not easily decomposed in sediments and bear many structural features characterizing their origin and subsequent biochemical transformations.

My thesis entitled "Sterols in Mangrove Sediments of the Cochin Estuary" is an attempt to characterize the sterol content of the mangrove sediments, their dietary status with respect to the natural flora and fauna present, their transformations in the sediment and assess contributions, if any to

the nursery character of the mangrove eco system. Samplings were done from two sites at Mangalavanam and Vypin. Mangalavanam is a patchy mangrove area in the heart of the city of Cochin and serves as a small bird sanctuary. This is an almost closed system with a single narrow canal linking to the estuary. Vypin, the largest single stretch of mangroves in Kerala, is regularly inundated by a semi diurnal rhythm of Cochin bar mouth. Perhaps, this is the only site in Kerala where one can see mangroves right along the accreting seacoast. However a lot of developmental pressure is threatening the very existence of these mangroves. Post monsoon sediment samples from these areas were used for the present study, as it is the period of maximum faunal growth and activity.

The thesis is divided into three chapters. Chapter I is the Introduction and it deals with the general purpose of the present study. It also deals with the general methods of isolation and characterization of sterols.

Chapter II is the Materials and Methods. This chapter deals with the nature and extent of the study area including the hydrographical and sediment characteristics and the method of analysis of sterols.

Chapter III is The Sterols and in it is discussed the general aspects of the sterol chemistry and the related analytical techniques for the study. The chapter further deals with the nature of sterols identified and their significance in the Mangrove ecosystems.

A brief summary of the work done is given at the end of the thesis and the references at the end of each chapter.

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Chapter I

Introduction

1.1 Mangroves- a perspective

1.2 Mangrove ecosystems of Cochin

1.3 Sterols in aquatic systems

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1.5.1 Extraction and isolation of sterols

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a. Derivatization of sterols

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c. Characterization and Quantification of sterols

1.6 Alternative chromatographic techniques

1.7 Aim and Scope of the present study

1.1 Mangroves- a perspective

Mangroves have long been a source of astonishment for the laymen and of interest for scientist (Bandaranayake, 2002). Mangroves are the tidal forests of coastal wetlands, existing in the intertidal zones of sheltered shores, estuaries, tidal creeks, backwaters, lagoons, marshes and mud-flats of the tropical and sub tropical regions of the world (ENVIS Report, 2002). Tomlinson (1986) has defined mangroves as halophytes that thrive in the intertidal zone found in tropical and sub tropical climates. The total area of mangroves in the Indian Ocean region is 84,984.56 km^2 equivalent to about 47% of the total area of world mangroves. Indonesia accounts for about 50% of the mangroves of Indian Ocean region. According to a Government of India estimate, the total mangrove area in India is put to be around 6000 km^2 (Nair and Sankar, 2002). Kathiresan and Rajendran (2005) put it around 4871 km^2 Venkataraman and Wafer (2005) in their study on coastal and marine diversity have put the area of Indian mangroves as 4827 km^2, of which about 57% are along the east coast, about 23% are along the west coast and the remaining 10% in the Andaman and Nicobar Islands.

Mangroves confer many direct and indirect benefits to the coastal population. Studies in Cuddalore district of Tamilnadu in India have indicated the definite advantage of coastal vegetation like mangroves in attenuating tsunami induced waves and protecting shorelines against damage (Danielsen *et al.*, 2005). The annual turnover of wood biomass is being utilized by the

common folk for firewood, charcoal etc. The foliage is used as a cattle feed. The most important utility is the litter production in mangrove areas that is estimated to be about 5-10tonnes/ha. This litter upon degradation becomes detritus, which is utilized by the prawns and fishes. Mangrove ecosystems are the world's most productive ecosystems (Kathiresan and Bingham, 2001). Mangrove leaves are significant sources of lipid compounds to estuarine ecosystems and the tidal waters transport the lipids and fatty acids adsorbed to particulate matter from mangroves to adjacent estuarine sediments and the ocean (Mefilinge *et al.*, 2005). (Bandaranayake, 2002) has surveyed the bioactive compounds of mangrove plants. Many of them contain active ingredients against common ailments but some species like Exoecaria agallocha contain antiviral components including those active against human immunodeficiency virus. Mangrove ecosystem eventually provides an excellent supply of organic detrital matter in the early food chain of coastal habitats. Krisnamurthy and Jayaseelan (1984) have compared the animal production in the Pichavaram (Tamilnadu) mangroves with adjacent area without mangrove. The prawn production in the area was 110 kg ha/year and in adjacent area 20 kg/ha/year, whereas the fish catch was 150 kg/ha/year and 100kg/ha/year respectively. In the Cochin estuarine system, the mangrove based water bodies are visited by more than eight species of prawns, twelve species of estuarine fishes and over fifty species of marine organisms. About eight to ten thousand tonnes of fishes are harvested annually from this area. The mangrove shelter area and detritus are the principal causes that sustain this

production also (Subramaniyan, 2000). Above all mangrove ecosystem contains species, which can donate the necessary genes that are highly valuable in the development of transgenic plants for adoption to climatic change. It is possible to isolate from mangrove species, the genetic material conferring tolerance to seawater intrusion and transfer them to other plants growing near coastal areas (Subramaniyan, 2002).

1.2 Mangrove ecosystems of Cochin

The Cochin estuary includes a system of interconnected lagoons, bays and swamps penetrating the main land and enclosing many islands in between, whose total area amounts to approximately 500 km^2 The backwater around Cochin is located between Lat. 9^0 40'-10^0 12' N and Long. 76^0 10'- 76^0 30'E. This estuary is connected with the Arabian Sea by a permanent opening, 450m wide, through which tides act within the estuary. The major sources of fresh water in the estuary are the two rivers, the Periyar in the northern part and Muvattupuzha in the southern part. In addition, several small tributaries, irrigation channels and innumerable drains contribute to the system. The entire Cochin estuary is a part of the large Vembanad-Kol wet land ecosystem, a Ramsar site. Mangrove ecosystems are found in isolated patches along the banks of this estuary. They cover an area of 2.6 km^2 Like many other mangrove ecosystems all over the world, these are also subjected to increasing human influences. Millennium Ecosystem Assessment Report (2005) states that 35% of the total mangrove area has been lost worldwide in last several

decades. The total mangrove area in the state of Kerala has decreased from 1000 km^2 a century ago to a mere 17 km^2 (Basha, 1991). Indian Remote Sensing Satellites are being used to estimate the present status and loss, if any in mangrove areas recently (Vijay *et al.*, 2005).

The important problems concerning the depletion of mangrove forests of Cochin can be classified into two categories. The first is from land reclamation and other developmental activities. The second is from pollution by industrial effluents as well as domestic sewage.

The wetlands of Kerala included a large mangrove swamp centuries ago and anthropogenic activities modified the system into settlements, agricultural fields, filtration ponds and prawn culture fields. The plots employed for prawn farming are of 2-10 ha in size and are contoured by low mud dikes planted all along with coconut and other cash crops. The water flow inside the ponds is regulated by sluice gates operated manually. Here, salt resistant paddy is cultivated during monsoon periods. This is known as 'Pokkali' cultivation. During inter monsoon period, when seawater enters inside, prawn farming is conducted. Prawn culture is another form of traditional agro silvi aquaculture followed in this country. The conversion of mangroves that has taken place and the ecological loss thereon is difficult to quantify.

A large number of heavy industrial establishments are situated on both sides of the river Periyar and on the southern bank of the river Muvattupuzha. These industrial concerns discharge their wastes into the estuary. Mangroves

act as the sinks for the heavy metals so discharged. Moreover large areas of mangrove areas are being reclaimed for the land requirement subsequent to the industrial development.

1.3 Sterols in aquatic systems

Lipids usually represent a small fraction of the total organic carbon, but their diversity and specificity make them useful compounds to study the sources and transformation of organic matter (Azevedo, 2003). The present study attempts to know the above aspects in mangrove sediments of Cochin estuary.

The Steroids are a widely active group of colorless and for the most part saturated natural products possessing the tetra cyclic carbon skeleton. The alcoholic derivatives of steroids are widely distributed in living things. The most widely distributed sterols possess 3β equatorial alcohols or have Δ^4 3-keto group.

1.3.1 Sterols as biomarkers

Many studies have highlighted the advantages of sterols as biological markers. Sterols are potentially excellent biomarker compounds due to their stability and the diversity of their structures. Their detection in marine sediments is common for this purpose (Parrish *et al.,* 2000). Biomarkers are geochemical organic compounds that have a carbon skeleton, which can be related to their biological precursors. An important class of biomarkers formed

by diagenesis and maturation of sterols is aromatic steroids (El-Gayar, 2005). In ecological studies the concept of biomarkers are meant as indicators signaling events in biological systems or samples following chemical exposure (Schlenk, 1999). Many pentacyclic triterpenoids like Taraxerol, Germanicol and phytosterols like, β-sitosterol have recently been identified as biomarkers for mangroves (Koch *et al.*, 2003; Versteegh *et al.*, 2004). The study of sterol distribution in sediments gives an idea about the contributing biota or dietary modifications brought about by the flora and fauna present in the environment. The sterols possess structural features such as positions of double bonds and pattern of side chain alkylation, which are restricted to a few group of organisms. Sterols are also excellent biomarkers for tracing diagenetic transformations in both recent and ancient immature sediments sterols are popular biomarkers, because as a group they represent a wide variety of molecular structures that are remarkably useful as source indicators and because alterations to the sterol skeleton can be readily followed with analytical tools available to organic chemists. The concept of biomarker is not a new one and involves the use of specific lipid components in tracing the origin of biological material. It has been remarked that the lipid compositions of recently deposited sediments are a result of input of biological material and its utilization by microorganisms, contributing towards the biomass of the sediments. (Perry, *et al.*, 1979; Nicholas *et al.*, 1982) Although lipids usually represent a small fraction of total organic Carbon, their diversity and specificity makes them useful components as biomarkers. In the paper entitled Review of

Sterol Biomarkers a summary of the studies on sterol sources till that time has been given (Volkman, 1986). Saliot et al. (1988) have pointed out that the biological activities, particularly the biochemical activities are indicated in interstitial water by the abundance and the distribution of lipids like branched fatty acids, odd carbon numbered n-alkanes and stanols. To minimize the problems due to the transformations, using of relative and absolute concentrations instead of presence/absence of several biomarkers has been suggested (Colombo *et al.,* 1996). The C/N ratio is also indicative of the source. Shi et al. (2001) during their studies on variability in the distribution of lipid biomarkers have made use of this concept to substantiate the evidences obtained elsewhere to distinguish between terrigenous and various marine organic matter. The rapid alteration brought about in the lipid compositions by the microorganisms, is called biological diagenisis and is brought about by the organisms present in the upper sediment. Past studies have shown that the degradation of distinct classes of biochemical tracers like fatty acids, sterols, lipids, proteins, carbohydrates and pigments is significantly higher in anoxic than in anoxic sediments (Chen *et al.,* 2001). This fact assumes significance in the present study since one of the mangrove sediments under study is anoxic. Steroids can be used to trace organic matter alterations involved even in petroleum sources even (Wakeham, 1987). Steroidal alcohols are comparatively more stable in sediments and hence they have a long geological record (Gagosian *et al.,* 1982). They are less active than fatty acids and alcohols, but more active than the bulk organic matter such that sterol

concentrations decrease during diagenesis relative to total organic Carbon (Wakeham, 2002). Also it should be noticed that the differences between phyla and individual species are more a question of actual concentrations than of overall spectrum of sterols present since basically the same range of sterols occurs in all phyla (Goad, 1978). Hudson et al. (2001) have found no decreasing trend in total or individual sterols down a thirty cms deep core from recent sediments in a cold ecosystem and have indicated overall preservation. There has been and is a continuous interest in the sterols of marine organisms ever since the early studies of Henze (1904) and Doree (1909) revealed the presence of sterols other than the common animal sterol. The works of Bergman (1962) has given a review of earlier works on sterols. Later Heilbron *et al.* (1926) chararacterized Fucosterol in marine algae.

1.3.2 Sterols as life sustaining agents

The biosynthetic studies on sterols have shown that many of the sterols found in invertebrates are either unchanged or modified dietary sterols to of algal origin. Goad (1978) in his article on sterols of marine invertebrates has summarized the sources and transformations of various sterols in marine environment. Kanazawa, (2001) while discussing the nutritive value, metabolism and composition of sterols in marine environment, has summarized the results of researches during the past two decades. In this study, the sterol compositions of marine invertebrates, crustaceans, mollusks, echinoderms, coelenterates, and sponges have been re-examined by using improved

analytical techniques. As a result, the sterol composition of marine invertebrates has been shown to be more complex mixtures including many new types of sterols with unusual steroid nuclei or with non-conventional side chains.

Several authors have pointed out that Crustaceans including prawns lack the ability to synthesize sterols (Goad, 1978; Kanazawa, 2001). The essential requirement of Cholesterol or other phytosterols like β-sitosterol as a supplement in the diet of the prawn species Paenus japonicus has been shown and reinforced by several workers and is discussed in detail by the above authors. Ramesh et.al. (1992) have pointed out that crude mangrove sterols when included in the diet of penaeid prawn Penaeus indicus, the species showed greater absorption rate and growth rate besides assimilation efficiency and net growth efficiency of prawn juveniles. They have further suggested that the positive effect on growth rate may be due to the fact that the Cholesterol acts as precursor of molting hormones. The isolation technique used by them separates only mangrove sterol mixture. So this study shows the importance of mangrove sterol mixture in the diet of prawns. When fed on other sterols like C-24 alkylated sterols the species showed very good survival rates but the growth rates were inferior to those specimens fed on Cholesterol. (Goad, 1978). A feeding experiment using artificial diets conducted by Kanazawa (1992) first demonstrated that *Penaeus japonicus* requires sterols for growth and survival, and reported a value of 0.5% dietary cholesterol for good growth. The optimum or required levels of dietary cholesterol for crustaceans reported are

approximately 0.1–2.0% of the dry weight of diet and may be age and diet dependent (Kanazawa, 2001). A growth study (Chen, 1993) using juvenile Penaeus monodon, fed on test diets containing graded levels of cholesterol ,0, 0.5 and 1% of diet) and purified phosphatidylcholine (0, 1.25, 2.5 and 5% of diet) in combination showed that the shrimp attained optimal growth when diets contained 1% cholesterol or 1.25% phosphatidylcholine. It has been indicated that the juvenile Macrobrachium rosenbergii, a fresh water species was capable of de novo cholesterol synthesis in contrast to other prawn species but still required a dietary source of 0.1% cholesterol for the maximum growth (Teshima *et al.*, 1997). The above studies have also shown that the crustaceans can convert the phytosterols to cholesterol biochemically. So these studies point towards the importance of dietary supplementation by mangroves, which is the natural habitat of prawns, though in an indirect way.

The sterols occurring in the sponges are thought to be derived from exogenous sources or by the modification of dietary sterols, because the sterol biosynthesis in the sponges seemed not to take place or to proceed at a slow rate. Therefore, the sterol components of sponges are likely to vary with habitat. The sterol composition of molluscs varies from class to class (Teshima, 1991). Chitons, the most primitive molluscs belonging to class Amphineura, contain Δ^7-sterol, mainly cholest-7-enol, in contrast to other classes of molluscs which predominantly possess Δ^5-sterols. When the phylum Echinodermata is considered the presence of considerable amounts of Cholesterol sulphate is a peculiar feature. Cholesterol sulphate is the major sterol even in classes of

phyla in which Δ^7 free sterols are dominant Gastropods and cephalopods contain primarily cholesterol with small amounts of C_{28} and C_{29} sterols. This shows the importance of cholesterol and other sterols towards the nursery character of mangroves for the prawn species, (Goad, 1978). Thus these studies have indicated that the mangrove sterols promote growth, conversion efficiency and biochemical constituents in prawns rather than synthetic sterols. Kanazawa, while reviewing the requirement and nutritive value, biosynthesis, metabolism, composition and structure of sterols in marine invertebrates has pointed out that the crustaceans and some molluscs require dietary sources of sterol for growth and survival because of the absence of de novo sterol-synthesizing ability. This also explains the fact that the Crustaceans have one of the most simple sterol compositions among the nonchordates (Kanazawa, 2001).

In an attempt, to find hypocholesterolemic substances without hormone activity, for humans, sterols isolated from marine organisms have been tried and found to yield promising results.. 7-Cholestenol and 24-methylenecholesterol from mollusks were found to significantly decrease the cholesterol level in both serum and liver of rats (Teshima *et al.*, 1974).

1.3.3 Sterols and pollution

Environmental changes can bring about change in sterol distribution pattern though such changes are infrequent in aquatic systems but they can occur in organic rich sediments. However if there is an exigency like an algal bloom, there will be a change in the nature of organisms and a consequent

drastic change in the sterol distribution ratio in aquatic systems as well as in sediments. Bacteria though in general are non capable of synthesizing sterols can bring about modifications in the sterol structures. Human fecal pollution is indicated by Coprostanol/ Cholestanol ratio being greater than one. However the sterols can be reduced under anaerobic conditions existing such as in mangrove sediments, which also changes the distribution ratio. Coprostanol, a sterol that is an indicator of fecal contamination, has been studied in order to determine the degree of fecal pollution in a part of Lake Constance, Germany during the past 200 years (Muller *et al.,* 1979). The concentrations ranged from 0.0 p.p.m. in the lower most part of the core below 12 cm to 5.87 p.p.m. in the uppermost 0–1 cm sediment layer. The analyses of chemicals within coral bands have been proposed as a method to investigate past historical events of pollution in the marine environment (Law *et al.,* 1994). To investigate this hypothesis coral colonies were collected from Kuwait coastal waters following the Gulf War to examine the hydrocarbon and sterol contents in different bands. Analyses of sterols revealed significant amounts of cholesterol and β-sitosterol, which generally declined rapidly from around 250 μ g/g in the surface layer to undetectable levels in the bottom layers. Correlations obtained between the marine sterols and the principal fatty alcohols produced in zooplankton also help to ascribe sterol sources (Yunker *et al.,* 1995). Same authors and triterpenoids have used Principal Component Analysis method to compare the sources of sterols.

1.4 Alternative Source Characterizing Methods

The stable isotope analysis ,$\delta^{13}C$), when combined with the biomarker studies can yield valuable information regarding the relative contributions from marine and terrestrial sources in an estuary (Fichez *et al.*, 1993). In a novel study, δC^{14}Canalysis and δ ^{13}C stable isotope analyses have also been utilized to determine the sources of individual lipids including sterols (Pearson *et al.*, 2001). While distinguishing between the particulate organic matter of riverine and mangrove origins, it was shown that the yield of lignin phenols and $\delta^{13}C$ as paired indicators is the best parameter of source assignment (Dittmar, 2001). Fatty acid distribution and compound specific isotope analysis of $\delta^{13}C$ have been engaged to establish the completely marine origin of the acids in the particulate matter from Alboran Sea (Tolosa *et al.*, 2004). To characterize the source of dissolved organic matter in a mangrove- dominated estuary. (Jaffe *et al.*, 2004) have developed a Fluorescent analytical technique.

1.5 Analytical Methodologies of Sterols

An examination of literature before 1960 indicates that for the most part new sterols identified in an exotic manner. Detailed studies revealed many, of them to be actually a mixture of co- crystallized sterols. With the advent of newer methods of analysis the structures of sterols have been unraveled, some of them with unique alkylation patterns in the side chain and some others with modified ring structures. Since 1960 many useful marine pharmacological

products have been isolated and this generated interests in the study of sterols also. Another stimulus was the development of highly improved separation techniques like glc, argentation chromatography and gcms. This type of analysis has led to the identification of sterols with less than 0.1% availability. These methods avoided the problems of co crystallization of sterols and allowed separation and identification of more than twenty sterols in a mixture. Kawakami and Montone (2002) have summarized the steps involved in the analysis of sterols from sediment samples as i) extraction of sterols from the matrix; ii) fractionation, also called clean up by adsorption column chromatography; iii) derivatization of the sterol into their trimethylsilyl ethers; iv) determination of the steroid by gas chromatography coupled to flame ionization detector, and v) conformation of the compounds with mass spectrometry. The steps, which are commonly employed for the extraction and clean up involve the use of large volumes of solvents. Consequently the procedure produces large quantities of wastes. The above authors have suggested an alternate procedure for extraction using ethanol as the solvent and simpler clean up procedures suiting specific types of analyses.

