BIOMINERAL LIPIDS IN LIVING AND FOSSIL MOLLUSCS.

by

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DECLARATION

I hereby certify that the work described in this thesis is my own, except where otherwise acknowledged, and has not been submitted previously for a degree at this, or any other, University.

Benjamin Stern

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ABSTRACT

It has been proposed that geochemical and biomolecular palaeontological information can be obtained from biomineral associated lipids. The location of lipidic material within the inorganic structure of molluscan shells has previously been unknown, with important implications for long term survival of lipids and post-depositional contamination from the environment. Discrete experimental stages have been investigated and the different mechanical and chemical methods combined for the removal of contaminating material prior to the release and analysis of surficial, intercrystalline and intracrystalline lipids. Three extraction protocols have been compared using Recent *Patella vulgata* shells. Sequential stages of cleaning and extraction treatments identify *n*-alkanes, cholesterol (free and bound) and bound fatty acids. The *n*-alkanes are indigenous to the shell, but laboratory contamination can be significant, and highlights the need for experimental blanks. Bound fatty acids are extracted from intercrystalline and intracrystalline fractions. Cholesterol is extracted throughout the sequential methodology. The extraction of these compounds after extensive cleaning treatments illustrates the protective role of the inorganic biomineral.

An experimental protocol for sequentially extracting protected lipids from the shells of Recent molluscs has been tested to distinguish the indigenous shell lipids from laboratory contamination and post-depositional ingress. The use of a calcium carbonate blank reveals the phthalate plasticisers extracted from the shells are due to laboratory contamination. Pristane, phytane and free fatty acids were rarely extracted which limits their use for interpretation. The *n*-alcohols, bound fatty acids, β-hydroxy fatty acids, cholesterol and other steroids are extracted from the shells in higher yields than the calcium carbonate blanks and are considered indigenous to the shells. Multivariate statistical analysis is used to compare the distributions of bound fatty acids and steroids extracted from different shell locations with the reported fatty acids and steroids for the soft tissues of the same species. The reported values for the soft tissues were used to indicate the original shell lipid composition. The shells lack the unsaturated bound fatty acids reported in the soft tissues. The saturated bound fatty acids of *Littorina littorea* shells also differ in the carbon number distributions to the reported saturated fatty acids of the soft tissues. Surficial shell extracts are characterized by steroidal ketones, representing sterols which have been oxidised by the cleaning treatments used. The steroids from both intercrystalline and intracrystalline shell locations in *Littorina littorea* are most similar to the soft tissues. However, the intercrystalline steroids are different to the intracrystalline steroids which may indicate a different original composition.

Potential Class level phylogenetic differences between the shells of Recent molluscs are revealed by their steroidal and bound saturated fatty acid compositions. The bivalves (n=3) have bound saturated fatty acids with a carbon number maximum of C_{16} whilst the gastropods (n=8) have a maximum of C_{16} or C_{18} and exhibit higher yields. β-hydroxy fatty acids may indicate phylogenetic differences below the Class level for the Gastropoda. Principal component statistical analysis of the shell steroidal composition indicates differences at the Class level. Steroidal markers indicating the dietary intake have been found in the shells.

The application of a methodology for the sequential extraction of lipids from molluscan shells has been used in a preliminary analysis of shell material for the presence of hydrocarbon pollutants. The shell *n*-alkanes require comparison of carbon number distributions and yields with an experimental calcium carbonate blank to ensure indigeneity. Different *n*-alkane distributions within two *Artica islandica* shell samples are attributed to the different sampling locations. Differences between *Patella vulgata* and *Littorina littorea* shells from the same environment have also been observed, indicating different *n*-alkane uptake by different species. Polyaromatic hydrocarbons and sterane biomarkers reported to be present in the soft tissues of *Patella vulgata* exposed to the *Braer* oil spill have been searched for in the shells of an exposed sample. These compounds have not been detected. No increase in the shell *n*-alkane yields or similarity in carbon number distribution with the spilt oil is observed. This suggests no hydrocarbon incorporation or depuration pathway into the shell.

Quaternary aged mollusc shells yield *n*-alkanes, *n*-alcohols, bound fatty acids and cholesterol. These have been extracted from both intercrystalline and intracrystalline locations within the shells. When compared with the extracts from Recent shells the yields of these lipids from fossil shells are significantly lower. The *n*-alkanes extracted from Quaternary shells are dominated by laboratory contamination, although some indigenous intracrystalline *n*-alkanes have been extracted. The bound fatty acids from intercrystalline sites within the fossils maintain their carbon number distribution but decrease in yields with increasing age; no diagenetic products were observed. The previously reported phylogenetic distinctions based on the bound fatty acids between the gastropods and bivalves are maintained for fossils. However, the information obtained from this analysis is limited by the small diversity of lipid distributions found in these fossil shells.

INTRODUCTION

Lipids in geochemistry

The study of the widespread organic compounds detected in the geosphere has led to the successful understanding of the generation and maturation of oil and coals, the biological origins and degradation of these organic compounds and the fractionation of different isotopes during these processes. The organic compounds detected in the geosphere originate from molecules synthesised by living organisms. It is the preservation of the carbon skeleton and stereochemistry of the fossil molecules, with no or small changes during the translocation from the living organism to the sediment, which can lead to the identification of the original source of these biological markers (also known as chemical fossils). The major sources of these compounds into the geosphere are bacteria, phytoplankton, zooplankton and higher plants. Lipids are defined as being soluble in commonly used laboratory solvents, such as dichloromethane and methanol. In geochemical studies it is generally the hydrocarbons, originating from lipids, which are used as biological markers (see Tissot & Welte, 1984; Engel & Macko, 1993 for a review). Biological markers are used in the field of geochemistry for the reconstruction of depositional environments, to correlate oil to oil and oil to source rocks, and are also used as indicators for early diagenesis and thermal maturation in the sediment.

The information content of hydrocarbons and lipids is less than that of other biological molecules such as proteins and DNA. However, the hydrophobic nature of lipids leads to enhanced preservation in the geological record; lipids do not require the exceptional conditions needed for the preservation of the more vulnerable proteins and DNA (Eglinton & Logan, 1991). The variability of the hydrocarbon and lipid biological markers is generally due to carbon chain branching and carbon number distribution for those forming homologous series (e.g. *n*-alkanes and fatty acids) and the individual compounds present (e.g. steroids).

Geochemistry of macrofossils

Most geochemical studies examine lipids incorporated into rocks and sediment. The direct association of biological markers with the hardparts of specific organisms is explored in the field of molecular palaeontology (Curry, 1987). It is these hardparts, such as molluscan and brachiopod shells, corals, bones and teeth which directly associate the organic material contained within these structures to the once living animal. The application to taxonomy of this fossil material has focused on the proteins (e.g. Lowenstein & Scheuenstuhl, 1991; Robbins *et al.*, 1993) and DNA (e.g. Marshall,

1988). However, hydrocarbon or lipid biological markers are also extracted from a variety of macrofossils yielding *n*-alkanes, fatty acids, phytadienes, ketones, cholesterol and other steroids. (Thompson & Creath, 1966; Das & Smith, 1968; Ivanov & Stoyanova, 1972; Ivanov *et al.*, 1975; Stoyanova *et al.*, 1980; Stoyanova, 1984; Curry *et al.*, 1991; CoBabe & Pratt, 1995; Evershed *et al.*, 1995; Logan *et al.*, 1995). The extraction of lipids from the hardparts of Recent or living samples has not been examined in such detail.

General description of molluscs and brachiopods

The phylum Mollusca has been chosen for examination in this study of lipids in macrofossils. These animals use controlled biomineralization to form a shell. The molluscs are classified as the second largest phylum within the animal kingdom (the insects having the largest number of species). Seven classes are within the Mollusca, of these the class Bivalvia (also known as Lamellibranchia and Pelecypoda) possesses two interlocking shells, whilst the class Gastropoda includes species with a single shell which is often coiled (see Tebble, 1966; Campbell, 1976; Graham, 1988). The taxonomy of some species are in a state of flux. Traditional techniques based on the morphology of the animal are now being combined with more recent molecular techniques, such as DNA sequencing, to ascertain the relationships between extant species.

One fossil brachiopod has been examined in this study. Members of the phylum Brachiopoda (sometimes called lamp-shells) have a shell morphology similar to the molluscan class Bivalvia but with shell symmetry perpendicular to the two valves, as opposed to a plane of symmetry which runs between the valves in bivalves (Rudwick, 1970).

Molluscs form a diverse group of animals. Habitats vary from terrestrial to freshwater and marine, whilst trophically they range from filter feeders, grazing herbivores to carnivores. Molluscs are a major component of the fossil record; some sediments are almost entirely molluscan shell fragments (Scoffin, 1987, page 22). The geological history of molluscs and brachiopods extends back to the Cambrian (570 Ma). The evolution of both groups has also been well studied.

Biomineralization

Phospholipids have been suggested to have a rôle in the process of biomineralization (Wuthier, 1973; Abolinš-Krogis, 1979), arising from their strong binding affinity for Ca^{2+} (Isa & Okazaki, 1987). The electrostatic attraction between Ca^{2+} and the carboxylic functionality of aspartic acid in shell matrix proteins suggests that the matrix acts as a template for mineralization (Weiner & Hood, 1975); similarly the free fatty acids may be electrostatically attracted to the biomineral. The polar lipids (such as those with alcohol functionalities) may also be attracted to the biomineral. Hydrophobic attraction between lipids and the hydrophobic matrix proteins may result in the lipid incorporation of lipids without functional groups.

Molluscan shells are complex layered structures consisting of calcium carbonate with a mean of 4.3% organic content (Price *et al.*, 1976). The shells increase in size with animal growth, but can be partially dissolved and remodelled by the animal (Lowenstam & Weiner, 1989, page 109). Shell formation is determined by phylogeny and takes place under cellular control. A specific organ, the mantle, is directly responsible for biomineralization. To achieve precipitation of the mineral, supersaturation of the ions to be deposited and nucleation of the crystals are required. The phylogenetic control defines the polymorph of calcium carbonate (generally calcite or aragonite), the alignment, size and shape of crystals which form the shell microstructure. A shell will often consist of a variety of crystal microstructures and both calcite and aragonite polymorphs (Carter, 1990a and 1990b). The external surface of the shell is covered in periostracum, which is a cross-linked proteinaceous layer. See Fig. 1 for representation of bivalve shell and soft tissues.

In general terms, biomineralization starts with the deposition of an organic matrix. The constituent macromolecules of the matrix are secreted by the outer cell layer of the mantle, the mantle epithelium, into the enclosed extracellular space between the mantle and the surface of the growing shell which is filled with extrapallial fluid. The matrix then acts as a substrate for mineral growth in the supersaturated extrapallial fluid. Other macromolecules are thought to be involved in regulating the mineralization process, either initiating or inhibiting crystal nucleation on specific crystal faces, and therefore directing crystal growth. The organic matrix and macromolecules are principally proteinaceous (see Lowenstam & Weiner, 1989; Mann *et al.*, 1989; Simkiss & Wilbur, 1989 for a review).

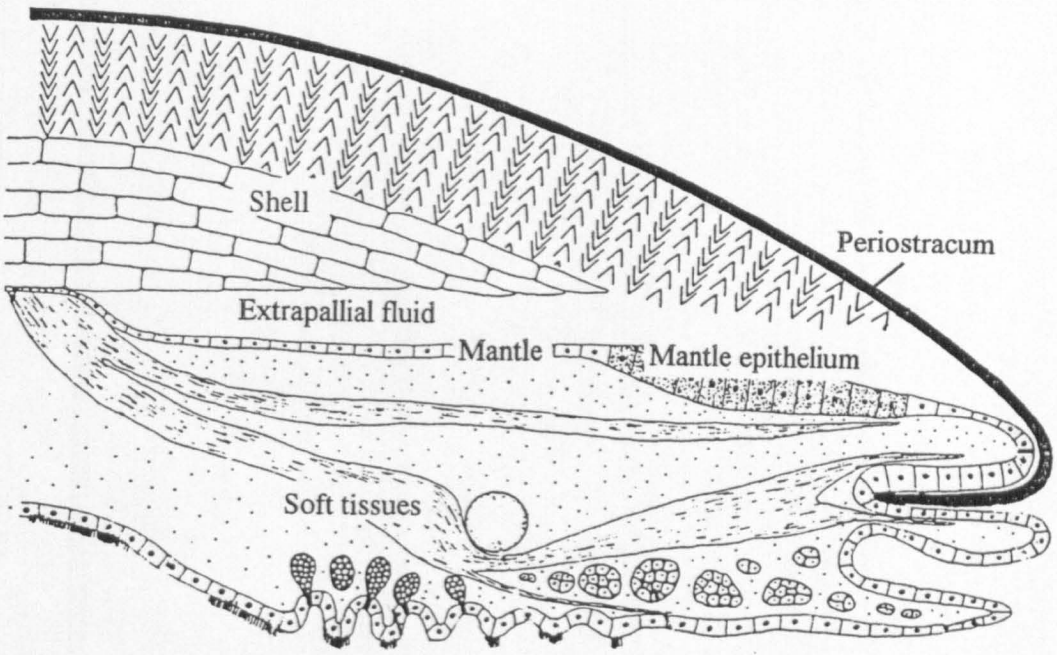


Figure 1. Idealised section through a bivalve valve showing both shell and soft tissues. Two different shell microstructures are indicated. Adapted from Simkiss & Wilbur (1989).

Intercrystalline and intracrystalline shell locations

Two distinct locations for organic material within the shell have been proposed, defined by their relationship to the inorganic crystals. A cartoon representing the location of organic material within the shell is shown in Fig. 2. Intercrystalline organic material is found surrounding the crystallites (Watabe, 1963; Lowenstam & Weiner, 1989). This is accessible to the external environment and makes the shells susceptible to non-indigenous contamination deposited into the shell; in addition there is the potential for loss of indigenous shell lipids from the shell. Intracrystalline organic material is occluded within the crystals (Crenshaw, 1972; Towe & Thompson, 1972; Towe, 1980 page 71). Organic material trapped at the crystal boundaries and imperfection sites, as suggested by Berman *et al.* (1993), is also considered here as intracrystalline. Towe (1980) suggested that the intracrystalline shell locations offered the best potential for the preservation of indigenous organic material. The different properties of these different shell locations have not been fully appreciated in previous studies of biomineral associated lipids.

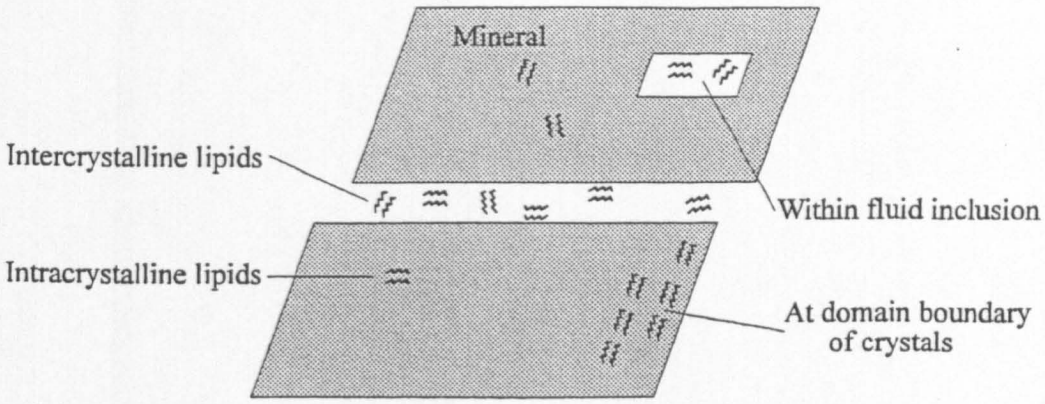


Figure 2. Diagrammatic representation of intercrystalline and intracrystalline shell locations.