1.5.1 Extraction and Isolation of Sterols

The extraction of organic components including sterols is a laborious process. One of the routine methods employed is the extraction with an organic solvent at its boiling point in a Soxhelet Extractor. For the present type of analysis, often a mixture of solvents or two solvents in succession are

employed. An alternate procedure is the use of a soniccator for the solvent extraction. In the latter case wet sediments can be used. This is best suited for the study of thermo labile materials such as the bioactive components. This fact is extremely important since all the different classes of compounds in a sample are routinely extracted together and then subjected to further separation using appropriate chromatographic procedures. The procedure may leave behind a small fraction of inextractable lipids. For their extraction a saponification may be necessary initially. However these sterols have the same profile as in the extractable lipids and the amounts are also relatively small (Nishimura, 1977). The extracted sample is usually subjected to saponifiction using methanolic or ethanolic KOH to liberate the bound or esterified sterols, if we are studying the complete set of sterols. Volkman *et al.,* (1993) have conducted both acid and basic hyrolysis of the neutral fraction and shown that basic hydrolysis liberates the bound sterols better than acid hydrolysis in all cases. The sediment samples containing sulphides should be redissolved in Hexane and passed through a column of Copper to remove the sulphur content (Blumer, 1957), a practice followed by subsequent workers. Huang and Mienschein (1976) have extracted sterol samples from a sediment sample by boiling with a mixture of Chloroform- Methanol mixture (2:1 v/v) for 48 hours. The extract was concentrated and was saponified with methanolic KOH. The non-saponifiable fraction was extracted into Chloroform. The sterol fraction was isolated using liquid chromatography (LC) over a column of Unisil adsorbent. Cranwell (1978) has given a detailed scheme of isolation of various

types of lipid components from the sediment. This helps to get an account of the bound and free sterols separately. He has combined the method of grinding (Bligh and Dyer, 1959) with Soxhlet extraction using Heptane solvent and then developed a scheme making effective use of methanolic KOH and other common solvents for the separation of various components. He has used Argentation Chromatography of sterol acetates for their isolation. Volkman, et al. (1981) during their studies on neutral lipids in intertidal sediment have extracted the lipids into a mixture of Chloroform and Methanol by sonication. Wakeham, (1987) has used Soxhlet extraction with Methylene chloride for the extraction of lipids from north Pacific sediments. Lipids were further fractionated on silica gel column with a series of solvents starting from Hexane. 4-methyl sterols were eluted using 15% ethyl acetate in Hexane and 4-desmethyl sterols with 20% Ethyl acetate in Hexane. Wakeham et al. (1984) during a work on particulate matter have used Toluene and Methanol for the extraction of a freeze-dried sediment sample and the partitioned the sterols using Liquid Chromatography (LC) as above. Brault and Simoneit (1987) used ultrasonication with Methylene chloride and methanol solvents for extraction. They further isolated the lipid components by LC over silica gel using solvents of increasing polarity commencing with Heptane; for sterols they used 15-20% Ethyl acetate in Heptane. Wakeham and Beier (1991) have used a mixture of Chloroform and Methanol in a soxhlet (24 hours) for extraction of lipids from the sediments. Santos, et al. (1994) have also used sonication technique with Dichloromethane solvent for the extraction of lipids from deep-sea. Pearson et

al. (2001) have extracted the total lipids by a transesterification procedure that involved heating with 5% Hydrochloric acid in Methanol at 700 ^{0} C for twelve hours and subsequent isolation using LC over over aminopropyl bound silica gel. The Alcohols, Hopanols and Sterols were obtained using a solvent mixture of 20-25% Ethyl acetate in Hexane. Leeuw et al. (1983) have given an elaborate procedure to isolate different classes of sterols from wet sediment of the Black Sea. The method involves an extraction using sonication in a solvent system containing a mixture of Dichloromethane and Methanol and then precipitation of 3β- sterols with digitonin. Gogou et al. (1998) have developed a one step method for the fractionation and analysis of molecular markers belonging to the lipid class, using Flash Chromatography. They have used. Methylene chloride as the solvent and obtained a recovery of about 78%-81% for sterols. Koch et al. (2003) also have made use of a saponification prior to the extraction of lipids with Dichloromethane. Chou and Liu (2004) have utilized a direct saponification technique, followed by solvent extraction to obtain sterol mixture.

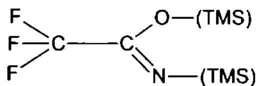
1.5.2 Gas chromatographic techniques

Gas Chromatography (gc) is the common method used in the identification of components of terrigenous or marine sediments. A gas chromatograph coupled to a Mass spectrograph (gcms) is the method of choice for identification of organic components. A gc with a conventional detector

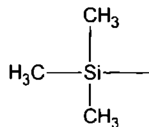
like the Flame Ionization Detector (fid) can be used to have a preliminary idea of the compounds present and is extremely useful for quantification.

a. Derivatization of Sterols

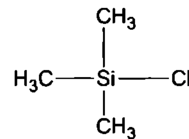
The compounds subjected to Gas Chromatographic Analysis should be volatile. Sterols, in some cases have been directly injected into GC column and analyzed (Goad and Withers, 1982). Derivatization is done so that we get a volatile derivative of certain compounds in a mixture and such that these derivatives are stable during the passage through the GC column. Acetylation using Acetic anhydride in Pyridine can be resorted to (Neher, 1969; Gagosian *et al.,* 1982). The identification would be very difficult in the case of present type of analyses consisting of a mixture of closely related sterols because the acetates fail to produce characteristic peaks in the higher mass range (Lee *et al.,* 1980). Hence silylation procedure was resorted to. The major problem in this procedure is the low shelf stability of the sample and the extreme care necessary for the exclusion of even traces of moisture from the reagents and the samples. It must be pointed out that the silyl derivatives are highly unstable in presence of moisture.



BSTFA



TMS (Trimethylsilyl-)



TMCS

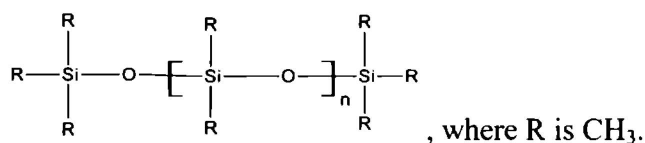
In the method suggested by Gleispach (1974) silylation is done at room temperature. According to the author, this procedure wills silylate 3α-and 3β-, 20α- and β-, 11β-, tertiary 17β- and 21-OH alcoholic groups. Many workers have prepared the silyl derivatives by heating at 60^0C for one hour. In many of the subsequent works, the same procedure has been utilized. Perry A.J (1981) has given a detailed account of the derivatizing reagents, commonly employed. He has suggested the mixture of BSTFA [2,2,2-trifluro-N,N-bis(trimethylsilyl)acetamide] and 1% TMCS [Trimethylchlorosilanes] at room temperature or at 60^0C as a common reagent that can be used for the derivatization of sterically unhindered OH groups of sterols. The alternate procedure that is also in current usage suggested by Hammargren et al. (1991) involves heating of 500 ng of the material with 50 µl of the reagent mixture and 50 µL of dry pyridine for one hour at 100^0C. Colombo et al. (1996) have heated the above mixture for two hours. A mixture of BSTFA and iso-octane at 100^0C has been used by Gogou et al. (1998). Koch et al.(2003) have used N-methyl, N-trimethyl trifluoroacetamide (MSTFA) along with Trimethyl iodosilane (TMSI) in Trimethylamine and Dichloromethane. MSTFA+ TMSI has been used for recent works such as those of (Chou and Liu, 2004).

b. Column materials

The adsorbent material used for column in Gas Chromatography is fused silica. This offers certain advantages over the conventional glass column. It has less oxide content, has high tensile strength and hence can be drawn into

exceedingly thin walled columns (0.2 mm i.d x 0.25 mm o.d).These columns are also to be desilanized to remove the active silanol functions, which otherwise may contribute towards chromatographic defects (Jennings, 1987). Long open tubular capillary columns in which the stationary phase is coated uniformly are used and this ensures a high number of theoretical plates for separation, thus increasing efficiency of the column to a greater extent (Ambrose D 1971).

The most commonly used stationary phase for separation of sterols is Methylpolysiloxane. To adjust the functionality, other groups may be substituted for the methyl groups



, where R is CH_3.

If we substitute about1% of the methyl groups with phenyl groups, we get columns like DB-1, PE-1 etc.; if 5% of groups are substituted, we get DB-5, PE-5 etc.

c. Characterization and quantification of sterols

Gagosian et al. (1982), while studying the sterols in waters of N. Atlantic Ocean, have used a temperature programming starting from 200-360 at 4^0C /min., He as the carrier gas and a column SE-30, 30m x 0.25mm (i.d). Nicholas et al. (1984) performed the analysis of sterols using a wall coated capillary column. Identifications were based on RR_f measurements, co-injection

with authentic standards and by comparison of mass spectra, published earlier.5α-cholestane has been used as an internal standard in almost all works. Volkman et al. (1990) have used methyl nonadecanoate as the internal standard. Misra et al. (1984) analyzed the sterol derivatives using a support column OV-17. Harvey et al. (1988) utilized OV-1 column 25-m.x 0.3mm i.d temp programmed from 100-300 at 2^0 min^{-1} An equivalent HP-1 column and a programming starting from 50^0C- 150^0C at 30^0C/min, then at 2^0C/min to250^0C and 300^0C at 5^0C/min has also been in use (Skerratt *et al.,* 1995; Nelson *et al.,* 2001). Hudson et al. (2001) have used the above column and He as the carrier gas. Nicholas, et al. (1982) have used an SE-30 SCOT column, with a temperature programmed from 150^0-280^0 at 20^0min^{-1}, also using He as the carrier gas. For the identification and quantification of sterols, previously published data on R_f and mass fragments using gcms and gc-fid as well as the data obtained by co-injection of standard compounds have been utilized (Robinson *et al.,* 1984; Brault and Simonet, 1987; Wakeham S.G, 1987; Wakeham and Beier, 1991). The columns used by the above authors were DB-5 column, Hydrogen being used as the carrier gas and 5α-cholestane as the internal standard for sterols. Leeuw et al. (1983) have made use of averages of peak areas of GC traces and Reconstructed Ion Chromatogram (RIC) traces of gcms for quantification and obtained reproducibility within 10%. Greenaway and Whatley (1990) have identified the compounds by Single Ion Reconstruction (SIR) technique of the Total Ion Chromatograms (TIC) of gcms, but only a mixture of two compounds can be analyzed at a time. Gogou

et al. (1998) have used He as the carrier gas for both (gc-fid) and gcms analyses. Santos, et al. (1994) have calculated the quantitative data by comparison of peak area of the internal standard with the peak areas compounds of interest, in TIC of gcms. For quantification Colombo et al. (1996) have utilized the average response factor of the six available sterol standards and obtained a recovery yield of 71$\pm$13%. They have also used the published data on retention times for the characterization of peaks. Koch et al. (2003) made use of peak intensities of specific ions in TIC for determining the relative intensities, not the absolute concentrations of sterols.

The sample, which can be separated in an open tubular column, is around $10^{-2} - 10^{-3}$ µl. However, the smallest sample that can be injected using a syringe is 0.1-0.5 µl. The universal solution to this problem is to use the Split mode of injection. The principle is to vaporize the injected sample, mix and homogenize it with the carrier gas and then vent a small portion only into the column (Ambrose D, 1971). The remaining portion is removed. The split ratios may be adjusted to get effective concentrations required for the appropriate detection technique. Another mode of injection in usage is the 'on-column injection', which is best suited for thermally labile materials. In this method, the sample is directly introduced into the top of the column. This ensures complete vaporization and passage through the column, but the traces of impurities are likely to cause problems due to column bleed (Jennings, 1987). In works on sterols, the split splitless mode of injections has been utilized by many workers (Skerrat *et al.,* 1995; Gogou and Stephanou, 2004). The on-column injection

has been utilized in several works (Wakeham and Canuel, 1990; Volkman *et al.*, 1990; Colombo *et al.*, 1996). This is a matter of convenience in the case of sterols, since it has already been pointed out that there is no thermal breakdown of the sterols in the hot injector (Volkman *et al.*, 1987).

1.6 Alternative chromatographic techniques

Another method of analysis of lipids constitute is TLC separation of the lipid components and direct analysis using FID (Volkman *et al.*, 1992; Volkman et al.,1993; Harvey and Johnston, 1995; Derieux *et al.*, 1998 and Geiss *et al.*, 2001). Identities of the major classes of lipids can be studied, but individual polar lipids are not identified. Sterols and fatty acids can be isolated from the neutral fraction of the extract concerned by TLC or the by LC and then analyzed using gc-fid as usual. By this procedure 4-methyl and 4-desmethyl sterols are effectively separated prior to GC analysis.

Giner et al. (2003), while studying the toxic sterols from a dinoflagellate species has effectively made use of High Performance Liquid Chromatography (hplc) technique for sterol isolation and analysis. The sterol fraction was fractionated by hplc using two C_{18} columns, 10 mm i.d. ×25 cm, 5 μm particle size linked together in a series. The columns were eluted with 100% Methanol at a flow rate of 3 mL min^{-1}, and sterols were detected using a differential refractometer detector. The relative proportions of sterols were determined from integration of the hplc traces. The fractions containing sterols were collected, and the solvent was evaporated with a stream of Nitrogen gas. They

characterized sterols by 300 MHz ^{1}H-NMR (in deuterochloroform) and gcms. The gcms data were obtained using a gas chromatograph equipped with an HP-5 capillary column cross-linked 5% phenyl methylsiloxane, film thickness 0.33 μm; 50 m $\times$ 0.2 mm id) using an injector temperature of 300° C, helium carrier gas (1.5 ml min^{-1},140 kPa) and a temperature gradient of 150 to 300° C at 10° C min^{-1} The GC was coupled to mass spectrograph operating at 70 eV in the electron impact mode.

1.7 Aim and Scope of the present study

Mangrove ecosystem is associated with coastal productivity enhancement. Apart from the physical protection, transfer of some biochemical precursors from mangroves is essential in the maintenance of the natural maricultural activities of the system. Among such organic components, the Sterols have a prominent role. It is well known that many marine organisms are incapable of biosynthesis of sterols. Such organisms satisfy their needs by uptake of mangrove sterols, often modifying these sterols to suit their needs. Hence the sterols can be considered as biomarkers. Relative stability in the sedimentary system and inter compartmental transfers make sterols attractive biomarkers. Very little work has so far been reported on these lines from India, though such works on mangrove as well as non-mangrove sediments are reported worldwide.

The present work aims at

- Isolation and identification of sterols present in two Mangrove ecosystems of the Cochin estuary.
- Assessment of the relative distributional characters of the sterols in the two ecosystems.
- Assignment of the source character of the sterols.

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Chapter II

Materials and Methods

2.1 Description of Sampling sites

2.2 Sampling Procedure

2.3 Analytical Methods and Results

a. Hydrographical Parameters

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2.3.2 Salinity

2.3.3 Dissolved oxygen

2.3.4 Alkalinity

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2.3.8 Grain Size Analysis

c. Sterol characterization

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2.3.10 Gas Chromatographic Analysis

2.1 Description of Sampling Sites

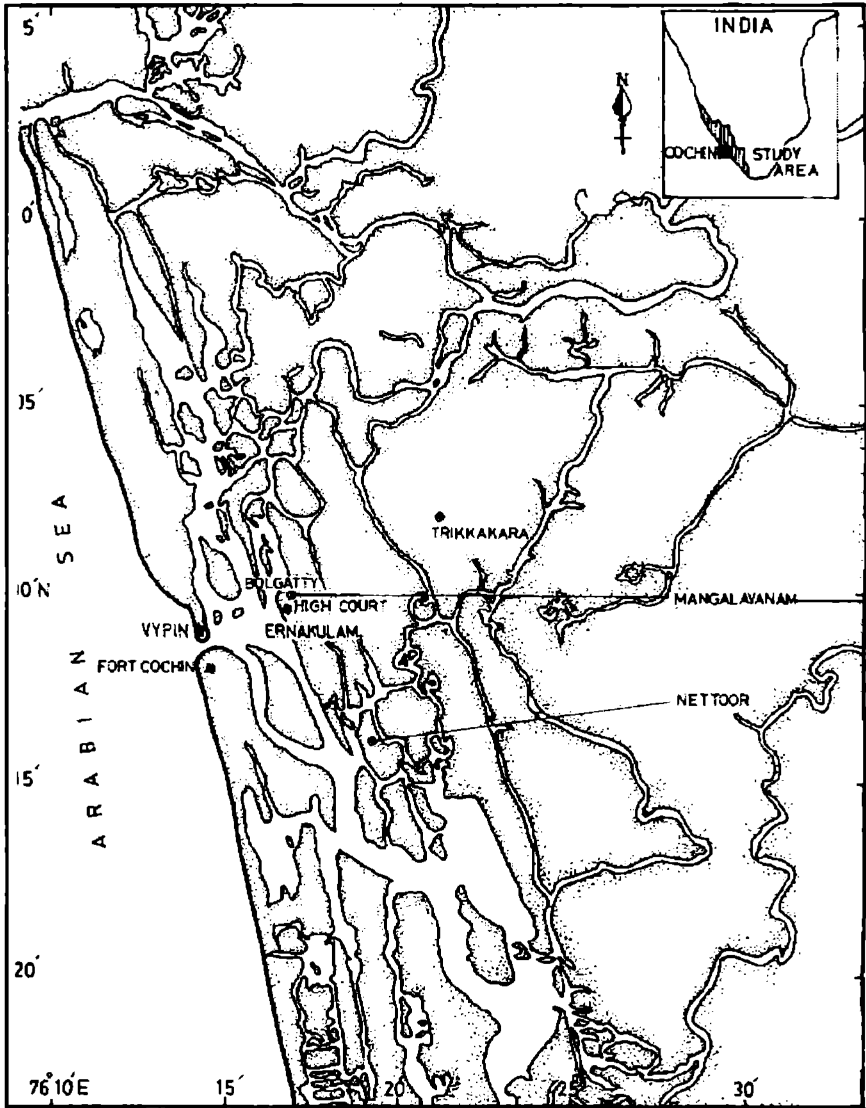


FIG. 2.1 MAP OF COCHIN ESTUARY SHOWING LOCATION OF SAMPLING SITES.

The area of investigation and the station locations are depicted in the location map. The stations were fixed so as to compare the complex environmental conditions prevailing in three different mangrove ecosystems. A brief outline of the characteristics of these stations is described as follows.

Mangalavanam

This is a patchy mangrove area in the heart of the city of Cochin behind the High Court of Kerala. It is 4.0 km off south to Kochi. This mangrove forest is a small bird sanctuary, as well. It is the home of many exotic and rare varieties of migratory birds. In this mangrove habitat under conservation undisturbed tree forms of Avicennia dominate in numbers with occasional patches of Acanthus, thus making up the total number of species to three or four. This is an almost closed system with a single narrow canal linking to the estuary.

Vypin

This is the largest single stretch of mangrove in Kerala and has an area of approximately 1.01km^2 (Geetha, 2003). This mangrove area is regularly inundated by a semi diurnal rhythm of Cochin bar mouth and the tidal waters bring in lot of fish seed of commercially valuable species of prawns. The mangrove vegetation consists primarily of Avicennia with Rhizophora constituting occasional growth, but in sea-accreted areas one can count about eleven species. Tree density is about 150-200/km^2 in the case of younger trees and 250-300/km^2 in the case of older ones. The canopy heights in well-preserved pockets range from 8-12 m (Subramaniyan V, 2000). Perhaps, this is

the only site in Kerala where one can see mangroves right along the accreting seacoast. However a lot of developmental pressures, including the proposed gas thermal plant, are threatening the very existence of these mangroves. Heavy developmental activities after the construction of the bridges connecting the mainland are threatening the existence of mangrove patches in the adjoining areas as well.

2.2 Sampling Procedure

Water and sediment samples were collected from the stations (Fig.2.1) at bimonthly intervals from November 2000 to November 2001. Water samples were collected using a clean plastic bucket from the adjoining water body (canal) as well as from the mangrove forest The dissolved oxygen and alkalinity of water samples were fixed in situ. Surficial sediment samples were collected from the forest using a clean plastic spoon. At Mangalavanam two collections were done, one during low tide and the other during high tide. The samples were taken in polythene bags and stored deep frozen until analyses. All analyses were done in duplicate. The preliminary studies showed that the sterol content is very low in the sediments. Hence about two kg of the sediment was collected during the post monsoon season of 2001, in monthly intervals and pooled together for analyses. The season was selected since the biological activity, related to the growth of prawns were maximum during the season (Kuttiamma and Kurien, 1982).

2.3 Analytical Methods and Results

Water and sediment samples were analyzed to ascertain the general parameters described herein.

a. Hydrographical Parameters

Water is of obvious importance in an aquatic system. There are a number of processes and factors that affect the rates of material transport, mixing and circulation in mangrove wetlands. These heavily vegetated inter tidal wetlands, when submerged are very shallow, with a maximum water depth of 2m in the swamps. Small tidal creeks, called mangrove creeks, drain the swamps. This demonstrates that a mangrove swamp is not an oasis, which is neither land nor sea, but is an important buffer deriving its wealth from both the land and the sea. It enriches the coastal waters and provides an important forestry and fisheries resource. For the effective management of this ecosystem an understanding of the hydrography of the mangrove swamps as well as that of adjacent water body is essential. pH was measured in situ and temperature was measured using a sensitive thermometer. Salinity of the water samples was estimated by Mohr- Knudsen method, (Muller, 1999). Modified Winkler method was used for the estimation of dissolved Oxygen (Hansen, 1999). Alkalinity of the water samples was estimated by the method of Koroleff, using Bromothymol blue as the indicator (Anderson *et al.,* 1999).

Values similar to those obtained during the present study have been reported in studies of the same area (Geetha, 2002 a; John, 2003 a)

Table I- Hydrographicl parametwers

Parameter	Mv (Forest-high tide)	Mv (Forest-low tide)	Mv (Canal-high tide)	Mv (Canal-low tide)	Vp (forest)	Vp (canal)
pH	07.5	07.2	07.45	07.5	07.4	07.4
Salinity °/00	10.5	12.3	11	09.3	21.6	25
DO mLL^{-1}	04.11	00	04.11	01.23	02.46	00.82
Alkalinity $mmolesL^{-1}$	00.77	00.89	01.04	01.04	01.40	01.25

2.3.1 pH

pH is a measure of the acidity or alkalinity of water. It indicates the level of dissolved carbon dioxide in the water. Photosynthetic activity, nature of dissolved materials, discharge of effluents, sewage out fall, solar radiation, temperature etc., influence the pH of a medium. Variation in pH due to chemical and other industrial discharges renders a stream unsuitable for the rearing of fish and other aquatic life (Webb1982). Since mangrove ecosystems support immense varieties of sea life, monitoring of pH variations in the system is highly essential to assess the life supporting character and the extent of the pollution in the system.

2.3.2 Salinity

Mangroves lie at the interface between land and sea. They dominate these ecotones because they have evolved several mechanisms that allow them to be successful under the highly variable salinity regimes. Mangrove areas in general have less salinity than the adjoining canals except during low tide at Mangalavanam.

2.3.3 Dissolved Oxygen

All aquatic animals depend upon the dissolved oxygen in water. The organic content of mangrove sediments is usually high (Shanmughappa, 1987; Kristensen *et al.*, 1988). The microbial decomposition of detritus in estuarine sediments consumes a lot of dissolved Oxygen resulting in increased demand on dissolved Oxygen in the water. The Oxygen is replenished due to tides and land run off. But any additional burden like pollution causes a further depletion in the dissolved Oxygen and becomes a direct threat to animals dependent on dissolved Oxygen. As a result, the variation in the dissolved Oxygen of aquatic ecosystems is very determining factor in the study of its productivity. At Mangalavanam the value comes down to zero during low tide except during monsoon, when there is inundation with floodwaters (Narayanan *et al.*, 2000).