Lipids in the soft tissues

Lipids contained within the shell are likely to originate from the soft tissues. The lipidic composition of molluscan soft tissues is thought to be related to a variety of factors such as diet, temperature, water depth, sex, sexual cycle, tissue type and phylogeny (Pollero *et al.,* 1979; Joseph, 1982; Piretti *et al.,* 1987). Molluscs are capable of *de novo* synthesis as well as incorporating lipids from their diets and altering this intake. These lipids are esters such as glycolipids, phospholipids, steryl esters and their free components such as fatty acids and sterols (Joseph, 1982).

Triglycerides (also known as triacylglycerols or neutral fats) are triesters of glycerol and act as an energy reservoir in animals, these lipids are therefore the most abundant lipid class. They are not used as membrane components. The fatty acid constituents can vary and are identified by their attachment position to the glycerol. Fig. 3a shows a generalised triglyceride structure. The structure of phospholipids (also known as glycerophospholipids or phosphoglycerides) is shown in Fig. 3b. These are major components of membranes. Phospholipids are polar due to the "head" group X, which can vary in its composition (see Voet & Voet, 1990, page 275). Sphingolipids are also major membrane components, the structure is shown in Fig. 3c, and a variety of X groups are similarly found (Joseph, 1982; Voet & Voet, 1990).

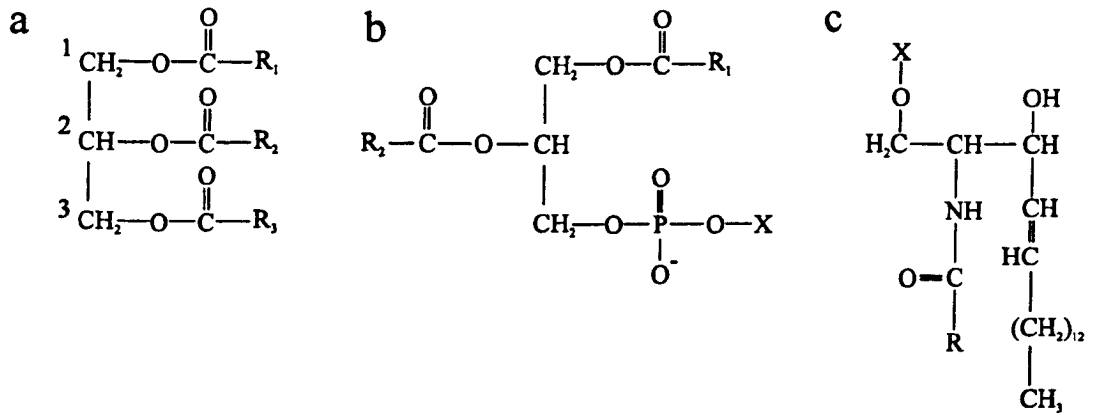


Figure 3. Generalised chemical structures of a) triglyceride, b) phospholipids and c) sphingolipids. A variety of "head" groups is indicated by X.

Hydrocarbons are also extracted using the same techniques as for other lipids and these components have a potential use as indicators of anthropogenic pollution. The soft tissues have been shown to bioconcentrate pollutants and molluscs have been used as sentinel organisms for organic pollution (Goldberg, 1980).

Scope and framework of thesis

This thesis consists of five chapters. Each is intended to be independent and is presented in the style of a scientific paper. It is proposed to submit these chapters for publication in a similar style to their current form. For the purpose of this thesis the references from each chapter are combined at the end of the thesis. The abstracts are combined at the front of this thesis and individually at the start of each relevant chapter. Appendices containing additional information not pertinent to a paper are provided at the end of the thesis. The development of the experimental protocols is described in Chapter 1, these are additionally described in Chapter 2 and have been briefly summarised in the later chapters. To avoid confusion between samples, the numbers assigned to the five experimental batches and those assigned to repeated analyses of shells of the same species are retained throughout this thesis.

Chapter 1 compares the effectiveness of different chemical cleaning techniques for the removal of organic material from the surface of Recent and live molluscan shells. These initial cleaning treatments are also compared to mechanical cleaning from a previously published methodology (CoBabe & Pratt, 1995). For the extraction of lipids from successively more protected locations within the shells (surficial, intercrystalline, intercrystalline bound, intracrystalline free and intracrystalline bound), two experimental protocols (A and B) have been developed here; both are used throughout this thesis. Initial investigations used experimental protocol A, which was limited by having no

quantification and yielding irreproducible yields of cholesterol. However, small scale experiments using protocol A showed that the extracted *n*-alkanes and bound fatty acids were unaffected and these data are therefore used. Experimental protocol B amends the problems found with protocol A, this allows quantification and some solvent extraction stages have been altered to increase the ease and speed of sample extraction. The two protocols (A and B) and a previously published methodology (CoBabe & Pratt, 1995) are compared using Recent *Patella vulgata* shells.

Chapter 2 examines the phthalate plasticisers, *n*-alkanes, pristane and phytane, free and bound fatty acids, β-hydroxy fatty acids, cholesterol and other steroids extracted from Recent shells. These compounds are tested to distinguish the indigenous shell lipids from laboratory contamination and post-depositional ingress and therefore this chapter examines the potential use of these compounds for further study. The bound fatty acids and steroidal components from *Littorina littorea* shells are compared to the lipids reported from the soft tissues of the same species. The reported values for the soft tissues are used to indicate the original shell composition and therefore the shell locations offering the best preservation of indigenous lipids are found.

Chapter 3 examines the bound fatty acids and steroidal components which are indigenous to the shell and thought to be of potential phylogenetic and dietary use. Class level phylogenetic differences are examined using both the bound fatty acids and the steroids. The steroidal components are also used as biomarkers to indicate the dietary intake of the molluscs.

Chapter 4 examines the shell *n*-alkanes, thought to indicate anthropogenic organic pollution. Although laboratory contamination is a major problem, the gradual accumulation of these compounds in the shell from chronic pollution is considered. Additionally, an example of live *Patella vulgata* shells exposed to the *Braer* oil spill has been analysed. Polyaromatic hydrocarbons reported in the soft tissues of the same sample, and other hydrocarbon biomarkers were searched for in the shell.

Chapter 5 examines the lipids in Quaternary aged fossil mollusc (and one brachiopod) shells sampled from New Zealand. *n*-Alkanes, bound fatty acids and cholesterol are found in these fossil shells. The lipids yielded from the fossil samples are compared to those extracted from Recent shells. The use of biomineral associated lipids for answering phylogenetic, environmental and diagenetic questions and their use to the palaeontologist is considered.

CHAPTER 1. DEVELOPMENT AND COMPARISON OF DIFFERENT METHODS FOR THE EXTRACTION OF BIOMINERAL ASSOCIATED LIPIDS.

ABSTRACT

It has been proposed that geochemical and biomolecular palaeontological information can be obtained from biomineral associated lipids. The location of lipidic material within the inorganic structure of molluscan shells has previously been unknown, with important implications for long term survival of lipids and post-depositional contamination from the environment. Discrete experimental stages have been investigated and the different mechanical and chemical methods combined for the removal of contaminating material prior to the release and analysis of surficial, intercrystalline and intracrystalline lipids. Three extraction protocols have been compared using Recent *Patella vulgata* shells. Sequential stages of cleaning and extraction treatments identify *n*-alkanes, cholesterol (free and bound) and bound fatty acids. The *n*-alkanes are indigenous to the shell, but laboratory contamination can be significant, and highlights the need for experimental blanks. Bound fatty acids are extracted from intercrystalline and intracrystalline fractions. Cholesterol is extracted throughout the sequential methodology. The extraction of these compounds after extensive cleaning treatments illustrates the protective role of the inorganic biomineral.

INTRODUCTION

Studies of organic material associated with Recent or fossil biominerals have generally not considered the location and environment of the organic material within the biomineral (Thompson & Creath, 1966; Das & Smith, 1968; Ivanov & Stoyanova, 1972; Ivanov *et al.*, 1975; Stoyanova *et al.*, 1980; Stoyanova, 1984; Curry *et al.*, 1991; CoBabe & Pratt, 1995; Evershed *et al.*, 1995). In molluscan shells, an intercrystalline organic matrix is found surrounding the crystallites (Watabe, 1963; Lowenstam & Weiner, 1989). This is accessible to the external environment which makes the indigenous shell organic material susceptible to ingress of exogenous material, digestion by endobionts and migration from the shell. Intracrystalline organic material is occluded within the crystals (Crenshaw, 1972; Towe & Thompson, 1972; Towe, 1980). Organic material trapped at the crystal boundaries and imperfection sites, as suggested by Berman *et al.* (1993), is also defined here as intracrystalline. The best potential for the preservation of indigenous organic material is at intracrystalline shell locations (Towe, 1980, page 71). Intracrystalline proteins have been described (Addadi & Weiner, 1989), but there have been few such studies of the less abundant

lipids (e.g. Clegg, 1993). In the study of molluscan shell lipids, the removal of non-biomineral lipids is the first essential task. Mollusc shells collected from a Recent environment can vary widely in age; over the animals lifetime, which can be greater than 200 years (Heller, 1990), and up to 10,000 years post-mortem (Kidwell & Bosence, 1991). This makes both the type, extent and duration of contamination extremely variable. Organic contamination can originate in three major ways; a) from the animal itself, such as the remains of soft tissues and ligaments on the interior and periostracum on the exterior surfaces of the shell, b) epibionts attached to the shell can contribute extraneous inorganic and organic material on and within the shell structure. Burrowing endobionts such as algae and fungi can penetrate deep within the shell leaving a network of tunnels and chambers (Goulbic *et al.*, 1975; Lukas, 1979). c) These processes greatly increase the exposed surface of the biomineral which is available for the selective adsorption of lipidic material from the surrounding environment (Suess, 1970; Zullig & Morse, 1988).

Previous attempts to remove shell organic material have highlighted a number of analytical problems and techniques for the removal of this contamination. None of these commonly used techniques for the removal of organic material from biogenic carbonates are problem free or totally effective when used in isolation.

The mechanical cleaning methods of abrading shells in a ball mill, causing fracturing along weakened areas caused by microborers, and after discarding the fine fragments a 10-15% partial decalcification to etch the carbonate surface have been employed by Hoering (1980). Mechanically removing the exterior surface, the hinge and muscle attachments of bivalve shells with a drill mounted steel brush has been used by CoBabe and Pratt (1995). Physical removal by heating biogenic carbonates above 100°C caused loss of water and organic material, although alteration of the aragonite generally occurred above 150°C; such a technique is known to have a destructive effect on the organic material (Gaffey *et al.*, 1991).

Chemical cleaning treatments are an alternative to mechanical removal of contamination from the surfaces of the shells. Amino acids adsorbed onto the calcitic surfaces of foraminiferal tests were removed by the use of hypochlorite (Stathoplos & Hare, 1993); different amino acid compositions were also found between the free adsorbed and chemically inaccessible fractions. In a comparison of the effects of hydrogen peroxide, sodium hydroxide and hypochlorite, full strength (5% active chlorine) hypochlorite treatment was recommended for the removal of all types of organic material with the minimum damage to the inorganic structure (Gaffey &

Bronnimann, 1993). The dissolution effect of hypochlorite on carbonate is less than that of deionised water (Pingitore *et al.*, 1993). However, changes in the inorganic elemental composition using hypochlorite and also with heating in sodium hydroxide were observed by Love and Woronow (1991). Treatment with boiling sodium hydroxide was found to remove carbonate, and that replacement was occurring with the deposition of calcium hydroxide (Pingitore *et al.*, 1993). The use of sonication with hypochlorite treatment for the removal of the organic matrix from bones and the subsequent disaggregation of the inorganic crystals has been recommended by Weiner and Price (1986).

Lipids are defined as being soluble in commonly used organic solvents, such as dichloromethane and methanol. For lipid extraction from solids, sonication of the shells immersed in solvent and Soxhlet extraction have been used (CoBabe & Pratt, 1995). However some lipids are either bound into insoluble organic material, or are physically trapped within the organic matrix of the shell. Saponification (hydrolysis under alkaline conditions), is used to release this material by breaking ester bonds, phosphate ester bonds, the amide bonds of ceramides (Klein & Kemp, 1977) and peptide bonds. This results in constituents with carboxylic acid and alcohol functional groups.

Three extraction protocols using *Patella vulgata* shells are compared for the extraction of lipids from different locations within molluscan shells. These combine some of the cleaning and lipid extraction techniques used by other workers. This results in the sequential extraction of surficial, free and bound intercrystalline, free and bound intracrystalline shell lipids and allows a comparison of the organic components in different shell locations. As the yields of lipidic material in shells are low, and lipids are ubiquitous in both the environment and the laboratory (Middleditch, 1989), the pragmatic removal of contamination where possible, with the use of experimental blanks provides a knowledge of contaminant types, distributions and yields. These are compared to the lipids extracted from the shells.

MATERIALS

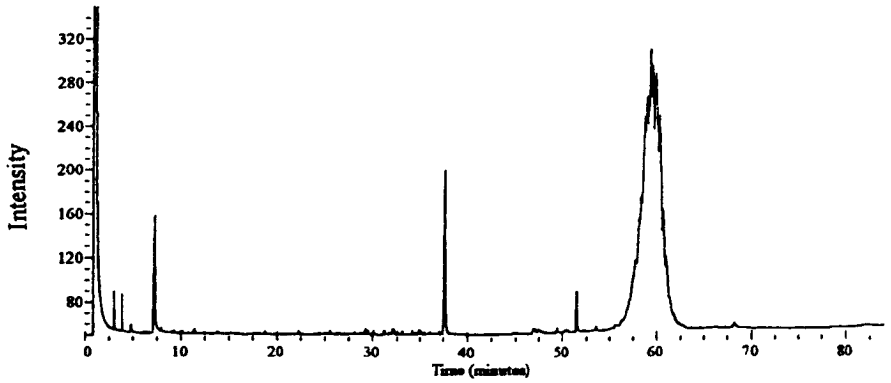
Live specimens of *Modiolus modiolus* were dredged at approximately 200 m water depth from the Firth of Lorne, Scotland. Until they were killed, the animals were held in tanks with fresh sea water for five days at the Dunstaffnage Marine Research Laboratories, Oban. The shells were separated from the soft tissues with a scalpel, rinsed in water and then dried in the atmosphere. Recent *Patella vulgata* shells were collected from the shoreline at Cullercoats Bay, Northumberland, England (Ordnance survey 1:50000 Landranger series sheet 88, 364713).

The following authentic and analytically pure standards were used in this study; *n*-nonadecane (C_{19} *n*-alkane), *n*-hexatriacontane (C_{36} *n*-alkane), 1-triacontanol (C_{30} *n*-alcohol), heptacosanoic acid methyl ester (C_{27} FAME), cholesterol and 20(29)-lupen-3B-ol (lupeol). (Selected mass spectra are shown in Appendix 1). They are commercially available from Sigma, except cholesterol (BDH) and the *n*-nonadecane was from an unknown source. Purity was tested by the gas chromatographic analysis of a standard solution.

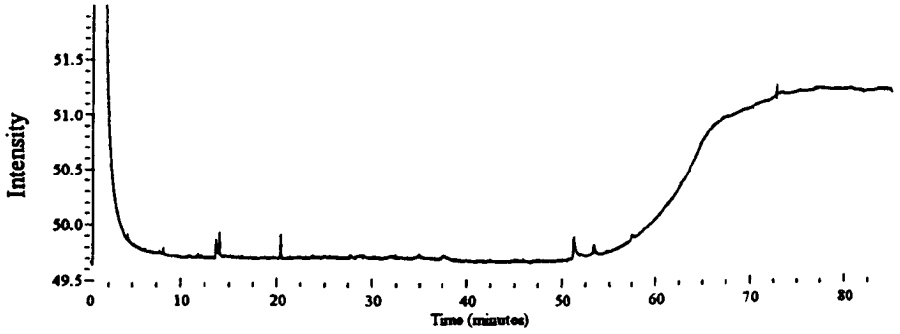
Removal of contamination from laboratory reagents