2.3.4 Alkalinity

Due to the burning of fossil fuel and the human impact on land biota the atmospheric concentration of CO_2 is steadily increasing (Keeling *et al.*, 1995). As the major greenhouse gas except water vapor, CO_2 interacts strongly with the radiation balance of the earth and its increasing concentration potentially influences the global climate. However, only about 45% of the total anthropogenic emissions of CO_2 remain air borne (Houghton *et al.*, 1990). The hydrosphere has long since been recognized as an important sink for a significant portion of the missing anthropogenic CO_2.

Mangrove wetlands, in general are considered to be highly productive systems. Alkalinity of the water is the result of the predominance of basic effectiveness over acid effectiveness. Various factors contribute to the alkalinity of a medium. Dissolution of $CaCO_3$, abundance of organic matter, temperature and partial pressure of CO_2 are a few of them. Mangrove forests have various mechanisms that contribute to the high production and recycling of organic matter. Leaves are a source of various chemical elements. The study of complex mechanisms involved in the biogeochemical cycles in these ecosystem require a constant monitoring of alkalinity of the medium.

b. Sediment Characteristics

Sediment samples were analyzed for its E_h, total organic Carbon, Nitrogen and Sulphur, mineral type and grain size.

2.3.5 Eh

E_h of the fresh wet sediment was measured using Zobell's solution for the calibration of the electrodes (Brassard, 1997). The Mangalavanam sediment gave a value of about - 450mv and Vypin sediment gave a value of –210mv. The potential usually drifted for the first ten to fifteen minutes and stabilized after this. The values indicate the reducing nature of the samples, with Mangalavanam being in the anoxic domain. It should be pointed out that the parameter was measured mainly to obtain general information on the redox conditions of the sediments with no further attempts to interpret the measured values.

2.3.6 Elemental Composition

Organic carbon determinations were carried out by the wet oxidation method in most of the earlier works (Geetha, 2002; Sebastien, 2002). In the present work, total organic carbon was determined by the method reported by Villanueva et al. (1997). The carbonates were removed by addition of a solution of 5% solution of Hydrochloric acid in water. The resulting suspension was centrifuged for 10-20minutes at 3000 rpm to avoid loss of fine particles. The decarbonated sediment was washed twice with double distilled water and freeze-dried. Chen et al. (2001) and Muri et al. (2004) also removed inorganic carbon by similar methods, but without the use of freeze-drying the samples. The Carbon, Hydrogen, Nitrogen and Sulphur were determined in the residue using a Vario EL III CHNS Analyzer. The experiment was repeated with sediment from which the extraction with organic solvents has been carried out.

This will give an idea of the extent of extraction of organic constituents from the sample.

Sample	N	C	S	H
MV before extraction %	0.43	3.65	0.82	0.92
MV after extraction %	0.42	3.52	0.70	2.06
Vypin before extraction %	0.41	3.21	0.84	2.68
Vypin after extraction %	0.27	2.19	0.43	0.71

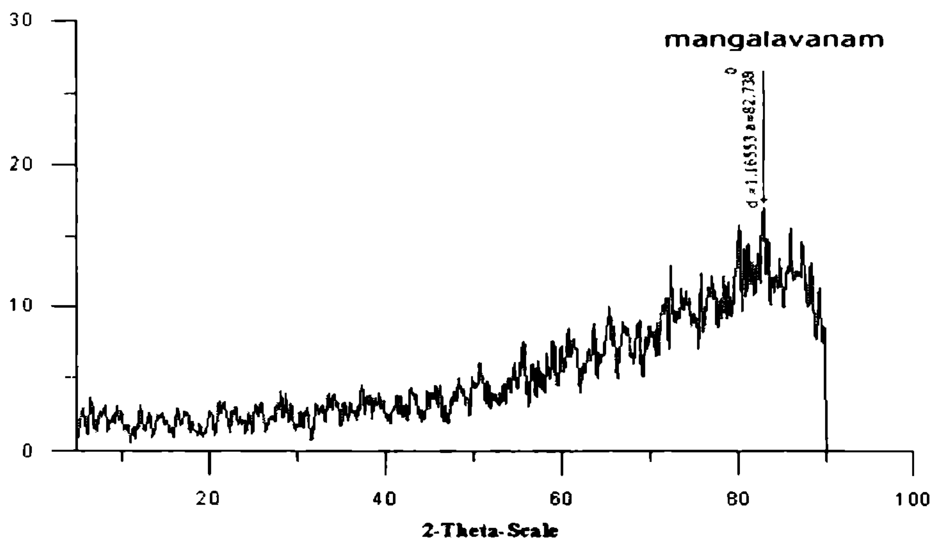
The concentration of organic Carbon varies from 0.17- 4.05% in the Cochin mangrove area (ENVIS report, 2002). Geetha (2002) has reported 5.0% and 10.72 of organic carbon at MV and Vypin respectively, while John (2003) has given a value between 9.42-10.604% of organic carbon at Vypin, all during post monsoon season. The higher values are obviously due to high salinity and consequent higher Chlorinity that will influence the determination by the wet oxidation method, which involves estimation using acid dichromate. The same authors have determined Nitrogen by the classical Kjeldahl method. Geetha

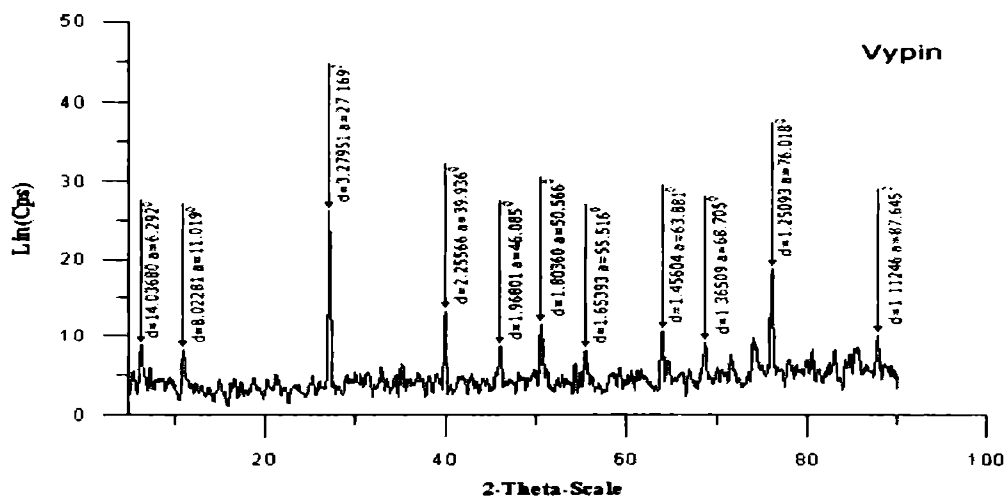
(2002) has reported a value of 1.42% of N at Mangalavanam and 1.131% at Vypin; John (2003) has given a value of 2.778 at Vypin.

2.3.7 XRD Analysis

X-ray diffraction pattern reveal certain characteristic d- spacings that can provide a unique fingerprint of the minerals present in the sample. When properly interpreted by comparison with standard reference pattern and measurements this fingerprint allows for identification of material.

An examination of the diffraction pattern and reference to the ASTM standards readily gives the presence of crystalline minerals of Chlorite and Quartz in the Vypin sediments. No such conclusions can be arrived at for the Mangalavanam sediment, which shows an irrational series of peaks. This may be due to either an appropriate degree of randomness to their interstratifications or a particle size gradient between top and bottom of the sample (Reynolds and Moore, 1997). It must be borne in mind that this sample is enriched in amorphous bioorganic materials like bird excreta, which always remain embedded in the sediment.


mangalavanam
d=1.16553 a=82.738
30
20
10
0
20
40
60
80
100
2-Theta-Scale


Vypin
Lin(Cps)
d=14.03680 a=6.292
d=8.02281 a=11.019
d=3.27951 a=27 169
d=2.25566 a=39.936
d=1.96801 a=46.085
d=1.80360 a=50.566
d=1.65393 a=55.516
d=1.45604 a=63.881
d=1 36509 a=68.705
d=1.25093 a=76.018
d=1 11246 a=87.645
50
40
30
20
10
0
20
40
60
80
100
2-Theta-Scale

2.3.8 Grain Size Analysis

The textural characteristics were studied by the method based on Stoke' Law suggested by Krumbien and Petty John (1938) and the following observations were made.

Sample	Sand %	Silt%	Clay%
MV	33.06	16.56	49.83
Vypin	39.32	25.95	34.00

Hence the sample can be classified as sandy mud, according to modified Folk's classification (Futterer, 2001). The same pattern has been obtained by the earlier workers (Geetha, 2003 b: John, 2003 b). Badaruddin *et al.*, (1996) have studied the textural characteristics of Kumarakom mangrove systems that have a high abundance of silt and sand with high levels of Calcium carbonate, due to the shell mining activities around the area. Sunil Kumar (1996) has remarked that inconsistent pattern with inconsistent concentration in accumulation of organic Carbon in the sediment with regard to vertical as well as horizontal distribution is discernible in the tidal areas of mangrove swamp. According to the same author the substratum of the Cochin mangrove area is categorized into clayey sand, silty sand and sandy silt. It must be remembered

that the grain size of Chlorite mineral not limited to clay fraction (<4μm), but in addition encompasses the entire silt fraction 4-63μm (Futterer, 2001).

c. Sterol Characterization

2.3.9 Isolation of Sterols

A perusal through the literature shows that there are a number of methods for the isolation of sterols. It is a matter of question of availability of facilities that determine the exact choice of method. The sediment samples collected were air dried in the dark at 30^0C and homogenized with a mortar and pestle (Parrish, 1998). About one kg of the powdered sediment was subjected to Soxhlet extraction using Chloroform and Methanol solvents for twenty-four hours (Wakeham and Beier, 1991). The combined extracts were evaporated in rotor under reduced pressure. The residue was boiled with 0.5N alcoholic potash for two hours under reflux. The boiled alcoholic solution was cooled and extracted into a 1: 4 mixture of Chloroform and Hexane to isolate the non saponifiable lipids, along with which the sterols are also present (Volkman et al, 1992; Volkman *et al.,* 1998). The aquous alcoholic phase consisted of 5% NaCl solution to facilitate the breaking of emulsion. The extraction was repeated for three times and the extracts combined after washing with water. The above steps ensure the removal of carboxylic acids. This will also ensure that components like triacyl glycerol, diacyl glycerol derivatives are removed so that they will not co-elute with sterols in the Ethyl acetate and n-Heptane mixture (Wakeham S.G, 1982). The extract was again evaporated in rotor under reduced pressure. The concentrate was dried over anhydrous Sodium sulphate.

Last traces of the solvent were removed by passing dry N_2 gas. The sample is then subjected to column Chromatography using 60-120 silica gel. The separation was done in two batches, the sample being divided into approximately two equal halves. Each column was prepared using 30 gms of the adsorbent packed into a column of about 30cms long and 2 cms wide. The solvent used for packing was n-Heptane. The sample was previously dissolved in Methanol and treated with about ten times its mass of Silica Gel and the solvent evaporated under reduced pressure and the adsorbed material was then applied at the top of the column after adding sufficient quantity of n-Heptane (Gilo and Claude, 1998). About 300 ml of the solvent n-Heptane was run through the column. This procedure eluted the hydrocarbons present in the sample. This was continued with mixtures of solvents the separation being followed concurrently by TLC done on silica gel coated plates using hexane as the primary solvent to ensure the completion of separation. The solvent systems used were n-Heptane followed by 30% Toluene, 20% Ethyl acetate, 50% Ethyl acetate, all in n-Heptane, and finally Methanol in succession (Brault and Simoneit., 1987). Sterols were eluted in 20% ethyl acetate mixture, the separation being followed by TLC done over Silica gel using an eluent of 13% Diethylether solution in n-Hexane and visualization of spots using Iodine vapours. The elution of sterols has been ensured by previous trials with a standard mixture of four sterols consisting of Cholesterol, Campesterol, Stigmasterol and β-Sitosterol.

The fraction obtained with 20% ethyl acetate in Heptane was proved to be sterol mixture by an earlier TLC using standard Cholesterol, Stigmasterol mixture for comparison. Previous trials have already demonstrated that the sterol yield is maximum with 20% Ethyl acetate. The sterol mixture obtained was evaporated under reduced pressure and dried using N_2 gas after combining the fractions obtained in two sets of separations. The samples were kept in a desiccator till analysis.

2.3.10 Gas Chromatographic Analysis

It has also been observed that the separation of individual sterols was not satisfactory using the above procedure. Hence the characterization has to be carried out using gas chromatographic analyses. Preliminary analyses were done using acetyl derivatives. About 0.1mg of the sample was acetylated by adding two drops of Acetic anhydride in two drops of Pyridine and keeping the sample for sixteen hours at room temperature (Neher R, 1969). The excess of reagents was removed by flushing in a stream of dry N_2 gas. A set of standard sterol acetate mixture was also prepared in a similar manner using four sterols, Cholesterol, Campesterol, Stigmasterol and β-Sitosterol. The standards and the samples were analyzed using gcms.

For silylation a little of the sample (about 100 μg) is dissolved in 0.2ml of dry pyridine, (silylation grade) followed by 0.2ml of silylating mixture consisting of (Bis-trimethylsilyl)-trifluoroacetamide (BSTFA) +1% Trimethylchlorosilane (TMCS) is added and kept for eighteen to twenty four hours at room temperature (Gleispach, 1974). Pyridine is not essential, as the silylating agent mentioned will

dissolve the sterols. A mixture of four standard sterols consisting of known weights of was also derivatized in a similar manner. On elapse of 18 hours, 0.1ul of the derivatized specimen was injected into Gas chromatograph attached on to an FID (gc-fid) and subsequently into a Gas chromatograph-Mass spectrograph (gcms).

Sterol identifications were based on GC retention times and individual mass spectra by comparison with previous data as well as available authentic standards as has been the existing practice (Gagosian *et al.*, 1982; Robinson *et al.*, 1984). The attention is mainly concentrated on peaks that can be clearly identified by a gcms limited mass range search as belonging to either the 3β-hydroxy stenols and their ring saturated counterparts. Such a course has been followed in the analysis of lipids by early workers (Gagosian and Heinzer, 1979; Marlow *et al.*, 2001).

The instruments used for analysis were GC-Perkin Elmer-model with FID and GCMS-Varian-12000 with single quadrupolar system. He at a flow rate of I.0ml/minute was used as the carrier gas and the column was 95% Polysiloxymethane coated, 30 meter long open tubular type with an internal i.d of 0.32mm. The column used for gas chromatography had a film thickness of 0.25μm and that used for gcms had 0.17μm. Further the mass spectrograph was set in the EI mode with electron energy of –70 ev the ion source pressure was 15mTorr, while the ion source temperature was 200.0 deg. The scan speed was 1 00amu/sec with a mass range of 40-500 amu. All analyses were conducted in duplicate. All solvent samples used were tested to ensure the absence of sterols, by prior injection. Reliability of the method was tested using baked samples of sediment spiked with Cholesterol and Stigmasterol.

The oven was programmed as follows

Temp. ©	Rate(C/min)	Hold (min)	Total (min)
150	00.0	01.00	01.00
200	20.0	10.00	13.50
270	10.0	30.00	50.50

The procedure showed a recovery of about 81% for Stigmasterol and 73% for Cholesterol. Venkatesan and Kaplan (1987) have reported a recovery varying from 40-82% for sterols in similar studies. Gogou et al. (1998) have reported any yield of about 78-81% for sterols using Methylene chloride as the solvent and one step Flash chromatographic separation of the components.

The acetate spectrum was done under following oven programme conditions.

Temp. ©	Rate (C/min)	Hold (min)	Total (min)
100	0.00	02.00	02.00
250	05.00	28.00	60.00

Alternative oven programmes have also been used, the details of which is given during the course of discussion.

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Chapter III

Sterols

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3.2 Biological Role of Sterols
3.3 Mangrove Biomarkers
3.4 Isolation of Sterols - Results
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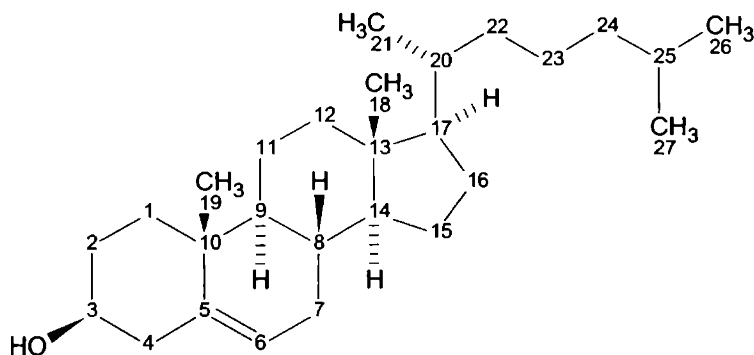
3.1 Structural features

Steroids are compounds possessing the skeleton of cyclopenta(a) phenantrene or a skeleton derived there from by one or more bond scissions or ring expansions or contractions. Methyl groups are usually present at C_{10} and C_{13}. An alkyl side chain may or may not be present at C_{17}. Sterols are steroids carrying a hydroxyl group at C_3 and most of the skeleton of Cholesterol, which is shown below. Additional carbon atoms may be present in the side chain. Stanols are sterols having no unsaturation in their structures.



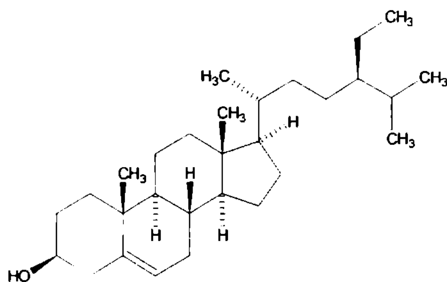
Cholesterol

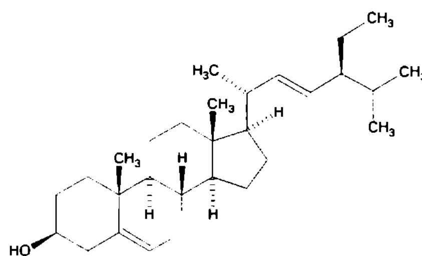
Steroids are numbered according to the recommendations of IUPAC (1989) and modifications thereon. An atom or group lying above the plain of the ring is shown β and that lying below the plane of the ring is termed α. If the conformation is not known then the prefix ε is used. In the formula shown all

angular groups and H atoms have been written out explicitly. However methyl groups may be shown without lettering if there is no ambiguity. Likewise, the hydrogen atoms at he bridgehead may be omitted if they are oriented 8β, 9α, 14α and methyl groups are oriented 10β, 13β. Side chain at 17 is β-oriented. The configuration of hydrogen or a substituent at the bridgehead position 5 is always to be marked α or β after numeral, this letter and numeral being placed immediately before the stem name.

Additional alkyl substituents attached to a steroid skeleton are numbered using the locant of attachment position and a superscript number indicating the number of atom from the attachment position. Additional carbon atoms at position 24 is numbered like 24^1, 24^2 etc. in modification of the earlier system of giving numbers like 28, 29 etc. These numbers are now assigned to additional methyl carbon atoms at positions C_4 and C_5 respectively. The orientation of the substituents at C_{20} and C_{24} in a C_{17}-side chain is described by a convention due to Fieser and Fieser (Shoppe, 1964 a; Rodd, 1970). When the side chain is oriented with longest C chain to the rear and a Fischer projection is made, the 20 or 24-substituent on the left side is designated β and on the right side as α. The 20α/20β nomenclature is continued because of the long tradition (Rodd, 1970). The R/S system of designating the absolute configurations at the chiral centers is followed here also. This system it must be emphasized, is not much useful to discuss the configurational relationships at the chiral center since a double bond may change the priority at the position and hence the R or S configuration. (Rodd, 1970; Volkman, 1986).

The sterols shown below are configurationally related, at C-24 but have different absolute configurations





β-sitosterol

(20R) - (24R)-24-ethylcholest-5-en-3β-ol

24α- ethylcholest-5-en-3β-ol

Stigmasterol

(20R)-(22E)-(24S)-24-ethylcholesta-5,22 dien-3β-ol

24α- ethylcholesta-5, 22- dien-3β-ol

3.2 Biological Role of Sterols

Common sterols found in higher plants are Campesterol,β-sitosterol,and Campesterol, β-sitosterol being the most abundant as shown by several workers (Crozier *et al.,* 2000). A characteristic feature of the plant sterols is the alkylation at C-24 with methyl or ethyl substituents. However Cholesterol is a minor sterol widely distributed in plants though not alkylated at C-24. β-sitosterol is the major sterol in the case of mangrove plants also. Campesterol and Stigmasterol are also present in appreciable amounts (Ghosh *et al.,* 1985;

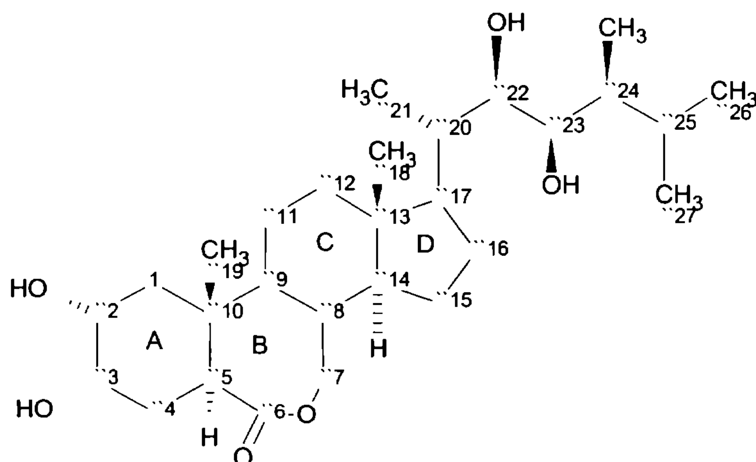
Hogg and Gillan, 1984; Misra *et al.*, 1984). Sterol products such as 24-methylene cholesterol, Isofucosterol have also been isolated from higher plants.