The low level of recovered lipids from the shells requires that organic contamination be minimised. This requires solvent extraction of reagents, careful laboratory protocol and a system for the characterization of distributions and yields of the background contamination. The composition and distribution of contamination from the laboratory and reagents is batch dependent, but generally includes abundant long chain *n*-alkanes and phthalate plasticisers. Fig. 1 shows gas chromatograms of dichloromethane (DCM) (Fig. 1a, 1b), hydrochloric acid (HCl) (Fig. 1c, 1d) and potassium hydroxide (KOH) (Fig. 1e, 1f) before and after the removal of contamination. The removal of organic contamination from solvent is achieved by distillation on a 30 plate Oldershaw column. Solvent purity was tested by taking 100 ml from each 2.5 l distillate, evaporating the solvent under reduced pressure with analysis by gas chromatography (GC). HCl (BDH, AnlaR 35.4%), 10M KOH solution (BDH, general purpose reagent, pellets) and laboratory distilled water were liquid-liquid extracted four times in a separating funnel with an equivalent volume of DCM. The fourth solvent extract was analysed as for the distilled solvent. The cellulose thimbles, cotton wool and alumina (aluminium oxide, BDH) were Soxhlet extracted with DCM / methanol (93 : 7 v/v) for 24 h. Glassware was cleaned by immersion in chromic acid (30 g $Na_2Cr_2O_7$ per litre of concentrated H_2SO_4) for a minimum of 4 h., rinsed in distilled water, dried and rinsed with the appropriate solvent three times prior to use. To avoid laboratory cross contamination, glassware was kept aside for the use with this project alone.

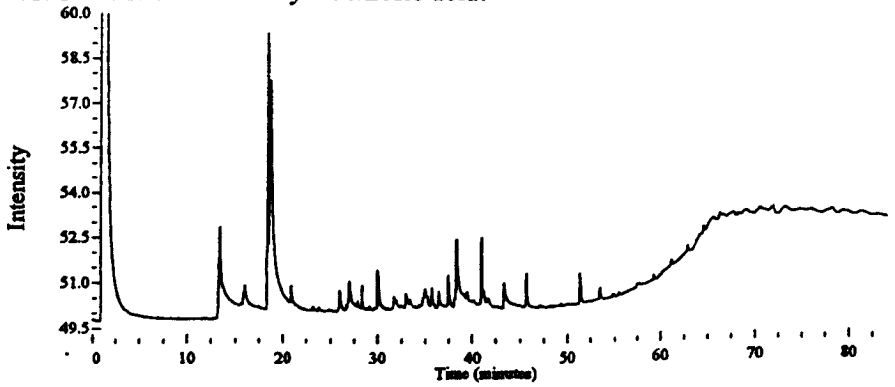
a) Undistilled dichloromethane



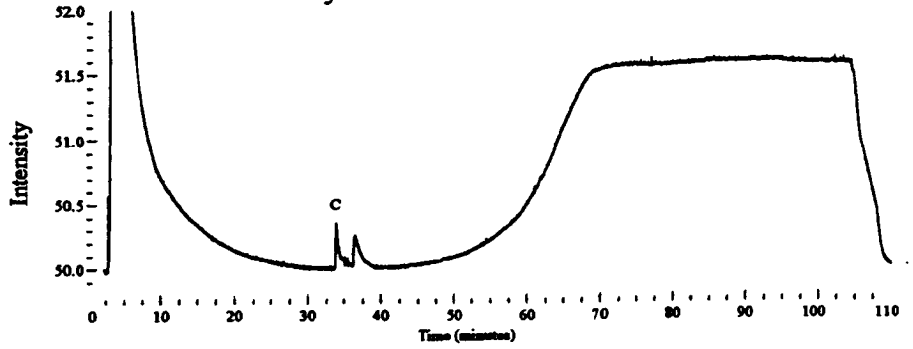
b) Distilled dichloromethane



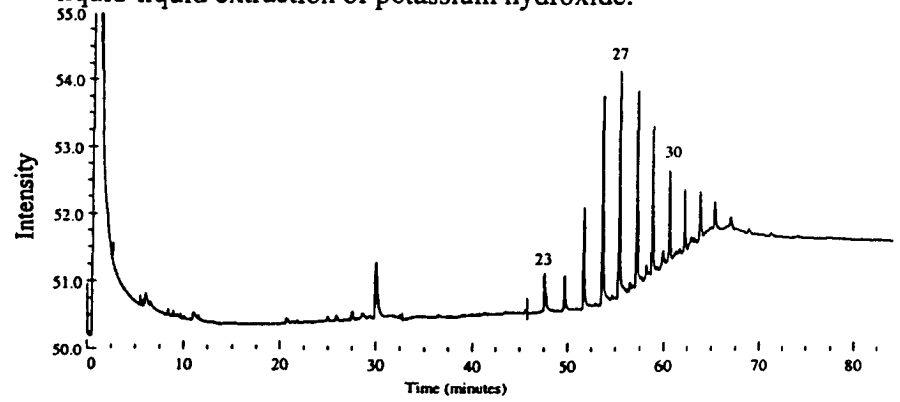
c) First solvent extract of hydrochloric acid.



d) Fourth solvent extract of hydrochloric acid.



e) First liquid-liquid extraction of potassium hydroxide.



f) Fourth liquid-liquid extraction of potassium hydroxide.

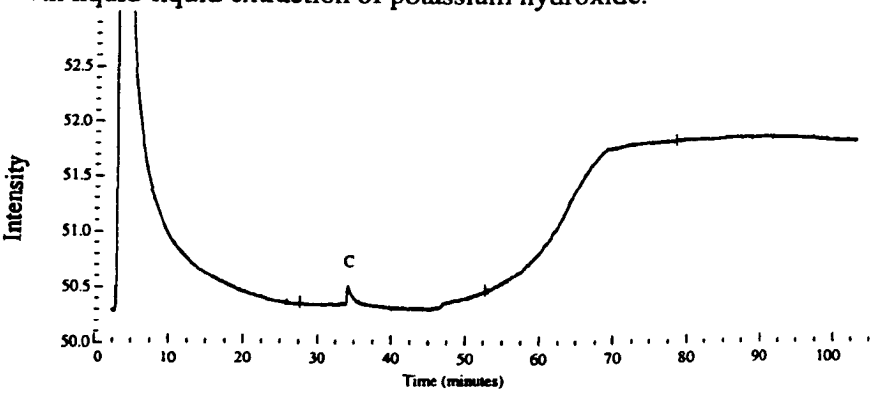


Figure 1. Gas chromatograms (retention time verses detector response) for laboratory reagents before and after removal of contamination. Numbers indicate *n*-alkane carbon numbers, c = contamination due to GC analysis only, other peaks are unidentified.

Extract preparation

All sample and calcium carbonate blank extracts were prepared by removing solvent with a rotary evaporator at 30°C, and further concentrated under a stream of nitrogen at room temperature. Total extracts were derivatised with excess (*ca* 20 µl) N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Fluka) (see Evershed, 1993) overnight, with excess reagent removed under a stream of nitrogen prior to analysis.

Gas chromatography / gas chromatography - mass spectrometry

A Carlo-Erba 5160 fitted with an OV-1 coated (0.4 µm thickness) glass column (25 m x 0.32 mm i.d.) was used for GC analysis of reagents. This was equipped with an on-column injector and a flame ionization detector (FID). Hydrogen was used as the carrier gas, cold on-column injection was used and the FID maintained at 310°C. The temperature of the oven was programmed from 50°C (2 min.) to 300°C (20 min.) at 4°C / min. For sample analysis a Carlo-Erba HRGC Mega series was employed, fitted with a HP-5 coated (0.25 µm film thickness) fused silica column (60 m x 0.25 mm

i.d.). This was equipped with an on-column injector and a FID. Hydrogen was the carrier gas, cold on-column injection was used and the FID maintained at 310°C. The temperature of the oven was programmed from 50°C (2 min.) to 300°C (40 min.) at 4°C / min. Data were collected using Multichrom (VG Data Systems, Micro Vax 3100).

Gas chromatography combined with mass spectrometry (GC-MS) was carried out using a Fisons 8060 GC connected to a Fisons Trio1000 mass spectrometer. The GC was equipped with an on-column injector and fitted with a HP-5 coated (0.25 μm film thickness) fused silica column (30 m x 0.25 mm i.d.) with 2 m deactivated fused silica 0.32 mm i.d. retention gap. Cold on-column injection was used and helium was the carrier gas. The temperature of the oven was programmed from 50°C (2 min.) to 150°C at 10°C / min. then to 300°C (25 min.) at 4°C / min. The column was directly inserted into the ion source. Electron impact spectra were obtained at 70 eV with full scan from 50 to 650 m/z, cycle time 1 s.

METHOD DEVELOPMENT

A comparison of three different extraction protocols (A, B and C in Fig. 2) using *Patella vulgata* shells from the same location was carried out. These protocols combine different methodological stages. The development of individual treatments are discussed below. The resulting extracts have been named according to Fig. 2.

1) Comparison of methods for the removal of macroscopic contamination from the surface of the shells

Hypochlorite and potassium hydroxide cleaning treatments

The effects of two different chemical cleaning methods are illustrated with the live *Modiolus modiolus* shells. These samples contained residual soft tissues, periostracum and epibionts. Four valves were placed in 2M KOH and four valves in hypochlorite (<5% available chlorine) for 24 h. The shells were then rinsed and left to soak in distilled water for a further 24 h. The residual KOH and hypochlorite solutions were each liquid-liquid extracted three times with 100 ml of DCM. Two valves from each treatment and two untreated valves were 20% decalcified in a stoichiometric amount of dilute hydrochloric acid. Vigorous stirring ensured an even decalcification of the surface.

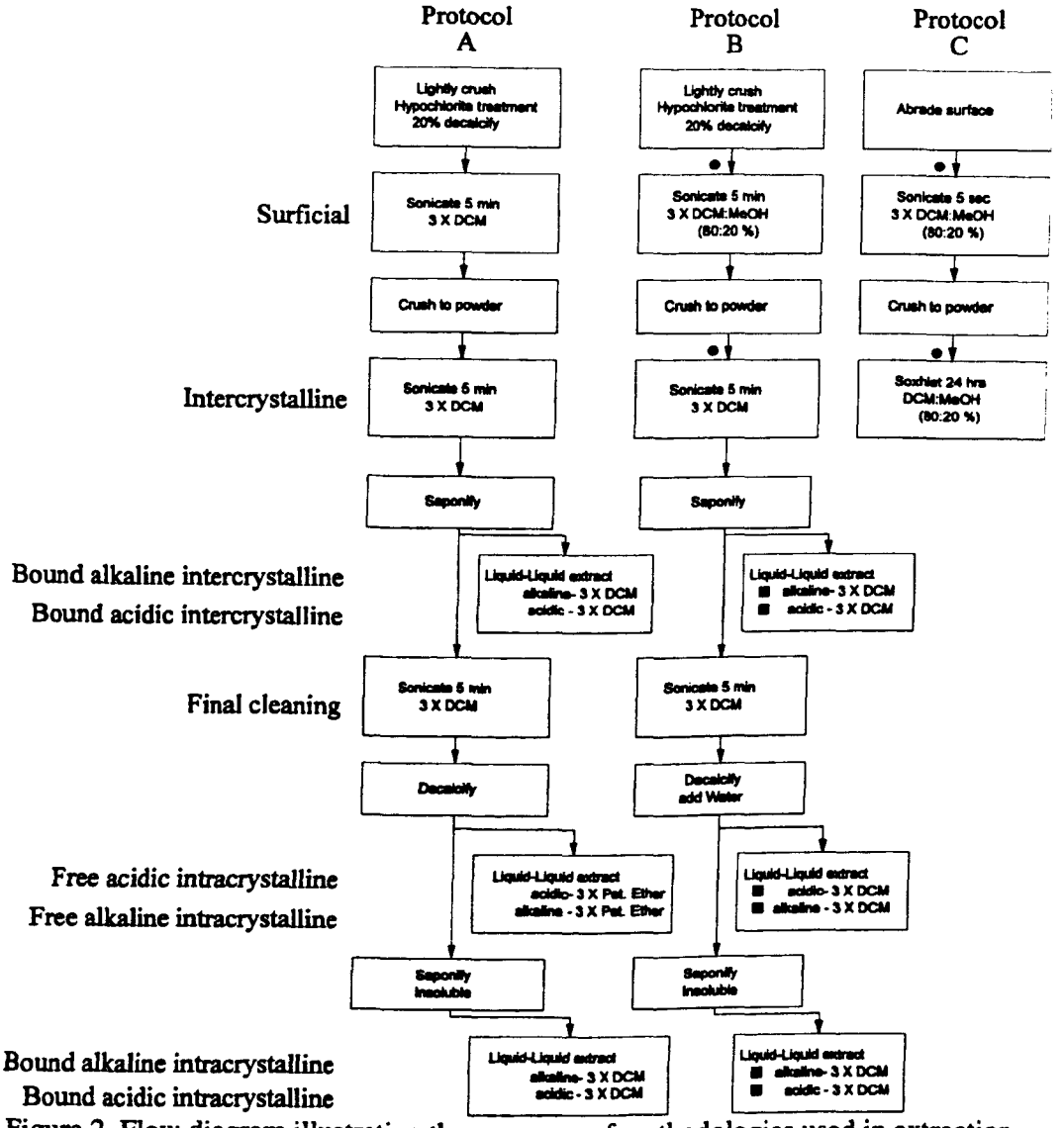


Figure 2. Flow diagram illustrating the sequence of methodologies used in extraction protocols A, B and C. Extract names apply throughout the text as on this figure. The addition of internal standards before solvent extraction is indicated by; ● = lupeol, ■ = C_{36} *n*-alkane.

Mechanical cleaning treatments

The entire inner and outer surfaces of *Patella vulgata* shells were mechanically cleaned using a hand held abrasive bit. This was found to be time consuming and a potential source of contamination due to excessive handling. Furthermore this method precluded the use of the powdered calcium carbonate blanks, was difficult to apply to fragile and coiled shells and did not completely remove endoliths which could be clearly seen in the abraded shells.

Shell mineralogy

To ensure that the shell mineralogy and microstructure were unaffected by the chemical cleaning treatments, subsamples of calcium carbonate blanks, original and treated shells were analysed by X-ray diffraction (XRD). Powdered samples were analysed on a Sietronics PW1710 diffractometer scanning over a 3° to 80° range with 0.02° steps, wavelength 1.540562Å (Cu) and a Kα strip of 0.5. The mineralogy was identified by reference to data given by Berry (1967). Selected shells were also gold coated using a Polaron E5000 100 with a cool head and scanning electron microscopy (SEM) analysis was carried out using a Cambridge instruments stereoscan 240.

The results from these initial tests led to the development of the methodologies used in extraction protocols A and B. The shells were scraped to remove any encrusting organisms, lightly crushed to *ca* 1 cm^2 fragments, then covered in hypochlorite (Clorox brand, >8% available chlorine) with two changes over three days and occasional sonication. The shells were then rinsed and soaked in distilled water for 24 h. This was followed by a 20% decalcification using a stoichiometric amount of dilute hydrochloric acid with vigorous stirring. Protocol C follows the methods described by CoBabe and Pratt (1995) and the mechanical cleaning method is compared with the chemical cleaning techniques developed here in extraction protocols A and B.

2) Solvent extraction of carbonate surfaces

To test the effectiveness of lipid extraction from a calcium carbonate surface, a standard solution consisting of C_{19} *n*-alkane (0.234 mg), C_{27} FAME (0.227 mg), cholesterol (0.13 mg) and lupeol (0.338 mg) was made up to 100 ml in a volumetric flask. 1 ml of this standard mixture was added to 10 g of inorganic calcium carbonate, the solvent was left to evaporate and the carbonate was extracted with three aliquots of 50 ml DCM with 5 min. sonication.

3) Effects of alumina and cotton wool

To remove small particles and aqueous droplets from the shell extracts in protocol A, the concentrated extracts were passed through a small volume of activated alumina over a plug of cotton wool in a Pasteur pipette. To test the effect of this treatment on the lipid composition, 1 ml portions of the standard solution used above, were passed through alumina over a plug of cotton wool and through cotton wool only. The results from these two experiments were compared to aliquots of the untreated standard solution.

In protocol A all extracts were passed through a small volume of activated alumina, in B and C the extracts were passed through a cotton wool plug only.