Stahelin and Newcomb (2000), while reviewing the role of sterols in plasma membrane of plant cells have made the following observations. Sterol esters and sterol glycosides are far more abundant in plants than in animals. In fact sterol glycosylation, a reaction catalyzed by UDP-glucose: sterol glycosyl tranferase is used as a marker for isolated plant plasma membranes. It should be understood that on the average the free sterols constitute about 35% of the root plasma membranes while the amounts of sterol glycosides is about 3-13%. Sterols form an important part of the plasma membranes of animal and plant cells and are important lipid constituents like phospholipids and glycolipids. The ratio among the different lipid classes is fairly constant in same type of animal tissues, whereas there is remarkable variation of lipid classes among organs of same plant, among identical organs in different plants. The membrane lipids due to their amphipathic nature form bilayer membranes and thus affect the transport pattern through the membranes. The sterols themselves do not form any bilayer, but function by affecting the fluidity of the plasma membranes. The hydrophobic domain of a sterol molecule is much larger than the uncharged polar head group since the cyclohexane rings of the sterols with the puckered conformations have only one or two polar groups at the end which even may be esterified. Fluidity alteration in plasma membranes is done in two ways. At low temperatures they disrupt the gelling of phospholipids by reducing the proportional amounts of lipids that can undergo phase transition,

and at higher temperatures interfere with the fatty acid tails thus decreasing the fluidity by reducing the lateral mobility of the polar lipids within the membranes In short membrane sterols act as membrane fluidity buffers. The plants also adjust the lipid composition according to seasonal requirements. These changes are very essential to withstand temperature variations.

The sterols are found in all eukaryotic organisms, where they play a structural role in cellular membranes and represent a significant proportion of the organism's membrane biomass (Bloch, 1992). Membrane sterols can move rapidly in the plane of the phospholipid bilayer and move out of the membrane without enzymatic assistance. Cholesterol anchored signaling molecules have been discovered in animal cells attached to the external surface of plasma membranes. No sterol-linked proteins are yet known in plants. In higher animals steroid receptors are examples of inducible transcription factors. The steroid hormones pass through the plasma membranes of their target cells to cytosol and attach to their cognate receptors, which may be the response elements in the upstream regions of genes.

Many of the plant sterols are precursors of Brassinosteroids(BRS). These are a set of plant steroids, which function as growth promoters. BRS are found in almost all classes of plants except microorganisms. The biological functions of these include increased rate of stem elongation, pollen tube growth, and proton pump activation, to mention a few. Many of these compounds have the skeletal structure of Campesterol with a lactide entity between 6,7 positions.



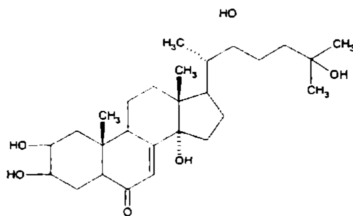
Brassinolide, an important BRS isolated from many plants and which has Campesterol as its precursor.

Recent studies have proved that the sterol glucosides participate in the synthesis of cellulose in plant cells. (Peng *et al.*, 2002). The glucolipid is used as a substrate to produce higher homologues of the Cellobiose type with β 1,4 linked glucosyl residues. The resulting disaccharide is split off and used as a primer for further elongation to Cellulose (Schrick *et al.*, 2004). While assessing the crucial role for sterols in plant growth and development, they demonstrated that the sterols are crucial for the cellulose synthesis in plants and any sterol biosynthesis inhibitor or any abnormality in the sterol structure will result in cell wall abnormalities

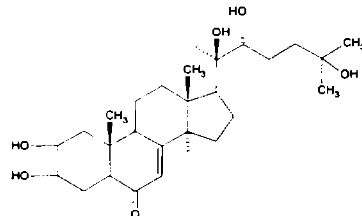
It should be noticed that the prokaryotes could not synthesize the sterols. However presence of certain sterol glycosides have been demonstrated

in a prokaryote Acoleplasma axanthior by Maykerry (1983). Moreover about thirty three percent of the total lipids in the bacterial genus Helicobacter is found to consist of Cholesterol glycosides (Haque., 1995) and hence these molecules may act as an important chemotaxonomic marker for the organisms.

In arthropods, molting process is under the control of steroid hormones called Ecdysones. This is also the case with insects. The site of Ecdysone synthesis in crustaceans is the Y organ as shown by earlier studies (Carlisle, 1965; Chang and O'connor, 1977). These Ecdysteroids have multiple functions in the control of development and are active in most organs, including the nervous system, even in the adult organisms (Delbecque *et al.*, 2004). Diwan (2005) in his review on shrimp endocrinology has reiterated the earlier observations that the female shrimp molts as a requirement for mating.



Ecdysone



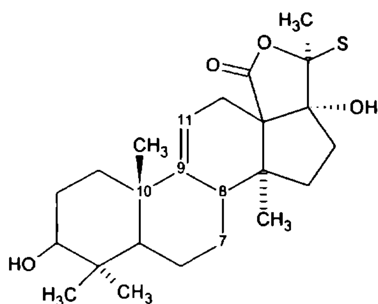
Ecdysterone /20-E

Among the ecdysones 20-E is the active form. Gillot (1988) has pointed out that Ecdysterone initiates the developmental activities within the spermathecae of many female species of insects and the ecdysones produced within the ovary will favor the release of the ovulation hormone. In female crustaceans also the spermathecal activity is correlated with stages in molt

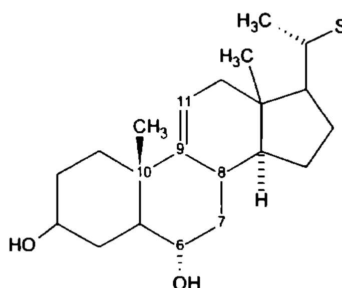
cycle and it is likely that it is under the influence of ecdysteroids. It is shown that Ecdysteriods promote oogonial mitosis in female crustaceans (Adiyodi and Anilkumar, 1988). Okumura (2004) has observed that 20-E has a direct positive effect on reproductive activity in male crustaceans including shrimps, while no correlation could be demonstrated between added ecdysteroids and ovarian development in the females.

Adams et al. (2003) have shown that the concentration of these hormones which a crayfish leaks into the surrounding water increases during the actual shedding of the exoskeleton and that the non molting crayfish sense this and stay nearer to the molting ones. This offers a protection against the natural enemies to whom the molting ones are extremely vulnerable. The presence of Ecdysteroids like 20-E has been demonstrated in many plants (Bathori *et al.,* 2000). Phylogenetic classification is not possible at present since the phytoecdysteroids are widely distributed among ferns, gymnosperms and angiosperms (Dinan, 2001). Schmitz et al. (2002) has demonstrated that the ecdysteroids are formed in plants like spinach by mechanical or insect damage to the roots, which suggest its role in plant defense. It is now stated definitely that the phytoecdysteroids play a pesticidal role in plants (Daniel *et al.,* 2005). Ingested phytoecdysteroids could lead to an abnormal ecdysone profile in the insects that may cause malformation, sterility or insect death. As these phytoecdysteroids are non hazardous to vertebrates, they are being considered as attractive, eco friendly insecticides without environmental pollution implications.

A possible function for unusual marine sterols is proposed whereby structural modifications render the sterols non-nutritious to marine invertebrates. The sterol composition of the "red tide" organism *Karenia brevis* was found to be a mixture of six novel and rare $\Delta^{8(14)}$ sterols and this reduces predation and thereby enhancing the ability of the dinoflagellates to form massive blooms (Giner and Djerassi, 1991; Giner *et al.,* 2003). Echinoderms contain many toxins and saponins, which are steroidal in nature and possess a 9(11) bond.



Holothurin skeleton
R-OH, H; S- sidechain



Asterosaponin skeleton
S- sidechain

Two important toxins from the Echinoderms are shown above. Holothurins possess a Lanostane skeleton, an Oxygen containing ring between C-18 and C-20 of the steroidal skeleton and an odd 9(11) double bond and an additional double bond at C-7 occasionally. Asterosaponins posses a 9(11) double bond besides an additional hydroxyl function at C-6. Holothurins from

sea cumbers are neural toxins that serve in the self-defense of the organisms against the enemies. A special organ called Cuvierian organ secretes these toxins. A closely related group of compounds, asterosaponins are found in starfish. These compounds serve as a spawning inhibitor besides acting as a self-defense agent in the organisms. This neural toxin is being used in folk remedies in traditional Chinese and Japanese medical systems. Its effects as an antifungal agent and cardio active agent are being investigated.

Steroids are components of bile salts, which are emulsifying agents. They are not much relevant in the present study since they have their roles in animals with complex digestive systems that do not form a part of the ecosystems under study.

Mineralocorticoids and glucocorticoids are present in higher animals and play an important role in Na^+ control and glucose metabolism. Corticosteroids like Cortisol, 11-deoxycorticosterone and Cortisone act as ante inflammatory agents and have a partial control over glucose metabolism in higher animals. These are detected in many lower marine organisms including invertebrates. The enzymes capasble of interconversion of progesterone to 11-deoxy corticosterone and Testosterones have all been found in Crustaceans. However their exact significance is to be ascertained (Adiyodi and Anilkumar, 1988). The hormones for their biosynthesis from Cholesterol as also from Mevalonic acid, without having to pass through a C_{27} intermediate are present in these invertebrates (Goad, 1978). Many of these steroidal hormones function

as gonadic developing factors and mediate sexual development and functions in non chordates as well as chordates.

3.3 Mangrove Biomarkers

Lipids in the leaves of mangroves distributed in different geographical areas are more or less similar (Bagchi *et al.*, 1988). It has been pointed out that fortification of the cell membranes with sterols appears to increase the tolerance of the organisms to the external stresses. Among the lipid classes, sterol ester was the largest constituent in almost all the mangrove species, ranging from about 175 –33% (Oku *et al.*, 2003). Proportions of Triterpenoid alcohols, which share a common biosynthetic pathway with phytosterols, increases with salinity in both leaves and roots of Kandelia candel, but only in roots of Bruegeria gymnorriza (both mangrove plants). This clearly demonstrates that salt stress specifically modulated the triterpenoid concentration in mangroves. The triterpenoids included Taraxerol, Lupeol, β-amyrin, both possessing a 9,19 cyclolanostane skeleton. In fact these compounds along with β-sitosterol are being considered as Mangrove biomarkers (Koch *et al.*, 2003). Taraxerol along with Rhizophora pollen has been selected as proxies for tracking past mangrove ecosytem (Versteegh *et al.*, 2004)

3.4 Isolation of sterols-Results

The lipids were extracted from the sediment by extraction with Chloroform and Methanol at boiling points for twenty-four hours. The extracts obtained were hydrolysed to liberate the bound sterols and acid components removed. The lipid mixture was subjected to LC using a system of solvents as shown below. All the

extractions were carried out in duplicate and the mean values recorded. The solvents were evaporated by distillation under reduced pressure. Final traces of the solvent were removed by passing a thin stream of pure, dry Nitrogen. The LC was done in batches and similar components combined together before determining the yield. This was a matter of convenience only. The sterol fractions were dissolved in Chloroform and taken out as and when required for analyses.

Components isolated using LC

Solvent system	Component eluted
n-Heptane (150ml)	Hydrocarbons
30% Toluene (150ml)	Halogenated compounds
20% Ethyl acetate (150ml)	Sterols, alcohols
50% Ethyl acetate (50ml)+Ethyl acetate (20ml)*	Steroidal ketones Polar compounds etc.
Methanol	Colouring matter and other unidentified compounds

*The two extracts were combined together since the same type of compounds eluted.

Yield of Different Components

Component eluted	Mass of the Component (gms)	
	Vypin	**MV**
Hydrocarbons	0.42570	0.27890
Halogenated compounds	0.09643	0.03400
Sterols, alcohols	0.51750	0.12297
Steroidal ketones, Polar compounds etc.	0.03696	0.02360
Colouring matter	2.99159	1.57230
Total	4.06818	2.03177
Total mass of sediment extract used	5.12	2.3682
Percentage of recovery after LC	79.46	85.79
Percentage of sterols on sediment extract basis	10.07	05.19
Recovery of sterol from sediment-mg/kg	517.5	122.97

It is noteworthy that the content of coloring matter is high in MV sediment. Only a few workers have reported the yield of sterols in sediments. Ghosh et.al

(1990) have reported an yield of sterols varying from 50- 200 ppm in sediments of Hooghly estuary. In the mangroves at santa catarina, only a maximum of 2.9 mg/kg of sterols have been reported (Mater *et al.*, 2004). However it is a riverine estuarine system, where the hydrodynamics may be quite different.

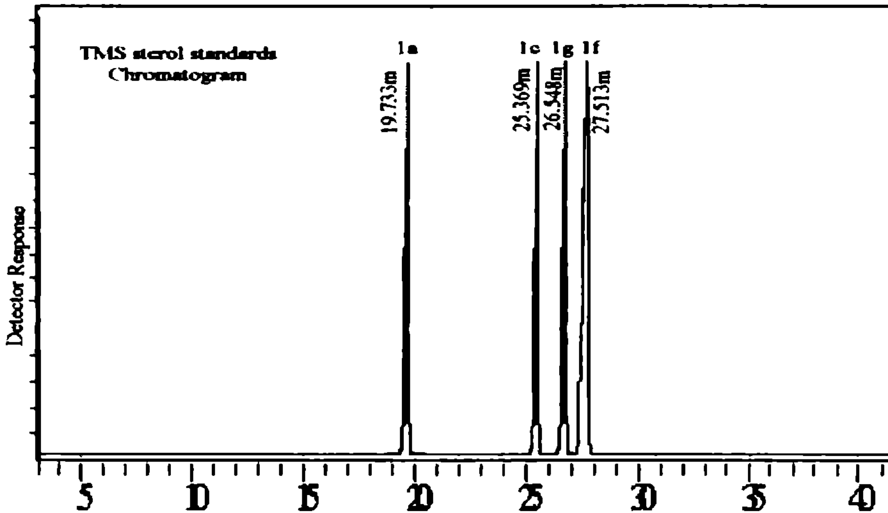
3.5 gc-fid Analysis

A trial experiment with known equal quantities of four standard sterols showed good recovery and quantification. The sterols used were Cholesterol (1a), Campesterol (1c), Stigmasterol (1g), β-sitosterol (1f), 0.1 mg of each as their trimethylsiloyl ethers.

The oven programme for the analysis was as follows

Temp.^{0}C	Rate (^{0}C/min)	Hold (min)	Total (min)
150	0.00	01.00	01.00
200	20.0	10.00	13.50
270	10.0	30.00	50.50

The gas chromatogram showed peaks of almost equal intensities. Only the relative concentrations of typical mangrove sterols like Cholesterol, Campesterol, Stigmaserol and β- sitosterol were determined in sample studies. The rest of the sterols were available in small quantities and the relative concentrations did not vary much.


TMS sterol standards
Chromatogram
Detector Response
1a
1c
1g
1f
19.733m
25.369m
26.548m
27.513m
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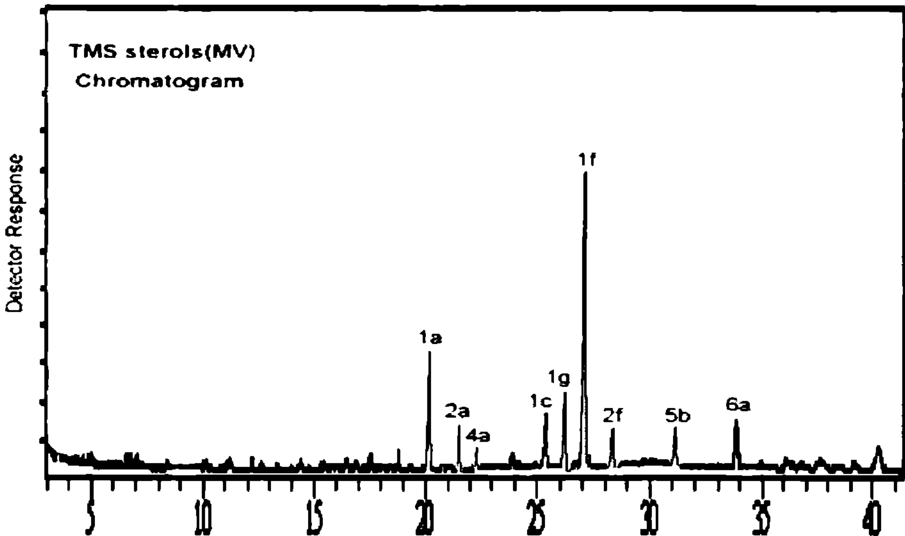
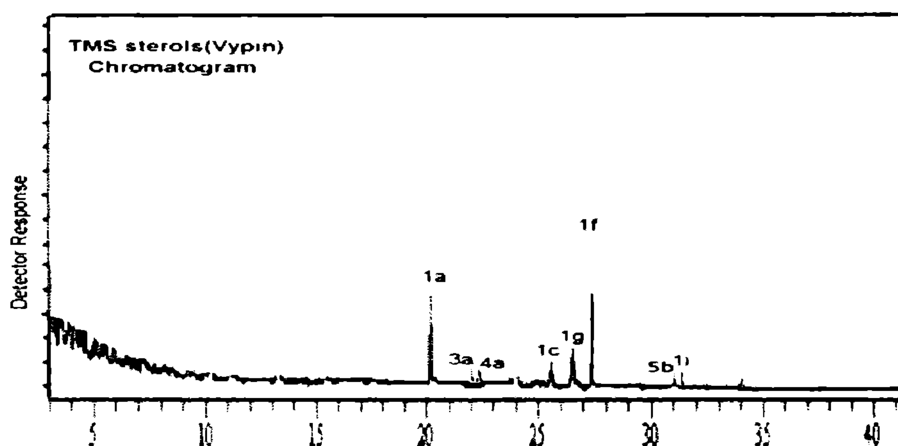

TMS sterols(MV)
Chromatogram
Detector Response
1f
1a
1g
1c
2a
4a
2f
5b
6a
5
10
15
20
25
30
35
40

Mangalavanam sample showed the presence of nine sterols, among which β-sitosterol (1f) was the most predominant, Cholesterol (1a) coming next at 39.35% of the former. Campesterol (1c) is 10.21% and Stigmasterol (1g) 13.45% of the β-sitosterol. All the rest of the sterols are having a relative concentration less than 10%. Lathosterol (4a) and Lanosterol (5b) are other two sterols present in both the systems. Cholestanol (2a), Stigmastanol (2f) and Dihydroagnosterol (6a) are unique to this system.



The Vypin sample also displayed a similar pattern with eight sterols. The highest concentration was for β-sitosterol followed by Cholesterol (81.11% of the former). Campesterol was 7.56% while Stigmasterol 9.14% and other sterols were all less than5% of the β-sitosterol. Lathosterol and Lanosterol have been identified here also. Coprostanol (3a), Spongesterol (2d) and Isofucosterol (1i) have been identified in this sediment.

3.6 gcms anlaysis

During the analysis using gcms it has been found that the intensities of mass spectral peaks are varying from the standard values stated. This may be presumably due to the combined effect of mass fragments coming in succession. This effect can be seen in the mass spectral pattern of the standard sterol ether mixture also as well as from the reconstructed ion chromatogram also. There is some difficulty in identifying Stigmasterol from the mass spectral pattern in the standard mixture of four sterols as the peaks of β-sitoserol are found to interfere. It must be stated that for a mixture of two sterols like Cholesterol and Stigmasterol, whose retention times differ much, almost the theoretical mass spectral pattern is obtained.

The oven programme adapted here was the same as that in the gc-fid analysis. Alternate temperature programmes have also been attempted in gc-ms studies in order to ascertain if better resolution could be obtained. It must be remembered that of the numerous references quoted elsewhere, for the gc separation, widely varying programmes have been adapted by different authors.

An earlier programme tried is shown below. This started at a lower temperature of 100^0 C. In this case Cholesterol derivative eluted after 60 minutes, while Stigmasterol derivative eluted only after 70 minutes. The trial established clearly the futility of starting the programme at a lower temperature. Hence all the analyses were carried out using former programme, unless otherwise required for some specific identification purposes.

Temp. ^{0}c	**Rate (^{0}C/min)**	**Hold (min)**	**Total (min)**
100	0.00	02.00	02.00
250	05.00	28.00	60

Another programme tried started at a temperature of 180^{0}C.

Temp. ^{0}C	**Rate (^{0}C/min**	**Hold (min)**	**Total (min)**
180	0.00	10.00	10.00
250	05.00	36.00	60.00

In this case there was considerable overlapping of the peaks of the sterol derivatives, especially towards the cholesterol end.

The acetate spectrum was done under following conditions. An alternate programme has also been attempted.

Temp. ^{0}C	Rate(^{0}C/min)	Hold (min)	Total (min)
100	0.00	02.00	02.00
250	05.00	28.00	60

.Temp. ^{0}C	Rate (^{0}C/min)	Hold (min)	Total (min)
200	0.00	05.00	05.00
275	05.00	40.00	60

It is found that Stigmasterol could be positively identified as acetate using this programme only while β-sitosterol could not be, while the opposite is true with the programme mentioned formerly.