4) Inorganic calcium carbonate blanks

The time taken for the sequential extraction of each experimental batch of samples is approximately 6 to 8 weeks. This increases the potential for laboratory contamination. Therefore to provide a knowledge of contaminant types, distributions and yields in protocols A and B, an inorganic calcium carbonate blank was examined with each experimental batch of 3 to 5 shell samples. For protocol C no calcium carbonate blank was used, similar to the method proposed by CoBabe and Pratt (1995). The use of the powdered calcium carbonate blank to simulate abrasive removal of the shell surfaces was unfeasible and methanolic solvents could not be used, as discussed below. The calcium carbonate (General Purpose Reagent, BDH) used in the blanks was previously Soxhlet extracted for 24 h. and then heated at 450°C for 24 h.

5) Solvent extraction of free lipids

Solvent extraction of the shells was achieved by the use of sonication (5 min.) with three times 100 ml of DCM (protocol A) or three times 100 ml of DCM / methanol (80 : 20 v/v) (protocol B) with 20 ml solvent rinses, combining to yield the surficial extracts (see Fig. 2). To release intercrystalline lipids the shells were crushed in a ball mill, to pass a 500 µm sieve. Shell powders in protocols A and B were solvent extracted as above with DCM only, to provide the intercrystalline extract. In protocol C the abraded shells were sonicated for only 5 s. in three times 100 ml of DCM / methanol (80 : 20 v/v). After crushing the shell powder was Soxhlet extracted with DCM / methanol (80 : 20 v/v) and activated copper turnings for 24 h. Soxhlet extraction was not used for other extraction protocols because of the time needed to complete repeated extractions.

The inorganic calcium carbonate powder used in the blanks formed a suspension when sonicated in pure methanol, and DCM / methanol mixtures in the ratios of 93 : 7 v/v (azeotropic mixture used for Soxhlet extraction) and 80 : 20 v/v. The suspension of the powder provided the high surface area coverage for efficient extraction but the solid and liquid phases could not be separated even after centrifugation (3700 rpm; 5 min.). This made the use of methanolic solvents impractical for the finely powdered shell and blanks and therefore only DCM was used for these.

6) Release and extraction of bound lipids

Bound lipids are present at the crystal surfaces (intercrystalline bound, Fig. 2) and in the insoluble organic material released after full decalcification (intracrystalline bound, Fig. 2). Alkaline hydrolysis of insoluble organic material during saponification cleaves O-acyl bonds and the methyl esters are produced during transesterification under the methanolic conditions. The free fatty acids are unaffected (Klein & Kemp, 1977), although methylation of the free acids can also occur during methanolic Soxhlet extraction, as in protocol C. As the carboxylic acids become dissociated under the alkaline conditions used for saponification, and are therefore likely to be retained in the aqueous phase, the aqueous phases are extracted under both alkaline and then acidic conditions which ensures the carboxylic acids are protonated and therefore fully extracted.

To release these bound lipids, shell powders (yielding intercrystalline bound lipids) or the insoluble residues after full decalcification of the shells (yielding intracrystalline bound lipids) were refluxed for 1.5 h. in a 2M KOH 80% methanolic solution, under a nitrogen atmosphere with constant stirring. Following this saponification, 50 ml of solvent extracted water was added to the aqueous supernatant and shaken with three times 100 ml DCM. Centrifugation (3000 rpm; 5 min.) was required to aid separation of the phases.

After full decalcification by the slow addition of concentrated HCl, two extraction techniques to obtain the intracrystalline free lipids were used (Fig. 2). i) For protocol A, liquid-liquid extraction with three times 100 ml petroleum ether and centrifugation. This solvent is less dense than the decalcified solution and further aided phase separation. ii) For protocol B, liquid-liquid extraction with three times 100 ml DCM, with the addition of 100 to 150 ml of solvent extracted water was used to decrease the density of the decalcified solution. This only required centrifugation of the lower DCM layer to separate the insoluble material. For all aqueous extractions, samples were extracted under both strong acidic and strong alkaline conditions, by the addition of HCl or KOH as required.

7) The use of surrogate standards for quantification

Protocol A was used to find the type and distributions of lipids extracted from the shells of molluscs; no quantification was used for this extraction protocol. However, the percentage distributions of each lipid class are discussed in this study due to the limited number of samples analysed. For protocols B and C quantification of surficial and intercrystalline cholesterol was carried out relative to the lupeol surrogate standard

added prior to solvent extraction (Fig. 2). The remaining extracts of extraction protocol B were quantified relative to the C_{36} *n*-alkane surrogate standard. The use of these surrogate standards in these sequential extractions is discussed in Appendix 2. A relative response factor (RRF) of 1.563 was used for lupeol to cholesterol. RRFs for C_{19} *n*-alkane (0.762), C_{18} FAME (0.924) and cholesterol (1.242) to the C_{36} *n*-alkane surrogate standard were used for the extracted alkanes, FAMEs and cholesterol respectively. With the exception of a RRF for lupeol to cholesterol of 1.563, data from protocol C have been quantified to the lupeol surrogate standard assuming a RRF of 1. See Appendix 3 for calculations.

RESULTS AND DISCUSSION

1) Comparison of methods for the removal of macroscopic contamination from the surface of the shells

The thin section of a *Patella vulgata* shell (Fig. 3a), shows the penetration of the microborings into the shell. The SEM photomicrograph (Fig. 3b) show the alignment of crystallites and the removal of the shell both on the surface and penetration of microborings within the *Patella vulgata* shells. Such microborings greatly increase the surface area of the shell which will increase lipid absorption and are potential sites for the entrapment of contaminating organic material.

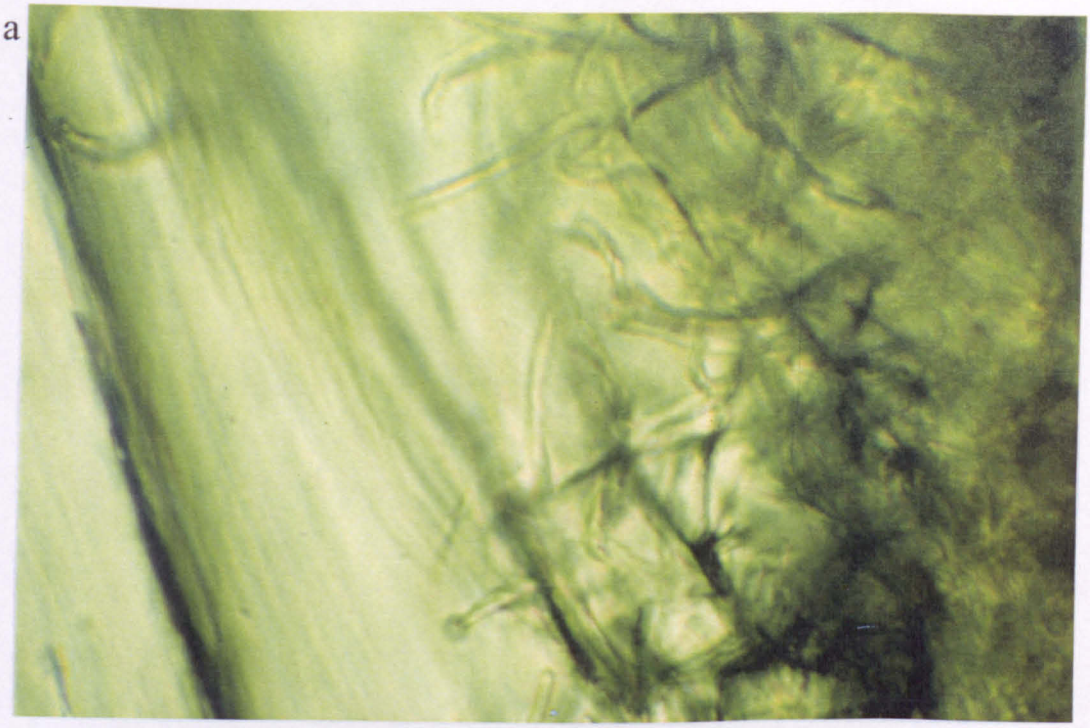
Fig. 4a illustrates the surface of a *Modiolus modiolus* shell freshly broken after a single hypochlorite treatment in both scanning electron (S. E.) and back scatter electron (B. S. E.) modes. The penetration of the hypochlorite from the surfaces of the shell is revealed by the deposition of a 'fluffy' layer onto the crystallites. The penetration of hypochlorite is complete through the nacreous layer (left hand side) and only partially into the prismatic layer (right hand side). The boundary of hypochlorite penetration in the prismatic microstructure is shown in Fig. 4b. As the nacreous microstructure was fully penetrated a non-bleached sample is shown in Fig. 4c for comparison with the bleached nacreous microstructure (Fig. 4d). The darker areas affected by the hypochlorite which are shown by the back scattered electrons indicates the deposition of an element with a higher atomic number than the carbonate, this however was not identified by XRD analysis, possibly due to the low amounts. The precipitation of sodium chloride crystals due to the partial evaporation of concentrated hypochlorite solutions has been observed by Boyde (1984). However, these results contradict those presented by Gaffey and Bronnimann (1993), who observed that a single hypochlorite treatment did not alter the surface texture of carbonate echinoderm plates. The rate of penetration of the hypochlorite solution in the bivalve shell appears to be controlled by