3.6.1 Results of gcms Analysis

Sterol	Structure	Availability
1.Cholesterol * Cholest-5-en-3β-ol ($C_{27}\Delta^5$)	**I a**	**Detected in both MV & Vypin samples**
2.Cholestanol 5α-cholestan-3β-ol ($C_{27}\Delta^{0}$)	**II a**	**Detected in MV sample**
3.Coprostanol 5β-cholestan –3β-ol ($C_{27}\Delta^0$)	**III a**	**Detected in Vypin sample**
4.Lathosterol 5α-Cholest-7- en-3β-ol ($C_{27}\Delta^7$)	**IV a**	**Detected in both samples**
5.Spongesterol (24S)- (22E)-24-methyl-5-α-cholest-22-en- 3β-ol ($C_{28}\Delta^{22}$; 24-methyl)	**II d**	**Detected in Vypin sample**
6.Campesterol * (24R)-24-methyl Cholest- 5-en-3β-ol ($C_{28}\Delta^5$; 24-methyl)	**I c**	**Detected in both samples**

7.Stigmasterol* (24S)-(22E)-24-ethyl cholesta-5, 22-dien-3 β-ol ($C_{29}\Delta^{5,22}$; 24ethyl)	**I g**	**Detected in both samples**
8.β-sitosterol* (24R)-24-ethyl cholest-5-en-3β-ol ($C_{29}\Delta^{5}$; 24ethyl)	**I f**	**Detected in both samples**
9.Stigmastanol (24R)-24-ethyl-5α-cholestan-3β-ol ($C_{29}\Delta^{5,22}$, 24-ethyl	**II f**	**Detected in MV sample**
10.Lanosterol 5α-lanosta-8, 24-dien-3β-ol ($C_{30}\Delta^{8,24}$)	**V b**	**Detected in both samples**
11.Isofucosterol* (24Z)-Stigmasta-5, 24(24¹) dien--3β-ol ($C_{29}\Delta^{5,24,}$ 24-ethenyl)	**I i**	**Detected in Vypin sample**
12.Dihydro-agnosterol 4,4,14α-trimethyl -cholesta- 5,9(11)- dien 3β-ol ($C_{30}\Delta^{7,9(11)}$)	**VI a**	**Detected in MV sample**

* Mangrove sterol. See Appendix I for structures of sterols

The retention times and mass peaks obtained in the Reconstructed Ion Chromatogram (RIC) during the analysis that are of diagnostic importance are given in the adjoining table. At least four important peak values reported earlier are used in each identification, unless resorted to the library set.

Diagnostic Peaks for Sterol derivatives

Sterol	Structure	Diagnostic peaks (TMS Ethers)	Diagnostic peaks (Acetate ester)
1.Cholesterol	I a	20.218mts 458, 368, 353, 255, 329, 255 247, 121	33.56mts. 368, 353, 326, 283, 260, 255, 213
2.Cholestanol	II a	21.52mts 460, 445, 370, 355, 230, 217, 106, 75	33.98mts 430, 370, 355, 315.
3.Coprostanol	III a	22.008mts 460(minor), 445, 370, 355, 230, 215, 216, 217	
4.Lathosterol	IVa	22.349mts 458,443,353,255,229, 213,129	
5.Spongesterol	II d		36.46mts 344, 329, 315, 257, 215
6.Campesterol	I c	25.623 mts 472,382,343,129	
7.Stigmasterol	I g	26.387mts. 484, 394, 355,129, 83	37.96mts. 394, 379, 351, 255, 81
8.β-sitosterol	I f	27.289mts. 486, 396, 381, 357, 356, 129	38.13mts. 396, 381, 255, 147
9.Stigmastanol	II f	28.451mts 488, 473, 398, 383, 230, 217, 215	
10.Lanosterol	V b	31.140 mts 498, 484, 241, 227, 157	
11.Isofucosterol	I i	31. 40mts 484, 394, 314 ,296, 229	
12. Dihydro-agnosterol	VI a	33.85mts 498, 408, 393, 369, 253, 240	

3.6.2 Fragmentation Pattern

The peaks expected by fragmentation of the sterols are given below (Brookes *et al.,* 1964; Nicholas *et al.,* 1984; Harvey *et al.,* 1988; Gagosian and Heinzer, 1979).

M^+Sterol	I a	I c	If	I g	II a	III a	II f	Ii	I j
M^+	458	472	486	484	460	460	488	484	484
$(M-15)^+$					445		473		
$(M-90)^+$ (Me_3SiOH)	368	382	396	394	370	370	398	394	
$\{M-(90+R)^+$ with or without H abstraction	255	255	255	255	257	257		255 253	
$\{M-(90+15)\}^+$	353		381		355	355	383	379	379
Mode B	247			261					
$\{M-(R+C_3H_5+Me_3SiO)\}^+$	215				215[1]	215[2]	215[1]		
$\{M-(R+C_2H_3+Me_3SiOH\}^+$					230	230	230	229 231	229
$C_6H_{13}SiO^+$	129	129	129	129				131	129
$(M-129)^+$	329[4]	343[4]	357[4]	355[4]				341[4]	341[4]
4C unit--A	145					142		145 143	
Sidechain frt-(F)				83				81	69
$M-(Me_3SiO+F)\}^+$								314	314
$\{M-(Me_3SiOH + H + C_{23}-C_{28})\}$								299 296	299 296

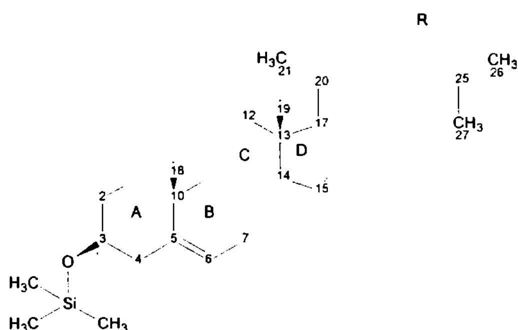
M^+Sterol	chl-4en	III d	24 Δ^7	IV a	V b	VI a
M^+	458	472	472	458	498	498
$(M\text{-}15)^+$				443	483	
$(M\text{-}90)^+$ (Me_3SiOH)	368					
$\{M - (90 + R)^+$ with or without H abstraction	255	257	255	255		253
$\{M\text{-}(90+15)\}^+$	353		367	353	393	393
Mode B						
$\{M\text{-}(R+C_3H_5+ Me_3SiO)\}^+$			213[3]	213[3]		
$\{M\text{-}(R+C_2H_3+Me_3SiOH\}^+$			229	229		
$C_6H_{13}SiO^+$						
$(M\text{-}129)^+$						369[4]
4C unit--A	142 143 145			147		
Sidechain frt.		69				

1-216 & 217 with reciprocal H transfer from C_{16} to C_{17} and removal of Me_3SiO without H atom abstraction from position 5 respectively

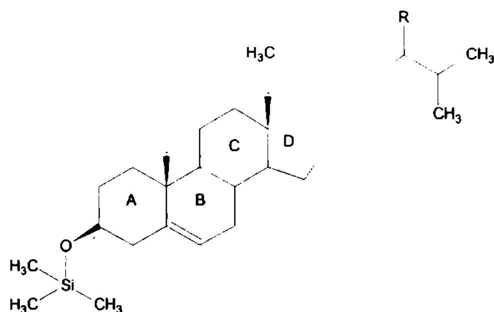
2-only 216 and not 217

3-19-CH_3 from m/z 229

4-typical of Δ^5-3β-ols



I) Cholesterol trimethylsilyl ether



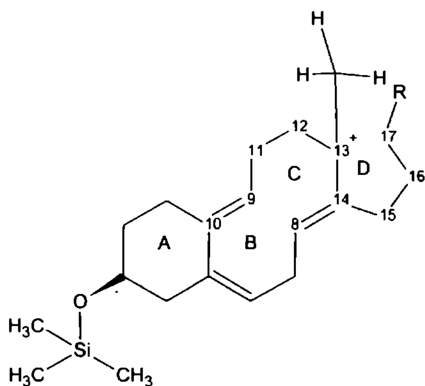
II) Cholesterol trimethylsilyl ether molecular ion

The principal ions born out of fragmentation of sterol ethers are shown in the above two tables. In EI spectrum, usually M^+ ions like II from Cholesterol are obtained though they are not the base peaks. Certain ions notably that of m/z 75 due to $(CH_3)_2\ SiO^+\ H$ from that of trimethylsilyl groups

and that at m/z 69 are of no diagnostic importance since they are almost ubiquitous.

A peak due to the molecular ion is always present with steryl 3-O-trimethylsilyl ethers. In majority of cases it is of relatively higher intensity, but it is absent in acetate derivatives of the corresponding sterols.

Some of the most important peaks for $\Delta^5$3β-OH sterol trimethylsilyl ethers are from the following cleavage patterns. $[M-90]^+$ is obtained by the loss of $[(CH_3)_3SiOH]^+$ Cholesterol (I a), and Cholest 4-en -5α-3β-ol give peaks at m/z 368, the corresponding stanols at m/z 370, etc due to this elimination.

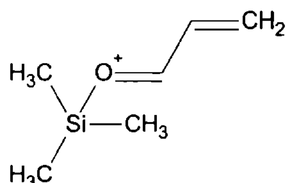


III)

$(M-15)^+$, $[M-(90+15)]^+$ are characteristic peaks of the steroidal skeleton and are obtained by the loss of angular methyl groups with or without silanol groups. According to Tokes et al. (1968) the C_{19} methyl group is lost preferentially over the C_{18} methyl group yielding peaks at m/z $(M - 15+89+1)^+$ Cholesterol (I a) gives the peak at m/z 353, β-sitosterol (I f) at m/z

381,Cholestanol (II a) at m/z 355etc. One of the reasons for this preferential expulsion is that the cleavage of highly strained 13-17 bond in the molecule prevents the rupture of a second bond connected to the same C_{13} quaternary center, thus reducing the chances of expulsion of C_{18} methyl group. Alternatively the activation of the 10-19 bond by the cleavage of 8-9 linkage in the ion will result in the expulsion of C-18.

One of the most significant peaks in Δ^5 3β-O trimethylsilyl ethers are strong peaks at m/z 129 along with a peak at m/z $[M-129]^+$ The former peak results by the loss of IV from ring A.

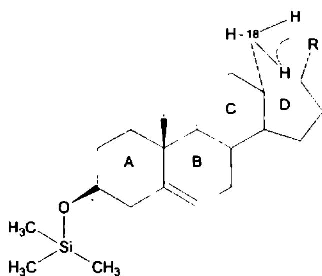


IV) m/z 129

Peaks at m/z $(M-129)^+$ occur for Cholesterol (I a) at 329, β-sitosterol (I f) at 357 (with H abstraction at 356) and Campesterol (I c) at 343 respectively.

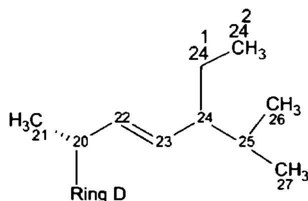
The derivatives of unsaturated sterols other than Δ^5 sterols give peak at m/z 129 with low intensities, while in Δ^5 sterols the average intensity of the peak is at least 68% of the base peak. In some cases of Δ^5 sterol ethers, this peak is even the base peak. The same peak is also obtained from 4-alkyl cholesterol derivatives and this shows that the ion is formed from the C_1-C_3 carbons of ring A rather than C_2-C_4 carbons (Brookes *et al.*, 1968). Dueterium labelled experiments have also proved the above fact. The accompanying [M-

129]$^+$ peak is typical of $\Delta^5 3\beta$-OH sterol derivatives though the peak at m/z 129 is given by other structural groups such as 17- trimethylsiloxy steroids.This observation is also true of trimethylsilyl ethers of compounds like Pregnenolone and 3β- keto compounds (Diekman & Djerassi,1967).



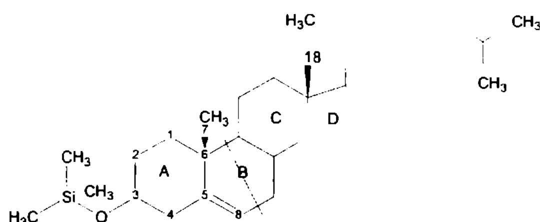
V)

A peak at m/z 255 is also seen in the case of many Δ^5 stenol ethers. The peak results from the loss of the side chain and elements of trimethylsilanol groups. The net loss is thus [M- (89+R-H)]$^+$ The pathway is as shown in Fig:V with the transfer of an H atom from the C_{18} angular group. The peak is not very intense in majority of the cases. This fact can be understood since the ejection of side chain in steroidal skeleton is not very significant as one bond is already broken in ring D (Tokes *et al.,* 1968). It should be noticed that the corresponding 5α-stanols have this peak at m/z 257. The same peaks are obtained in the case of acetates also. However in Stigmasterol (I g) ($C_{29}\Delta^{5,22}$ C-24- ethyl) this peak is of larger intensity. In β-Sitosterol (I f) where the double bond at C_{22} is absent, the former peak is of much less intensity.



VI)

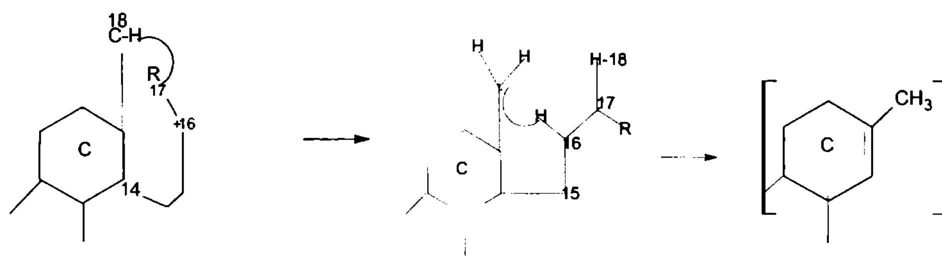
The Stigmasterol (1 g) is characterized by the presence of a base peak at m/z 83 in addition to the above peak. This is indicative of a cleavage of a C_6 group α to the Δ^{22} double bond. The latter peak appears from the fission of C-24 to C-24^2 in (VI), whose mechanism is not known fully.



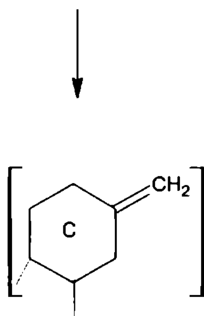
VII)

Another peak of importance is [M- {(Osi $(CH_3)_3$-C_8H_{11}+CH_3)]$^+$ which adds up to a net loss of 211 mass unit from the molecular ion. This is obtained by the fragmentation of ring B along with the loss of C_{18}angular methyl group (see VII). The complete retention of the C-7 and C-15 labels is indicative of a ring B fragmentation as shown here. The fact that the same peaks appear in the acetate spectrum, the partial loss of the 8β and 14 α labels and retention of a major portion of 5α, all substantiate the above pattern of splitting of the ring B.

The fission of ring B has been studied extensively with Deuterium labeled Cholestane (M^+, 372) giving peaks at m/z 262. Cholesterol (I a), Campesterol (I c) and β- Sitosterol (I f) give this peak at m/z 247, 261 and 275 (in acetate) respectively.



m/z-216 of Δ^0 stanols



m/z-215 of Δ^0 stanols

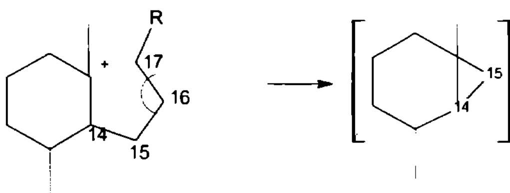
A peak appears at m/z 215 in the spectrum of acetates of $\Delta^5 3\beta$-OH sterols and at m/z 217 for corresponding 5α-H stanols. Corresponding peaks appear in trimethylsilyl ether derivatives also. The origin of this peak is a

tricyclic nucleus resulting from the fragmentation of ring D, consequent loss of C-15 toC-17 with or without H transfer along with the side chain, and the elements of hydroxyl derivatizing function (89/90units-89 for Δ^5 and 89 + 5(H) for Δ^0 compounds). An outline of the scheme of formation of the tricyclic nucleus as $[M^+-(89+R-C_3H_5/C_3H_6)]$ is shown above. In the case of 5 (H) stanols it may be $[M^+-(90+R-C_3H_6)]$. The mechanism of this elimination is as shown above. The intermediate formed by the fragmentation of Ring D abstracts an H atom from the 18 angular methyl group and decomposes to give an olefin with or without H transfer as shown.

The 5β stanol ethers generally give M^+ ions of low intensities but gives a base peak at $(M-90)^+$ A peak is obtained at m/z 217 besides at 215. On the other hand 5α-H sterols give a base peak at m/z 215 and several peaks of higher intensities with comparable peak heights. A peak is also given at m/z 216 presumably by the exchange of H from$C_{16} \rightarrow C_{18}$ in the intermediate.

A peak at m/z 149 is characteristic of steroids saturated at position 5. It is due to the loss of rings C and D together with an extra H from the charge retaininig side. The labeling studies show that it is a complex process in that there is about 70% loss of C-5 and C-8 label. Furthermore the participation of the H transfer is affected by configuration at C-5 since the partial splitting of the peak at m/z 149 into 151 is the only major difference between spectra of 5α and 5β Cholestanes and their derivatives. Peaks at m/z 144 and 145 also are

seen in several 5α-H stanol ethers and are attributable to fragments arising with and without transfer of H atoms by the cleavage of C_1 to C_{10} and C_4 to C_5.

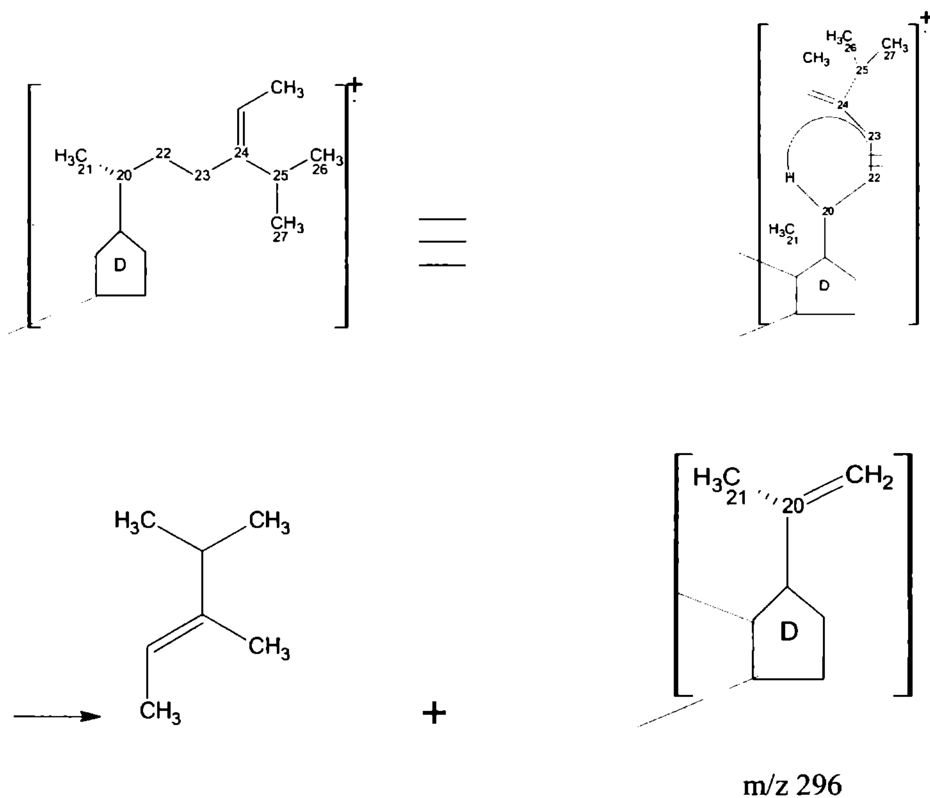


m/z-230 for 5αH stanols

Another major peak is at m/z 230 representing the loss of C_{16} and C_{17} in addition to the side chain and the elements of the derivatizing function. The peak consists of an ionized cyclopropane, which is an energetically favored segment of the steroidal skeleton.

A base peak at m/z 314 is characteristic of sterols like Fucosterol (I j) and 24, Isofucosterol (Ii). It originates from the simultaneous elimination of side chain fragments along with elements of trimethylsilanol group It is a typical feature of the mass spectrum of sterol ether with a $24(24^1)$ double bond and nuclear unsaturation (Djerassi *et al.,* 1979). In the case of 24- methylene or ethylidine sterols there is an intense peak at m/z 296 due to the tendency of $\Delta^{24(24^1)}$ derivatives to undergo McLafferty rearrangement. These sterols like Isofucosterol(Ii) and Fucosterol (Ij) undergoes the elimination of side chain fragments from C_{23} - $C_{24}{}^1$ along with an H and the elements of trimethylsilanol group to give the above peak. The existence of a similar peak for 24-methylene

sterols has been mentioned (Kates *et al.*, 1977). The elimination is shown below.



An intense peak at m/z 314 is given as typical for these 24- ethylidene sterols in the works of Urbina. et al. (1997). This peak has been described earlier by Rahier and Benveniste (1989). Also it can be noted that all the peaks of Isofucosterol (Ii) are of higher intensities than the corresponding peaks for

Fucosterol (Ij) except perhaps the base peak. A peak at m/z 83or 81 is absent for the latter (Sheikh 1974).

In the case of Cholest-4-en-3β-ol the spectrum is strikingly different from that of Cholesterol in the following respects (Brookes *et al.,* 1968). The base peak is at m/z 143 accompanied by a peak at m/z 142. These are ascribed to the fragments comprising of the trimethylsilanol group and a four C unit derived from ring A.

Regarding the Δ^7-3β-ol trimethylsilyl ethers the molecular ion is relatively more abundant and often is the base peak. The peak at m/z 255 is also relatively more intense. A peak at m/z 269 has been reported for Δ^7 stenols. Homolysis of the activated C_{15}-C_{16} bond and elimination of elements of trimethylsilanol groups from the molecular ion yields a neutral olefin and a formally ionized cyclopropane at m/z 229. Fragments are seen at m/z 213 and representing further degradation of rings most probably by the loss of 18 angular methyl function with an H abstraction (Knights and Berrie, 1971). Analogous peaks are obtained at m/z 243 and 227 in the case of 4α-methyl sterol derivatives.

Derivatives of $\Delta^{8(14)}$-en –3β-ols is generally similar to those of the Δ^7 compounds. Molecular ion peak, and peaks at m/z 343 and 227 in the case of 4α, 24 dimethyl cholest-8 (14) –en 3β-ol can all be explained similarly as the corresponding peaks of Δ^7 compounds. The base peak is obtained at m/z-98.

$\Delta^{(8(9))}$ sterols like Lanosterol (Vb) give intense peaks at (peak.M^+-15), M^+-(90+15) besides the M^+ being the only ones of any significance.

Dihydroagnosterol (Vi a) which is $\Delta^{7,9(11)}$ 3β-ol gives an odd peak at m/z 240 which is unique to this type. The molecular ion constitutes the base peak besides a peak at m/z 253.

3.7 Source Characterization of Sterols -General Scenario

There is a chance of wide variability in sterol compositions among animals, especially collected from different localities. Therefore to read into the physiological implications of sterol compositions, nature of the flora and fauna present, the physical and chemical nature of the water body and the probable chemical transformations, all should be looked into. An examination of the sterols identified in the samples show that they can be grouped into three classes according to their sources.

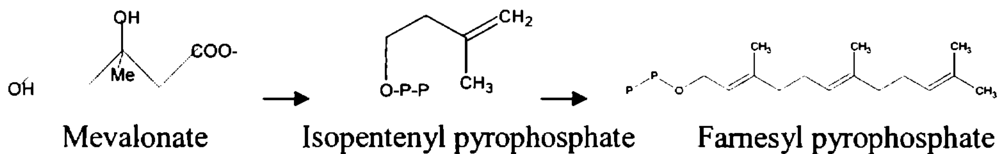
3.7.1 Biochemical Origin of Sterols

Any one attempting to discuss the biochemical importance, significance and interaction of sterols should have an idea of the biochemical origin of sterols. Regarding the biosynthesis of sterols, it is well known that the common precursor to sterol biosynthesis is Squalene obtained from Acetyl coenzyme A.

All plant sterols are synthesized from Cycloarterenol, derived from Squalene, which is the common intermediate of sterol biosynthesis. Cholesterol on the other hand is formed through Lanosterol, for which the common precursor is again Squalene, which itself is biosynthesized from Mevalonic acid. This is a common precursor except in a few microorganisms like

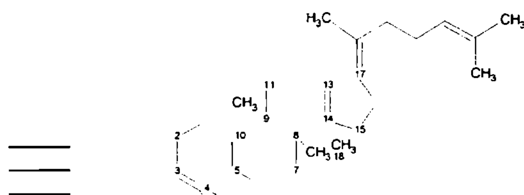
Plasmodium where there is an alternate route to sterol biosynthesis other than from Mevalonic acid. (Block H.J, 2004).

The biosynthetic route of sterols has been studied extensively using isotopically labeled acetic acid during earlier studies (Bloch and Rittenberg, 1942; Woodward and Bloch, 1953). $^{14}CH_3$ $^{13}COOH$ was used for the studies and the important intermediate was shown to be Mevalonic acid (3:5 dihydroxy- 3-methylpentanoic acid). The Mevalonic acid is formed through a series of reactions starting from the activation of acetate to Acetyl Coenzyme, which involves an acetyl kinase enzyme. (Shoppe, 1964 a). Another important enzyme, which is involved in this part of the reaction, is 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMG-CoA reductase). This enzyme is considered to be catalyzing the rate limiting step in the sterol synthesis (Mayes, 2000). The Mevalonic acid is phosphorylated and is converted to active isoprenoid unit Isopentenyl pyrophosphate by decarboxylation.



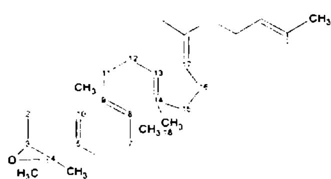
Mevalonate Isopentenyl pyrophosphate Farnesyl pyrophosphate

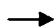
Three isoprenoid units condense to form Farnesyl pyrophosphate.

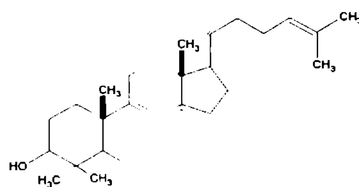


(Squalene)

Three molecules of this compound, through a set of reactions is converted to Squalene, which then cyclises with two 1,2 shifts, one from C_{14}-C_{13} and the other from C_{18}- C_{14} forming Lanosterol. This then demethylates and rearranges itself to Cholesterol through a series of steps.

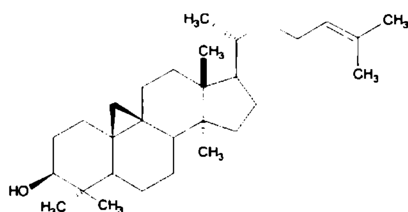






Squalene 2,3 oxide

Lanosterol (in animals)



Cycloarterenol (in plants)

It must be emphasized that the presence of molecular oxygen is essential in at least three steps for sterol biosynthesis. These are formation of Squalene 2, 3 oxide, C-4 demethylation and Δ^5 bond introduction in such cases as Cholesterol.

To understand how the sterol component of an animal originates, there are four important factors to be considered. The first and foremost fact to be verified is whether any *de novo* sterol synthesis takes place in the animal. This is done usually by providing [^{14}C] acetate. However, if Acetyl kinase is rate limiting sterol synthesis may not be observed since acetate requires prior conversion to acetyl coenzyme A for incorporation into the sterol biosynthetic pool. Another point to be considered is the regulatory role of HMG- CoA reductase. The activity of this enzyme is reduced in presence of high intake of dietary sterols or in the presence of excess Mevalonic acid, thus restricting the intake of labeled acetate into sterol pool. This problem is often circumvented by giving [2-^{14}C] mevalonate whose incorporation into the sterol pool is not under the control of HMG-CoA reductase

Another major problem in determining if any *de novo* synthesis of sterol is taking place by the injection of labeled precursor is the dilution effects. The extent of dilution of the radioactive material required to keep the animals healthy is so great that any labeled specimen of sterols in the final analysis escapes detection. Equilibration with bulk water is very high so that any labeled specimen of [C^{14}] directly injected into the animal does not enter the sterol biosynthetic pool.

Invertebrates in contrast to mammals have rather slow sterol turnover rates and incubation for a few days even may not demonstrate any sterol biosynthesis. The labeled [C^{14}] may escape through the more competing pathways of fatty acids and polyisoprenoid products. Like all other species of animals the extent of *de novo* synthesis of sterols in invertebrates also depend on aquarium temperature, age of the animal, and its sexual maturity.

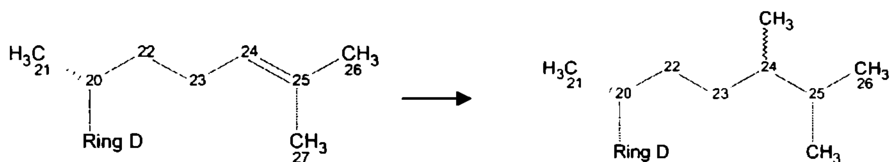
Many studies demonstrating sterol synthesis simply estimates the incorporation of labeled mevalonate into the total sterol content. This does not distinguish between sterols of dietary origin and sterols, which are, synthesized *de novo*. Besides, the modifications of dietary sterols and the passage of sterols from the symbiotic algae or other associated organisms like fungi or bacteria to the host animal are also to be considered.

Many fractional crystallization steps involved during the isolation may often remove a minor component, which has been labeled. Moreover no single technique could completely separate the complex sterol mixture. But with modern methods of separation like preparative hplc over C_{18} reverse phase system and gcms methods coupled with data handling system, the analysis of sterol mixtures has been revolutionized. The application of such strategies has facilitated the effective analysis of mixtures of sterols from biosynthetic studies possible. Even the identification of biosynthetic intermediates present in trace amounts can be done, thus ensuring accurate and comprehensive identification of labeled precursors and products. Such strategies have made Sterols, one among the most well traced biomarker compounds.

3.7.2 Inter Conversion of Side Chains

The side chain of the Sterol can be modified only through a limited number of biochemically feasible steps This gives an idea of the range of side chain modifications possible in a sterol of given nuclei that are possible by dietary modification in a given sterol. The study has led to the computer-simulated structures possible in marine sterols with a given nucleus. If we combine all the possible side chains generated similarly by the computer under the constraints, with the seven most common sterol nuclei, we can see that about one thousand and one hundred complete sterol structures are produced. It is exceedingly unlikely that about 50% of the structures envisaged exist actually (Djerassi *et al.,* 1977). The authors have remarked further that even if 10% of this actually is detected in nature, a remarkable variety of exciting biosynthetic leads will have been uncovered which have so far no precedent in terrestrial sterol biosynthesis.

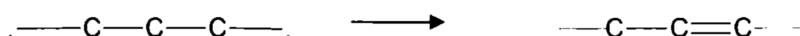
A simple rule that saturation can occur at any double bond leads to the formation of Cholesterol from Cycloarterenol in plants.



The set of permitted rules besides the above one that are relevant in the synthesis of 24-alkylated sterols from Cycloarterenol in the case of plants can be shown to be

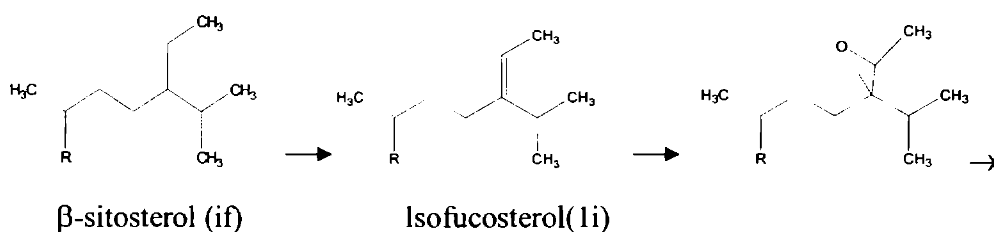
i) Methylation can occur as represented below, but with the constraint that it never occurs at tetra - substituted Carbon

ii) Double bond generation occurs as shown, but subsequent migration of the double bond is prohibited.

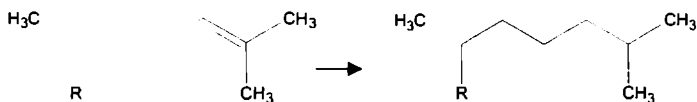


An introduction of a double bond at the side chain methyl group, leading to the formation of 24- methylene sterol entity and finally further methylation of the double bond leading to the familiar β- sitosterol, all can be explained by the above two rules. Further generation of a double bond at position 22 leads to Stigmasterol entity.

Another important conversion that is highly relevant in the present study is the removal of 24-ethyl side chain through a $24\text{-}24^1$ epoxide intermediates



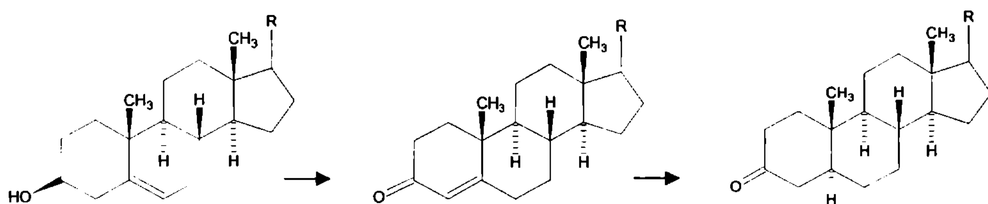
β-sitosterol (if) Isofucosterol(1i)

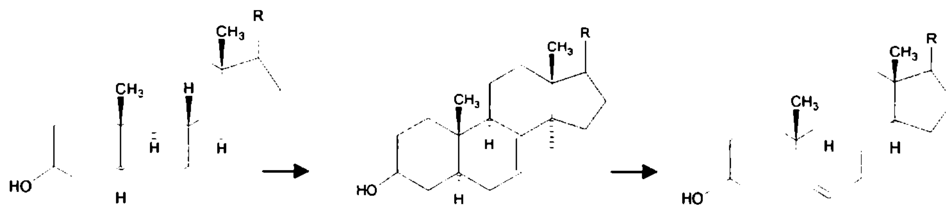


This explains the conversion of β-sitosterol (1f), the principal mangrove sterol into Isofucosterol (1i), which reaction is found in Crustaceans. This incidentally is an example of the dietary modification of a mangrove sterol by an organism.

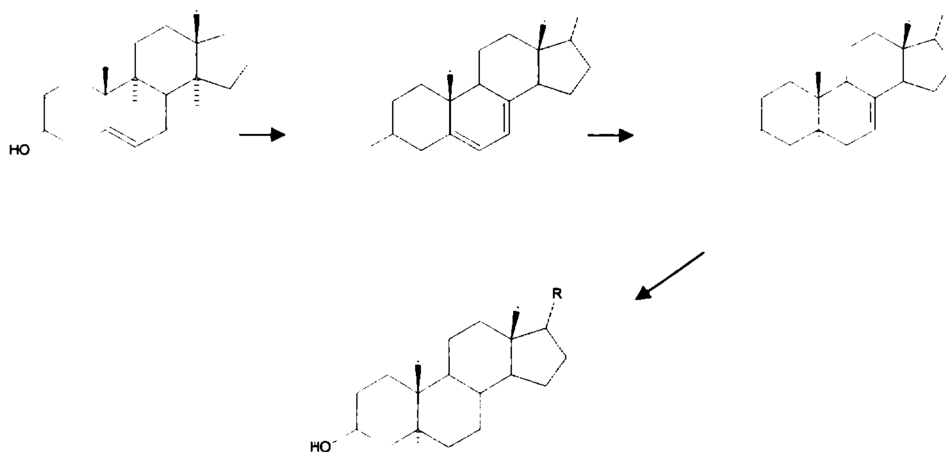
3.7.3 Inter conversions of Sterol nuclei

An important nuclear conversion, which is of significance in the present study, is the conversion of a Δ^5 sterol into a Δ^7 and $\Delta^{5,7}$ sterol. Several studies have demonstrated the presence of a 4-en-3-one, and corresponding 5α- stanol as intermediates. The origin of Δ^7 C_{28} and C_{29} sterols in starfish by this route has been substantiated by the conversion of [4-^{14}C] sitosterol to 5α-stigmastanol and 5α-stigmast-7-en- 3β-ol in certain species of starfish. (Smith and Goad, 1975; Voogt and Van Rheenen, 1976).





An alternative route to Δ^7 sterols has been demonstrated in starfish species by Goodfellow (1974). This operates through the production of 7-dehydrocholesterol as shown. Goad (1978) has described the observation in his earlier studies that when A. rubens was given an injection of [3α-$_3$ H, 4-^{14} C] Cholesta-5, 7-dien-3β-ol and incubated for 96 hours, the species produced 5αcholest-7-en- 3β-ol with about 85% retention of Tritium. This observation clearly indicates the reduction of Δ^5 bonds without prior production of oxosteroid.



In other words the series of reactions $\Delta^5 \rightarrow \Delta^{5,7} \rightarrow \Delta^7$ is operative in starfish. This is quite contrary to the opposite sequence in mammals by which the Cholesterol is biosynthesized.

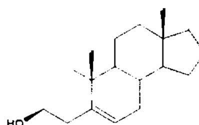
3.8 Sterols in the systems under study

A close examination of the results of the gas chromatographic studies reveals the presence of twelve sterols, which can be divided into three groups. The significance of these sterols with special emphasis on their possible biological origin and subsequent transformations are discussed underneath.

Group	Sterols
(i) Mangrove sterols	**Cholesterol**, Campesterol, β-sitosterol, Stigmasterol, and **Isofucosterol**
(ii) Sterols that can either be obtained from other organisms or produced by modification of the Mangrove sterols	**Cholesterol**, Cholestanol, Lathosterol, Stigmastanol, **Isofucosterol**, Lanosterol and Dihydroagnosterol
(iii) Sterols that are allochtonous to the system.	Coprostanol, Spongesterol

3.8.1 Group (i) Sterols

These sterols are present in mangrove plants and can be considered as autochthonous to the system. Isofucosterol, though a minor mangrove sterol can be considered as a group (ii) sterol as well.

a. Cholesterol (I a) 

Cholesterol has been detected in sediments of both the regions. It is detected as both acetate and trimethylsilyl ether. The retention time and the peaks obtained in mass spectrum matches with that of the standard sterol used (See Appendix IV). The important diagnostic peaks are 458, 368, 329, 255, and 129. This sterol has also been identified as the acetate ester.

This is ubiquitous in almost all the marine sediments and water samples studied earlier. Generally it is considered to be a zoosterol, since it is widely distributed in zooplanktons and animals. Besides it is present in certain types of microalgae, diatoms etc. (Goad, 1978). Cholesterol is a common sterol in marine animals also, especially Crustaceans in their fecal pellets (Wakeham and Beier, 1991). It has also been shown that the 20-E, the steroidal hormone responsible for ecdysis is biosynthesized from Cholesterol by shrimps, which in turn must be obtained from dietary sources (Diwan, 2005). It is present as a minor sterol component in mangrove plants as well as land plants as pointed out earlier. In mangroves the amount of sterols reported by different workers varies widely. To take an example the amount of Cholesterol expressed as percent of total sterols varies from about 0.5% (Hogg and Gillan, 1984) to 2.7% (Misra *et al.*, 1984) in the species of Rhizophora mucrunata. This conspicuous

variation may be due to the difference in locality of study, seasonal and often climatic differences existing in the study area. In the latter study it has also been shown that even the method of hydrolysis of the steryl esters is responsible for such variations. The former authors have reported the maximum amount of Cholesterol as 7.1% occurring in Acanthus ilicifolius. The latter authors have given a value of about 6% in Bruguiera gymnorrhiza, while these authors give a value of about 5% in A. ilicifolius. These species have been reported in these areas (Subramaniyan, 2000; Muralidharan and Rajagopalan, 1993; Pretha and Rajagopalan, 1993). In the present study Cholesterol is considered to be autochthonous since it is a component of mangrove though a minor one and since it is a major faunal dietary transformation product, as we shall see later. Zooplankton fecal pellets are additional sources of Cholesterol (Volkman, 1986; Harvey *et al.*, 1994). From the estimation of relative concentrations of the sterol in the two sediments, it can be seen that the Vypin sediment is richer in this sterol than the MV sediment, both on the basis of β-Sitosterol, the most abundant mangrove sterol. This may be due to the greater life supporting and hence the greater variety of life forms at the former site and also from the carcasses and fecal pellets of higher animals from backwater and marine sources. Such interferences are also responsible for the presence of a purely marine sterol, Spongesterol, as we shall see towards the end of the discussion. Moreover it has been shown that major peaks of abundance of zooplanktons in mangrove areas are obtained during post monsoon periods (Chandrasekharan 2000). The paenid prawn activity is maximum during the

post monsoon period in this estuarine zone (Kuttiamma and Kurien, 1982). It has already been pointed out that Mangalavanam is not at all congenial to higher forms of life due to the partial anoxic nature (Narayanan *et al.*, 2000). Cholesterol content is about 40% of β- sitosterol here. Like all mangrove areas this system is also having many species of zooplanktons, which constitute important steps in the food pyramid of mangrove waters (Kundu *et al.*, 1987; Sunilkumar, 1993).

b. 24-alkyl Sterols

As far as the C-24-alkylated sterols are concerned a CH_3 or C_2H_5 group at C-24 is the most dominant character. The stereochemistry at C-24 is also important. Using the ordinary gcms analysis it is not possible to distinguish between the C-24α- and C-24 β-epimers (Volkman, 1986). The author has further pointed out that that the different plant groups produce sterols with either C-24α- or C-24β- stereochemistry but rarely both. Sterols differing in C-24 stereochemistry can only be separated using very long polar capillary columns using Gas chromatography (Volkman, 1986). The C-24 epimers have the same RRT values in gc experiments, but it is possible to do the separation using hplc (Kokke *et al.*, 1984). Such an attempt to resolve the epimers has not been attempted by most of the earlier workers on sediment sterols as exemplified by numerous references quoted herein. Hence it has not been resorted to in the present study also.

The C_{29} sterols collectively comprise the majority of sterols in the system. Distinguishing between sterols derived from vascular plants or marine

phytoplankton is a really difficult task. It has been remarked earlier that the C_{29} sterols are the most abundant sterols in surface sediments, but are the most difficult to interpret as source markers since they are present in both marine algae and higher plants (Marlow *et al.,* 2001). The ratios of the sterols 24- methylcholesta -5-en 3β-ol, 24-ethylcholesta–5,22-dien-3β-ol and 24-ethylcholest-5-en-3β-ol are indicative of the origin of sterols (Volkman, 1986). If substantial amounts of these sterols are present, it is pointing towards terrigenous organic matter (Harvey, 1994). Like the C-28 sterols, C-29 sterols will also exhibit epimerism at C-24 and is difficult to distinguish using ordinary capillary columns as remarked earlier in the case of C_{28} sterols. Green algae, dinoflagellates and sponges synthesize sterols with C-24β configuration such as Clionasterol (1 e) and Poriferasterol (I h) whereas the diatoms and vascular plant sterols have C-24α configuration (Volkman, 1986). The β- Configuration is present in some species of algae & sponges indicating probably the dietary origin (Goad, 1978). Mangrove leaves contain 24α- ethyl sterols, β-sitosterol and Stigmasterol. The C_{28} sterol 24-methylcholesta-5, 22E-dien-3β-ol has been considered a diatom sterol, but Volkman et al. (1998) has questioned the validity of this practice and has suggested 24-methylene cholesterol as a better marker for diatoms. This sterol has not been identified in the present context. So the diatom contribution could not be ascertained that way. However this sterol is not unique to diatoms, as it has been reported in the dinoflagellate FCRG 51 (Nicholas *et al.,* 1984). It has also been pointed out that the 24-alkyl sterols are also produced by cyanobacteria and ice diatom communities

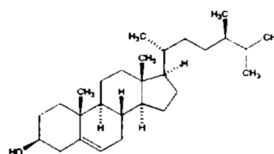
(Nicholas *et al.,* 1990). Total count of cyanobacteria is high in Cochin mangrove stations also (Joseph, 2006).

The sterols identified in this class include, Campesterol, β-sitosterol Stigmasterol and the non-mangrove Spongesterol. The list also includes a C-24-ethylidene sterol namely Isofucosterol.

It has been suggested by many workers that the ratio of Cholest-5-en-3β-ol/24-ethylcholest-5-en-3β-ol, Cholest-5-en-3β-ol/ 24-ethylcholesta-5, 22 (E)-dien-3β-ol + 24-ethylcholest-5-en-3β-ol, all indicate and are best used to establish source or preservation trends within a series of samples under study (Hudson *et al.,* 2001). This ratio is being engaged in recent works for the assessment of relative contributions of marine and terrestrial sources towards the sterol content of mangrove sediments (Mater, *et al.,* 2004). They have commented that it is a definite conclusion that if 24- ethyl cholest-5-en-3β-ol is less abundant than the other two sterols, then the algal (non marine sources) are the important contributors to the sediment. At Mangalavanam, Cholesterol/24- ethylcholest- 5-en –3β-ol is approximately 0.4, while at Vypin the ratio is nearly 0.8, both values much less than the suggested 2.5. The above authors also got values less than one in areas where the principal contribution could be assigned to mangrove sources rather than phytoplankton origin. Another ratio that is commonly used also demonstrates the above fact is the ratio of 24-methylcholest-5-en-3β-ol: 24-ethylcholesta-5, 22-en-3-β-ol: 24- ethylcholest-5-en-3β-ol. In the present context the ratios are 1:1.3:10 and 2.1:2.5:10 in the case of Mangalavanam and Vypin sediments respectively. The

high abundance of 24-ethylcholest-5-en-3β-ol indicates high mangrove contribution, but the difference in the relative contributions is noteworthy. This suggests the relative difference in sources of organic matter.

The importance of this class of sterols has received the attention of many biochemists. For the arthropods it has been demonstrated that C-24 dealkylation takes place and C-28 & C-29 Δ^5-sterols are converted to cholesterol (Goad, 1978; Teshima, 1991; Kanazawa, 2001). When the prawn Paenus japonicus and the brine shrimp Artemia salina were fed with diets containing C-24 alkylated phytosterols as the only sterol source, the sterol isolated from the species was predominantly cholesterol. It has been previously shown that the Crustaceans are not capable of de novo sterol synthesis (Teshima, 1991). The crustaceans can perform C-24 dealkylation reaction, which had been previously demonstrated in insects (Rees, 1971). It is also shown that not only the Crustaceans but also Coelentrates and Mollusks have the ability to dealkylate the C-24 sterols to Cholesterol (Kanazawa, 2001). It is noteworthy that about eleven species of Crustaceans and nine species of mollusks have been identified in the Cochin mangrove forests (Sunilkumar, 1991).



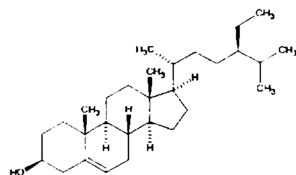
b.1 Campesterol (I c)

This is a typical mangrove sterol as already mentioned. Previous studies (Hogg and Gillan, 1984) show that A.ilicifolius contains maximum amounts

(about15.5%) while the minimum amount is present in Avicennia maria (0.9%). the abundant species in cochin mangroves is however being A.officinalis (Sunilkumar and Antony,1994; Subramaniyan, 2000). The sterol is found in both the sediments. It has been identified from relative retention times and mass spectral speaks of the authentic standard and published data. The diagnostically significant mass spectral peaks are at m/z values of 472, 382, 343, 369 etc. (Harvey *et al.*, 1988).

Campesterol is a sterol, which is converted to BRS in plants. Campesterol and its 24 epimer are not much reported in marine samples. The source of C_{28} sterols is less specific since they are relatively abundant in both algae and terrestrial higher plants. With the previous discussion, it can be reasonably concluded that the source of this sterol is mangrove litter.

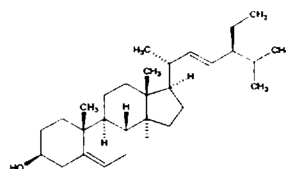
b.2 β-Sitosterol (I f)



This mangrove sterol constitutes above 60-75% of the total sterols in different mangrove species. (Hogg and Gillan,1984; Misra. *et al.,* 1984 and Ghosh *et al.,* 1985). This is generally considered to be a vascular plant marker. Among the marine organisms sponges contain the C-24 epimer Clionasterol. β- sitosterol has been identified in all the sediment samples examined and also during preliminary investigations, when smaller quantities of sediment were

taken. Identification could be done in even acetate ester. The identification was based on authentic standard values and published data (See Appendix IV). The important mass spectral peaks for TMS ether obtained in this case are at m/z values of 486(M^+), 396, 381, 357, 356, 129 etc (Brookes *et al.,* 1968).

The presence of β-sitosterol is taken to be the evidence for contribution from land plants in marine samples. However C_{27} sterols including β-sitosterol found in lacustrine and mangrove sediments cannot be treated as allochtronous. The stereochemistry at C-24 should be known to justify this full. (Volkman, 1986). But in the present study the source of 24-ethyl sterols has unequivocally traced to mangroves.

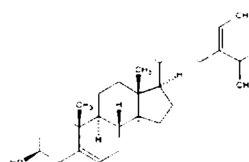


b.3 Stigmasterol (I g)

This sterol is also a mangrove sterol but unlike the previous one the relative concentrations vary from species to species. The highest concenteration is found in Avicennia species (about 28.6%) and the lowest concentration in Rhizophora mucronata (about 4.4%). In fact Avecennia is chemotaxnomically characterized by a high stigmasterol /campesterol ratio (Hogg and Gillan, 1984). C-24 alkylated sterols are found in marine microorganisms as well as terrestrial plants but the C- 24 stereochemistry may be different

(Volkman, 1986). Stigmasterol has been identified in both the sediments in the present study. The identification is based on relative retention time and mass spectral peaks of the authentic standard and on published data (See Appendix IV). The diagnostic peaks are at m/z values of 484, 394, 355, 255, 83 etc. (Brookes *et al.,* 1968). The discussion on 24-methyl sterols establishes the identity of the sterol as 24α-ethyl cholesterol.

b.4 Isofucosterol (I i); Fucosterol(I j)



Ii

These two sterols are geometrical isomers. The presence of the former is shown in Vypin sediments. The diagnostic peaks are almost the same. The peaks are obtained at m/z values of 484, 394, 379, 314, 299, 81 etc. and identification is also based on some of the values given for the acetate spectrum (Gagosian and Heinzer, 1984) and the values given for the 24-methylene derivative (Kates and Tremblay, 1977). In previous studies also where these sterols have been reported no effort has been taken to distinguish between the two, either one of them being reported. In this case the possibility is for Isofucosterol since it is a mangrove sterol. Also all the peaks of Isofucosterol are of higher intensities than the corresponding peaks for Fucosterol except perhaps the base peak and a peak at m/z 83or 81 is absent for the latter (Sheikh, 1974). Volkman *et al.* (1987) during their studies of lipids in the

sediments off Peru have pointed out that the Fucosterol co-elutes with 24-ethylcholesterol

Isofucosterol is a mangrove sterol, but the amounts reported in various species are highly varying. The highest content of this sterol has been reported in Rhizophora mucrunata(about 9%) while the lowest value is reported in Sonnaratia casseoralis (about 1.3%) The Bruguiera species also contains about 2.0% of this sterol. A study on free sterols present in the mangrove leaves done show that Isofucosterol is absent in A.illicifolius, B.gymnorrhiza, and R.mucronota, contrary to previous observations. This clearly shows that this sterol is present in the combined form in mangroves (Ghosh, *et al.,* 1985). In the present study, however sterols have all been liberated since hydrolysis with alcoholic potash has been resorted to, during the isolation.

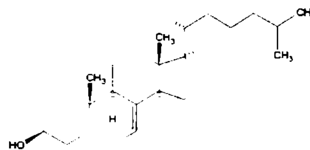
3.8.2 Group (ii) sterols

This group includes those sterols that have originated either directly from the flora and fauna present in the system or by the dietary modification of the mangrove sterols by them.

b.4 Isofucosterol (I i); Fucosterol (I j)

The E- isomer Fucosterol is the principal constituent of brown algae (Volkman, 1986). Iso fucosterol is an intermediate in the dealkylation of C-24 sterol to cholesterol or Desmosterol. The mechanism has been worked out in insects and probably operative in crustaceans as well and is shown section 3.7.2. This augments the fact that the sterol identified here is Isofucosterol and

not Fucosterol since nonchordates like mollusks and crustaceans are all present in only vypin system



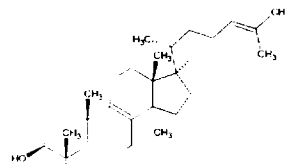
b.5 LathoSterol (IV)

In the present study only Lathosterol is identified as Δ^7 sterols. It is present in both Mangalavanam and Vypin sediments. The identification is based on published data on mass spectral peeks (Brookes *et al.,* 1968). The base peak at M^+ is missing. The peaks that were of diagnostic importance are at m/z values of 443, 353, 255, 229, and 213,129.

There are very few reports of Δ^7Sterols in surface sediments. The most important marine invertebrate sources of Δ^7 sterols are Chitons (Volkman, 1986; Kanazawa, 2001). Cyanobacteria are also potential sources this sterol is present as a minor component in certain diatoms (Volkman *et al.,* 1980).

These sterols come from several species of green algae. Sponges and asteroids, which feed on these algae are also sources of Δ^7 sterols in marine environment. The normal route of formation of Δ^7 sterols is the blocking of the Cholesterol biosynthesis at an appropriate stage and is thought to occur in chitons. Extensive studies have been done in Starfish species to study the possible biosynthetic routes of Δ^7 sterols. Starfish species like Asterias rubens was shown to synthesize these sterols. It has been shown that Cholest-4-en-3-

one is an intermediate during this conversion and that Cholesterol can also be converted into Cholesten-5α- 3β-ol during this process (Goad, 1978). The steps involved in the conversions have been discussed under section 3.7.3. This should be correlated with the fact that C_{27}, C_{28}, and C_{29}, sterols are associated with small amounts of corresponding 5α stanols. The presence of $\Delta^{5,7}$ sterols have been shown in mollusks which constitutes the food for starfish. Similar reactions are thought to be responsible for the formation of $\Delta^{5,7}$ sterols in these animals also. It should be noticed that mollusks have been reported in the Vypin systems (Sunilkumar, 1991). The presence of $\Delta^{5,7}$sterols in these animals is significant in that they are precursors of $\Delta^{5,7}$dienes and D vitamins, which are crucial in Calcium metabolism and exo skeleton formation. Besides Ecdysones, the hormones which help development as well as the principal hormone for molting process in arthropods including crustaceans are all having Δ^7 sterol or sterone basic structural pattern. In Mangalavanam the formation of Δ^7 sterols could have been brought about through microbial population. The reduction of Δ^5 sterols to corresponding stanols is microbially mediated by anaerobic bacteria.in highly reducing sediments (Nishimura and Koyama, 1977; Nishimura, 1978). The bacterial count is high during the post monsoon period in the Cochin mangrove sediments (Raman and Chandrika, 1993). Hence the formation of Lathosterol is well explained. It deserves special mention that the mechanism for microbial conversion of stenol to stanol involves $\Delta^{5,7}$ sterols (Wakeham, 1987).



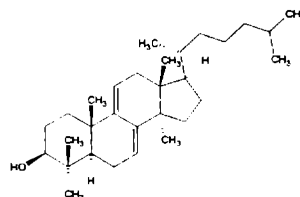
b.6 Lanosterol (Vb)

This is a C_{30} sterol and is found in the two sediments. The diagnostic characteristics are based on published data (See Appendix IV). The important peaks are obtained at m/z values of 498, 393, 241, and 227,187, 95 (Brookes *et al.*, 1968).

It is an important biosynthetic intermediate during cholesterol synthesis. In fact de novo synthesis of sterols from the radioactive ^{14}C mavelonate is indicated by the presence of radioactive Lanosterol. Hence small amounts could be reported in many species like microalgae (Volkman *et al.*, 1993).

Presence of Lanosterol is well demonstrated in mollusks (Goad, 1978). Moreover it should be noticed that during the conversion of this sterol to Cholesterol, three molecules of Oxygen are required (Section 3.7.1). The mangrove ecosystems are having reducing environment and hence these three steps might have been blocked. Further studies in this respect are to be undertaken. In this context it is noteworthy that although 4α- $\Delta^{8(14)}$ sterols could not be confirmed, their presence has been indicated in the systems during the present study. These sterols are present in trace amounts in many eukaryotic organisms, and represent the dominant sterols in methanotrophic eubacteria, the

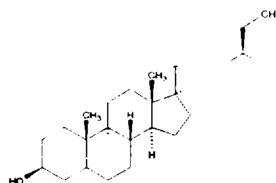
only prokaryotic organisms irrefutably known to produce sterols (Giner *et al.*, 2003). Methanotrophic bacteria have been reported in mangrove sediments of Pichavaram (Ramamurthy *et al.*, 1990), though such reports are lacking in the mangroves of Cochin estuarine zone. In some species of methanotrophic bacteria, 4, 4dimethyl and 4α-sterols dominate (Bouvier *et al.*, 1976; Schouten *et al.*, 2000). Many of the tri terpene alcohols that are being considered as mangrove markers are all having a basic Lanosterol skeleton (Oku *et al.*, 2003; Koch *et al.*, 2003) and their interconversion to this sterol also deserves future consideration.



b.7 Dihydroagnosterol (Vib)

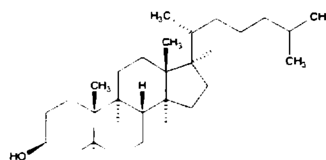
It is found in Mangalavanam. It is found always associated with L:anosterol (Shoppe,1964b). The diagnostic peaks obtained are 498, 408, 393, 369, 253, and 240 and is found to agree with the published data. (Brookes et al.al., 1968). The origin of sterol and transformations are similar tothe previous member. It should be mentioned that the presence of this sterol is quite odd and remarkable in the systems. The structure is also unique in that the nucleus is having double bond in the 9(11) position.. It has not been much reported or discussed in marine or lacustrine sediments previously. Hence it points towards

an hitherto unknown source or microbial transformation in highly anoxic sediment that is rich in organic detritus.



b.8. Stigmastanol (II f)

This sterol has been identified in the highly anoxic sediment of Mangalavanam (See Appendix IV). The important diagnostic peaks were at m/z 488, 473, 398, 383, 230, 217 and 215. This is most probably formed by the reduction of Stigmasterol, like Cholestanol from Cholesterol as we shall see in the next section.. No attempt has been made to distinguish the sterol from its C-5 or C-24 epimers. Previous studies in which this sterol is reported has likewise not attempted for this differentiation. (Nishimura and Koyama, 1977). The identification is based on relative retention time and published data on the mass spectral peaks. The diagnostic peaks are at m/z values 488, 473, 398, 383, 230, 217 and 215 (Brookes *et al.,* 1968). The reduction of the double bond at C-22 of Stigmasterol, besides the Δ^5 bond indicate a chemical reduction in the highly anoxic sediment (Wakeham and Beier, 1991).



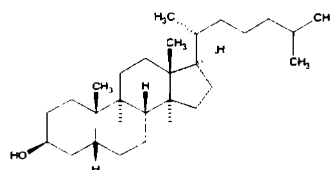
b.9 Cholestanol (IIa)

5α-cholestan-3β-ol is found in Mangalavanam. The identification is based on published data of the relative retention time and mass spectral details (See Appendix IV). The important peaks are obtained at m/z values at 460, 445, 370, 355, 230, 215 and 217 (Brookes *et al.,* 1968). In MV sample the mass spectral peaks were all of medium intensity with the presence of a conspicuous molecular ion, indicating 5α-(H) stanol. The stanols are reported in anaerobic sediments, where cholesterol can be reduced to 5 α H and 5β -H stanols. (Volkman *et al.,* 1998) The reduction can be microbially mediated (Nishimura and Koyama, 1977; Leeuw *et al.*, 1983;Wakeham, 1989). The former authors have further pointed out that the stenol –stanol reaction rate increases as the redox potential in lake sediments decreases. It has been shown by incubation experiments that an intense decrease in redox potential alone is not enough for the reduction of stenols, but the presence of autochthonous and metabolizable organic matter should also be present in sufficient amounts (Wakeham, 1982). He has shown further that no inter conversion of 5β-stanol into 5α-stanol takes place under the conditions. Similarly the same stanols found in contemporary lacustrine sediments have also been detected in small concentrations in marine algae zooplanktons and land plants showing that in lacustrine sediments stanol degradation virtually will not take place in high reduction zone (Nishimura, 1978). Some authors have categorically stated that the most obvious manifestation of sterol diagenesis in the anoxic zone is increased concentrations of 5α-H stanols (Wakeham and Beier, 1991). The redox potentials measured as

Eh values for the MV and Vypin sediments are –395 and –210 respectively. So detection of 5α stanol in MV sediments suggests the reduction of Cholesterol in the highly reducing environment. The reduction is mainly microbially mediated since the intermediates like Δ^7 sterols have been isolated. Authigenic filamented pyrites have been reported at Mangalavanm (Rosily, 2002), the presence of which can be taken for the anoxic conditions conducive to the above reduction (Jeng and Huh, 2001). Hence the source of this sterol can be traced to reduction of the precursor by anoxic organisms in regions of very low Eh. A possibility of partial chemical reduction cannot be ruled out since the ratio Stigmastanol/Stigmasterol and Cholestanol/Cholesterol is not widely different as is evident from the chromatogram of section 3.5 (Wakeham and Beier, 1991).

3.8.3 Group (iii) Sterols

This group includes sterols that come to the system from external sources. The sources may be due to anthropogenic inputs, marine inputs or land run off.

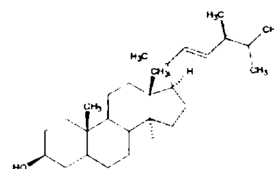


c.1 <u>5β-H- Chotestan – 3β-ol (III a)</u>

The diagnostic mass spectral peaks are same as that of the Cholestanol, but with less intensity. Also it is marked by the conspicuous absence of molecular ion at m/z 460 (Brookes *et al.,* 1968).

The presence of 5β-cholestanol at Vypin is proved by isolation using a second column chromatographic clean up of the sterol mixture sample on silica gel and using an elution mixture of 15% ethyl acetate in n-heptane. All the peaks in the mass spectrum were of low intensities with the M^+ ion at m/z 460 absent. The relative retention time also was slightly higher in this case. The canal connecting mangrove forest at the site of collection and the main water body is passing through human habitats and hence the presence of 5β-cholestanol is accounted for. 5β-Cholestanol is invariably a marker for human feacal pollution (Parrish *et al.,* 2000). It has been pointed out that some marine mammals also as the source of 5β-cholestanol (Volkman *et al.,* 1998), but this is not significant in the present study.5β-sterols are also having algal origin. However Hudson *et al.*, (2001) have suggested that the presence of 5β-stanol can be taken as evidence for human fecal contamination since about sixty percent of the total sterols in human feces consist of Coprostanol. These authors have also suggested a Coprostanol/ Cholestanol ratio greater than one to indicate sewage pollution. Mater *et al.,* (2004) have remarked that Coprostanol/ Cholestanol + Coprostanol ratio being greater than 0.7 is a better indicator of sewage pollution. While considering the fecal sterol pollution in Taiwan River a better indicator of sewage pollution will be the ratio of concentrations of Caprostanone x 3 Caprostanol/epi-Caprostanol (Chou and Liu, 2004). These

authors have considered the contributions from pig, duck cattle apart from human fecal pollution. The absence of 5α stanols at Vypin suggests the origin of the 5β-stanol from the fecal pollution. The ratio Coprostanol/Cholestanol is nearly 0.1 here. This lower ratio could be due to circulation and flushing pattern on which the concentration of Coprostanol depends (Mudge *et al.,* 1999). According to these authors the contribution from epi-choleserol need be considered only if treated sewage plants are near by. There are no sewage plants near by vypin. Therefore the fact that epi-coprostanol has not been detected in Vypin has no significance in the present context. Hence it could be reasonably concluded that 5β- cholestanol is from pollution due to anthropogenic fecal matter.



b.12 Spongesterol (IId)

The sterol is identified as its acetate. The important diagnostic mass peaks appear at m/z 344, 329, 315, 257, 255, 215, 213 and 69 (Gagosian and Heinzer, 1984). Structurally this is the side chain dehydogenation product of Campesterol. However it is classified as a marine sterol (Shoppe, 1964). It is present at Vypin and is identified as acetate. Their presence has been reported in some species of dinoflagellates (Harvey *et al.,* 1988) like Scrippsiella

trochidea, whose presence has not been reported here. This sterol is not reported in Mangalavanam. The presence of this sterol is not fully accounted for. Vypin is in quite proximity to the Arabian Sea and the forest is subjected to direct tidal flushing from the sea. It should be remembered that the salinity of the forest and adjoining canal are relatively high even during the monsoons (Geetha *et al.,* 2006) indicating seawater intrusion. Hence the source of the sterol maybe traced to marine sources.

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* not seen in original

Summary

Mangroves are halophytes that thrive in the intertidal zones of tropical and sub tropical climates. The area of mangroves in India is about 47% of the total area of mangroves in the world. Millennium Ecosystem Assessment Report of United Nations Organization states that 35% of the total mangrove area has been lost worldwide in last several decades. The mangrove area in the state of Kerala has decreased from 1000 km^2 a century ago to a mere 17 km^2.

Mangroves confer many direct and indirect benefits to the coastal population. The ecosystem eventually provides an excellent supply of organic detrital matter in the early food chain of coastal habitats. There is a definite increase in the fish and crustacean catch in the areas adjacent to mangroves.

The present study was an attempt to characterize the sterol content of the mangrove sediments, their dietary status with respect to the natural flora and fauna present, their transformations in the sediment and assess contributions, if any to the nursery character of the mangrove ecosystem. Many studies have highlighted the advantages of sterols as biological markers. The study of sterol distribution in sediments gives an idea about the contributing biota or dietary modifications brought about by the flora and fauna present in the environment. Crustaceans including prawns lack the ability to synthesize sterols. The mangrove ecosystem serves to supplement such life-sustaining agents besides providing physical shelter, especially for the juveniles.

Two mangrove ecosystems of the Cochin estuary were fixed for the study so as to compare the complex environmental conditions prevailing in

different mangrove ecosystems. One was Mangalavanam, a patchy mangrove area in the heart of the city of Cochin. This is almost a closed system with a single narrow canal linking to the estuary and is highly anoxic in nature. The second system was at Vypin. This mangrove area is being regularly inundated by a semi diurnal rhythm of Cochin bar mouth and the tidal waters bring in lot of fish seed of commercially valuable species of prawns.

The preliminary studies showed that the sterol content is very low in the sediments. Hence about two kg of the sediment was collected during the post monsoon season of 2001, in monthly intervals and pooled together for analyses. Water samples were also collected from the systems and adjoining canals. Water sample were analyzed for common hydrographical parameters such as pH, Alkalinity, Salinity, and Dissolved Oxygen. Sediment samples were analyzed for physical characteristics as well as the sterol content. The former included Eh, Textural characters and XRD analyses with a view to have an idea of the nature of minerals prsesent. The dried sediments were subjected to extraction with boiling Chloroform and Methanol. Sterol mixture was isolated from the extract using Liquid Chromatography. The sterol mixtures were derivatized and subjected to Gas Chromatographic analyses, for identification and partial quantification of the individual sterols.

The sterols identified were twelve in number and were grouped into three classes. Group (i) Sterols are those sterols that are present in mangrove plants and can be considered as autochthonous to the system. Among the five sterols coming under this head, Isofucosterol could be traced only at Vypin,

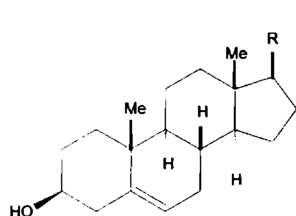
while Cholesterol, Campesterol, Stigmasterol and β-sitosterol could be obtained from the two samples. Group (ii) sterols include those sterols that have originated either directly from the flora and fauna present in the system or by the dietary modification of the mangrove sterols by them. Among these seven sterols Cholesterol and Isofucosterol are common to group (i) and group (ii). Cholestanol, Stigmastanol and Dihydroagnosterol could be detected only at Mangalavanam. Lathosterol and Lanosterol were present in both the sediments. Group (iii) sterols are sterols that come to the system from external sources. The sources may be due to anthropogenic inputs, marine inputs or land run off. The two sterols, Coprostanol and Spongesterol detected under this category were at Vypin.

The possible sources of the sterols and their relevance in the present systems of study are discussed, with a view to ascertain the role played by the individual mangrove ecosystems in the transformations of sterols. This also gives an assessment of their importance to the flora and fauna present in these two systems, which differ much in their life sustaining capabilities. The origin of all the sterols at Mangalavanam could be traced either to mangrove origin or microbial transformation products. At vypin the origin of sterols could be traced to mangroves or bio transformation products by higher organisms and exogenous sources due to anthropogenic and marine input.

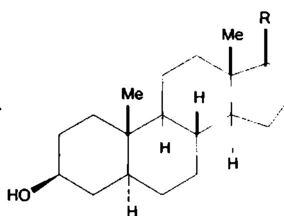
Appendix

Appendix I

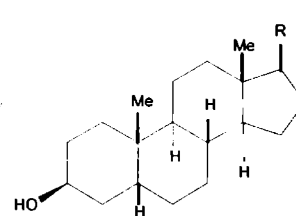
The structures of the sterol nuclei and side chains of sterols isolated during the study are noted below.



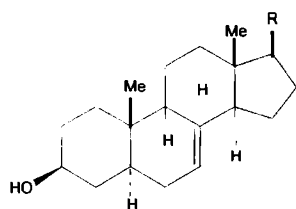
I



II



III



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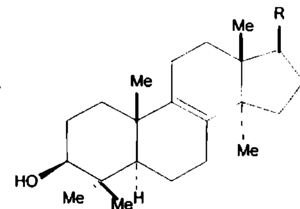

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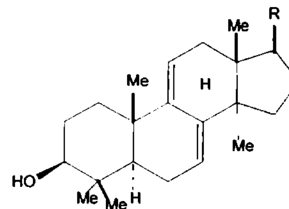
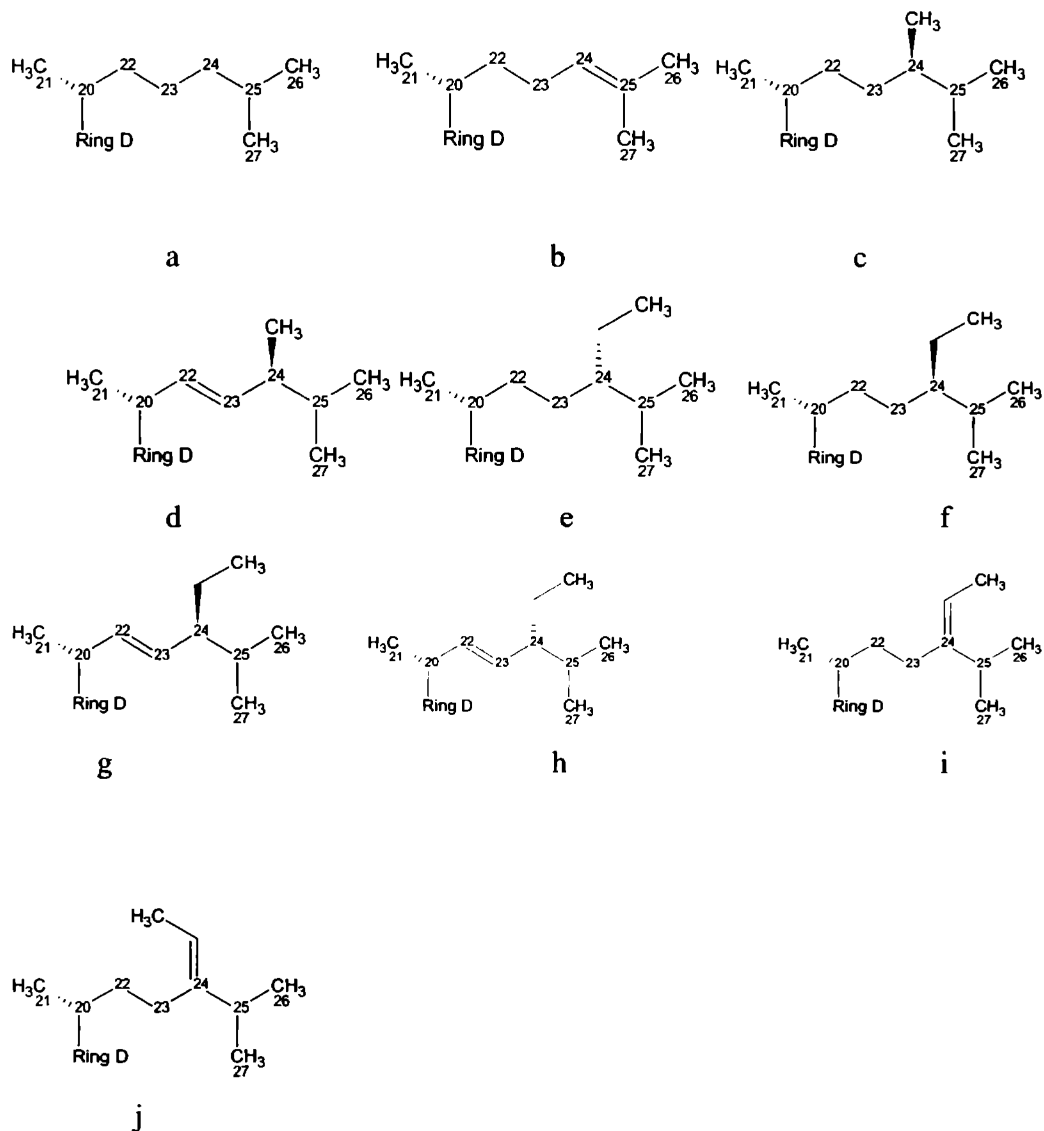


VI



H3C
21
20
22
23
24
25
CH3
26
CH3
27
Ring D
a
b
c
d
e
f
g
h
i
j

Appendix II

Name of sterols	Str:	Expected peaks for sterols	
		TMS ethers	Acetate esters
1.Cholesterol	I a	458(M+)(34%), 368(8%), 353(3%), 329(95%), 247(18%), 129(BP), 121(19%), 107(15%), 95(15%)	368(M^+-60) (58%), 353(35%), 260 (25%), 255(32%), 247(38%), 213(34%), 145(100%)
2.Cholestanol	II a	460(M+, 77%), 445(68%), 370(33%), 355(34%), 230(36%), 217(40%), 216(45%), 106(41%), 75(37%)	
3.Coprostanol	III a	460(M+,2%),355(25%)257(14%),230(10%), 216(10%), 215(25%), 142(8%), 135(7%), 108(22%), 106(20%)	
4.Lathosterol	IVa	458(M^+,100),443(18%),353(10%),255 (100%),229(18%),213(14%), 161(18%),147(10%),135(7%),107(13%)	
5.Spongesterol	II d		442(M^+) (6%), 315(10%), 257(20%), 255(27%), 215(13%), 213(18%), 69(100%)

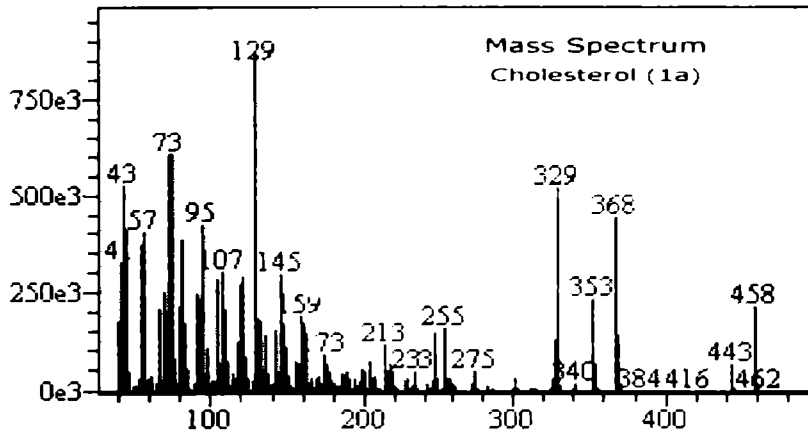
6.Campesterol	I c	472(M$^+$3%),382(11%),343(17%), 261(7.5%)255(13%,129(100)	
7.Stigmasterol	I g	484(M$^+$, 42%), 394(52%), 355(23%), 255(53%), 139(46%), 129(67%), 83(100%), 69(28%), 57(28%)	394(M$^+$-60)(34%), 351(5%), 282(5%), 255(33%), 228(7%), 213(15%), 81(100%)
8.β-sitosterol	I f	486(M$^+$, 37%), 396(80%), 381(30%), 357(93%), 356 (21%), 275acetate), 255(16%), 129(100%), 121(20%), 107(16%), 95(15%)	396(M$^+$-60)(44%), 381(14%), 329(4%), 288(10%), 275(12%), 255(18%), 213(22%), 147(100%)
9.Stigmastanol	II f	488(M$^+$37%), 473 (46%), 398 (44%), 383 (36%), 230(34%), 217(43%), 216(68%), 215(100%)	
10.Lanosterol	V b	498(M$^+$, 44%), 483( ),241(9%), 227(8%), 187(9%), 135(18%), 109(26%), 95(12%), 83(7%)	
11.Isofucosterol	I i	484(M$^+$, 53%), 394 (9%), 379(8%), 314(100%),296(43%), 229(59%), 281(60%), 255(16%), 81(77%)	
12.Dihydro-agnosterol	VI a	498(M$^+$, 100), 393(23%), 408(15%), 369(11%), 295(10%), 253(4%), 240(24%), 161(8%), 157(7%)	
13.Fucosterol* (not detected)	I j	484(M$^+$, 50%,), 394 (27%), 379 (10%), 314(100%), 296(40%), 281(50%), 229(31%), 213 (32%), 81(70%)	

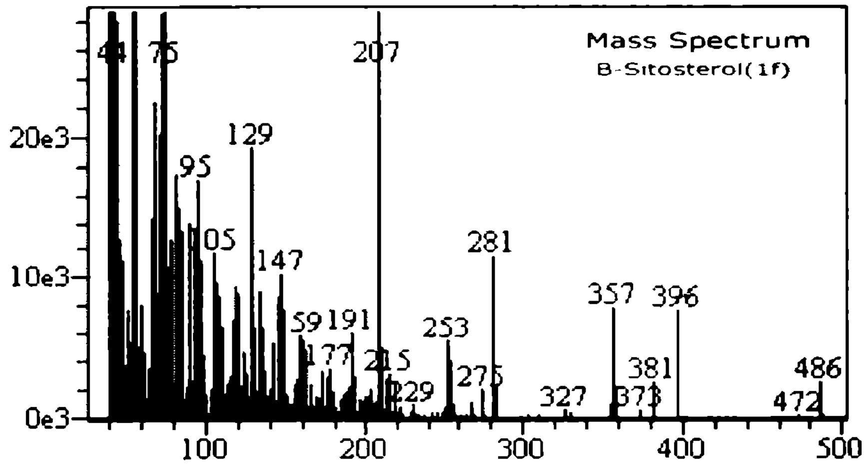
Appendix III

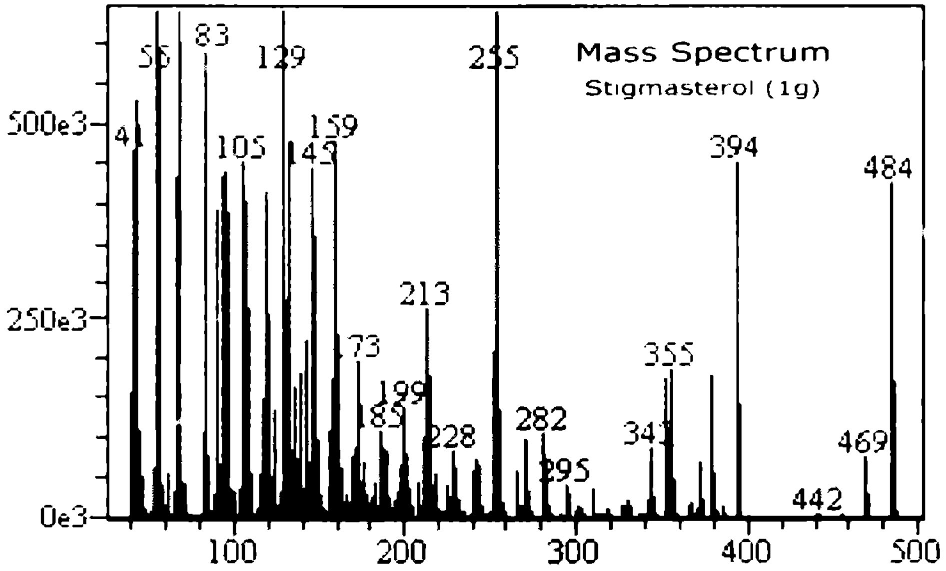
Sterol ether	Retention time	Relative retention time	Retention time -Std	Relative retention time std
1.Cholesterol	20.218	1	19.733	1
2.Cholestanol	21.52	1.0643		
3.Coprostanol	22.008	1.088		
4.Lathosterol	22.349	1.105		
5.Spongesterol	nd	nd		
6.Campesterol	25.623	1.2673	25.369	1.286
7.Stigmasterol	26.387	1.305	25.491	1.292
8.β-sitosterol	27.289	1.3497	26.548	1.345
9.Stigmastanol	28.451	1.407		
10.Lanosterol	31.140	1.541		
11.Isofucosterol	31.40	1.553		
12. Dihydro-agnosterol	33.85	1.674		

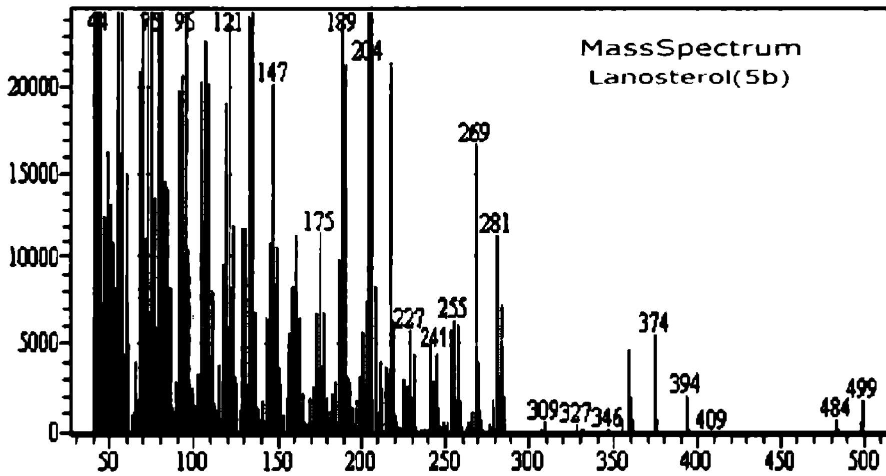
Appendix IV

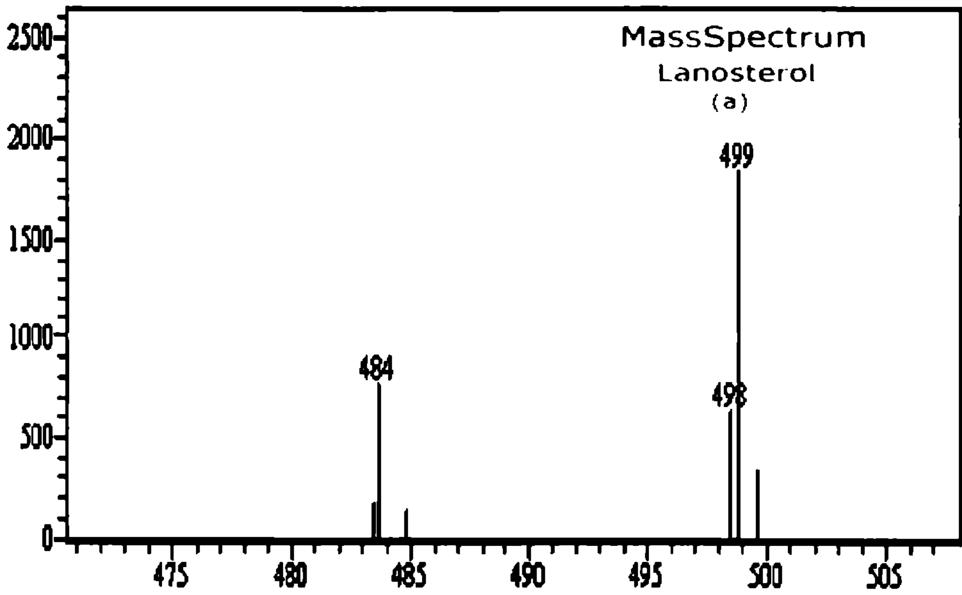
Mass spectra of selected sterols

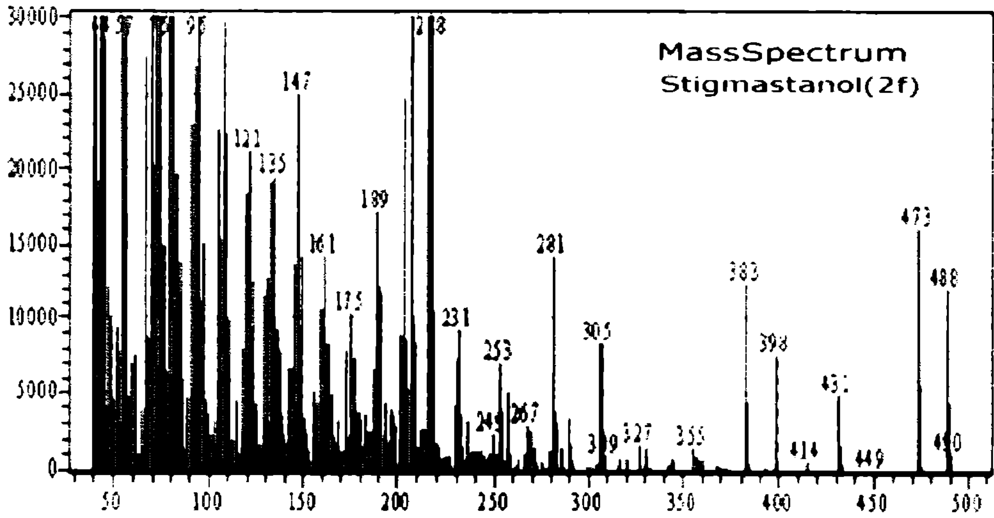


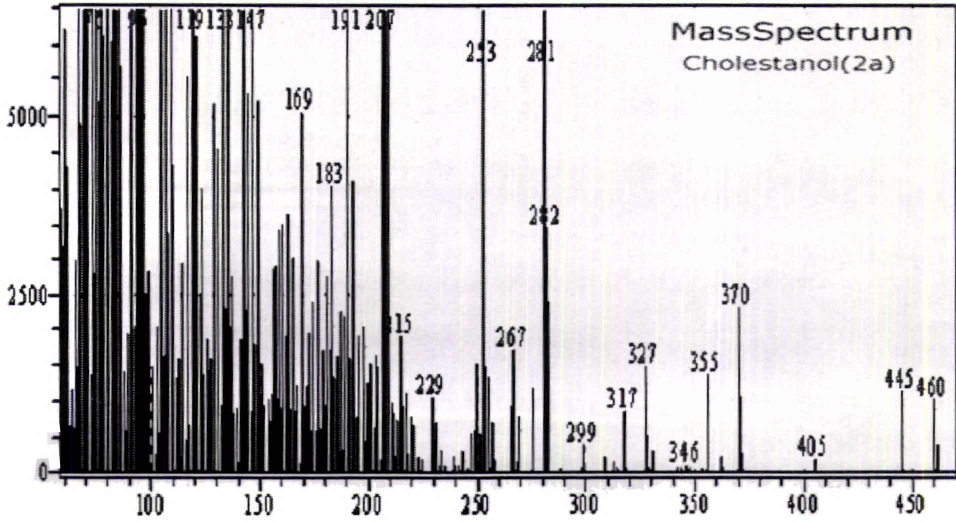



Mass Spectrum
Stigmasterol (1g)
83
105
159
213
228
282
355
394
484
469
442
500e3
250e3
0e3
100
200
300
400
500


MassSpectrum
Lanosterol(5b)
121
147
189
204
269
175
281
227
255
241
374
394
409
484
499
20000
15000
10000
5000
0
50
100
150
200
250
300
350
400
450
500


MassSpectrum
Lanosterol
(a)
499
484
498
2500
2000
1500
1000
500
0
475
480
485
490
495
500
505


MassSpectrum
Stigmastanol(2f)
147
189
281
231
253
305
383
398
473
488
30000
25000
20000
15000
10000
5000
0
50
100
150
200
250
300
350
400
450
500



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