the porosity of the microstructures. The ease of penetration of the hypochlorite solution into the intercrystalline spaces illustrates the necessity for the thorough removal of the intercrystalline lipid fraction which may have been modified by the ingress of extraneous lipidic material from the environment.

XRD analysis (Fig. 5) shows there is no change in bulk mineralogy during the hypochlorite, soxhlet, sonication, crushing or saponification treatments. This is particularly significant for *Modiolus modiolus*, which has a nacreous layer composed of metastable aragonite (Carter *et al.*, 1990). The heat generated by crushing and saponification must therefore below the threshold for phase change from aragonite to calcite (Gaffey *et al.*, 1991).

The large scale effects of chemical cleaning treatments of the shells are shown in Fig. 6. When compared to the untreated shells the macromolecular material on the surface of the shell is loosened although not fully removed by hypochlorite treatment. The use of hypochlorite to oxidise and disrupt the periostracum, remaining soft tissues and epibionts as well as penetrating the intercrystalline spaces allows for more efficient acid etching of the shell surface (Fig. 6). The destruction of the intercrystalline matrix in molluscs by hypochlorite has been noted by Carriker (1979), this greatly increases the fragility of the shells as shown in Fig. 6. This phenomenon has also been observed in brachiopods (Collins, 1986) and other molluscs (Glover & Kidwell, 1993). In all trials the KOH solution was found to be ineffective for cleaning the shells of macroscopic organic material.

a



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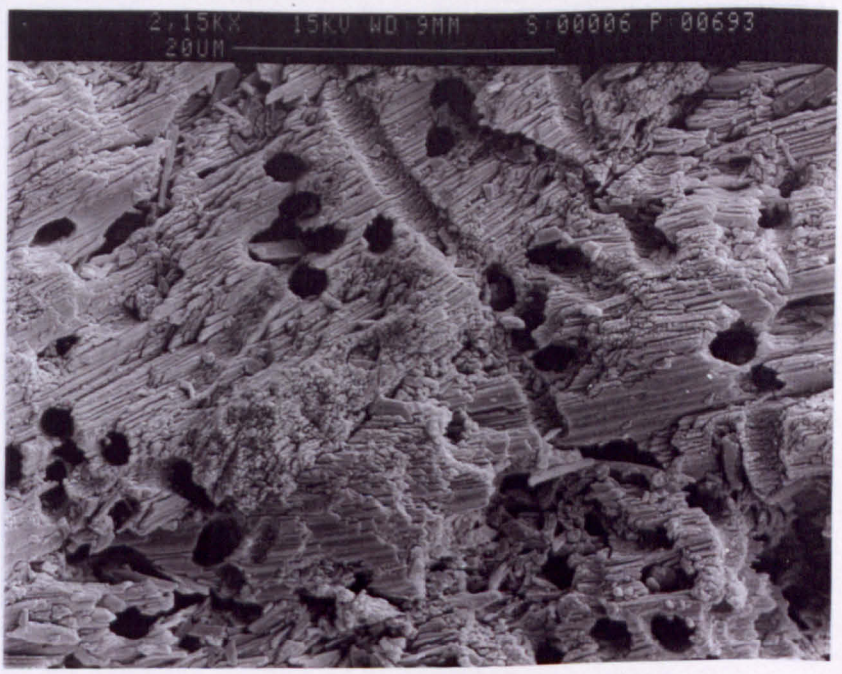


Figure 3. a) Thin section of Recent *Patella vulgata* shell, transverse cut through shell, outer surface at right of picture, microborers revealed as network of tubes originating from the surface. b) SEM photomicrograph of microborings on the outer surface of Recent *Patella vulgata* shells after a single hypochlorite treatment.

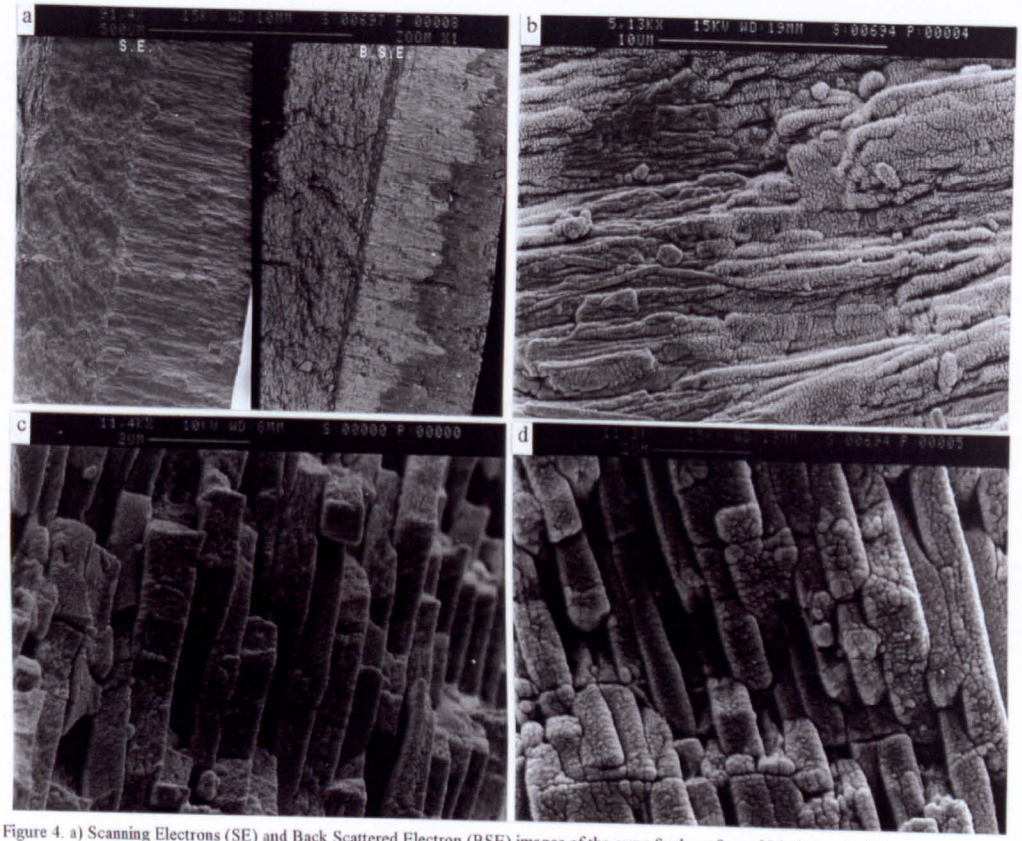


Figure 4. a) Scanning Electrons (SE) and Back Scattered Electron (BSE) images of the same fresh surface of *Modiolus modiolus* sample after a single hypochlorite treatment. From left to right: inner surface, aragonitic nacreous microstructure, calcitic prismatic microstructure, outer surface. b) SEM photomicrograph at boundary of hypochlorite penetration in to prismatic microstructure. c) SEM photomicrograph of untreated nacreous layer. d) SEM photomicrograph of nacreous tablets exposed to a single hypochlorite treatment.

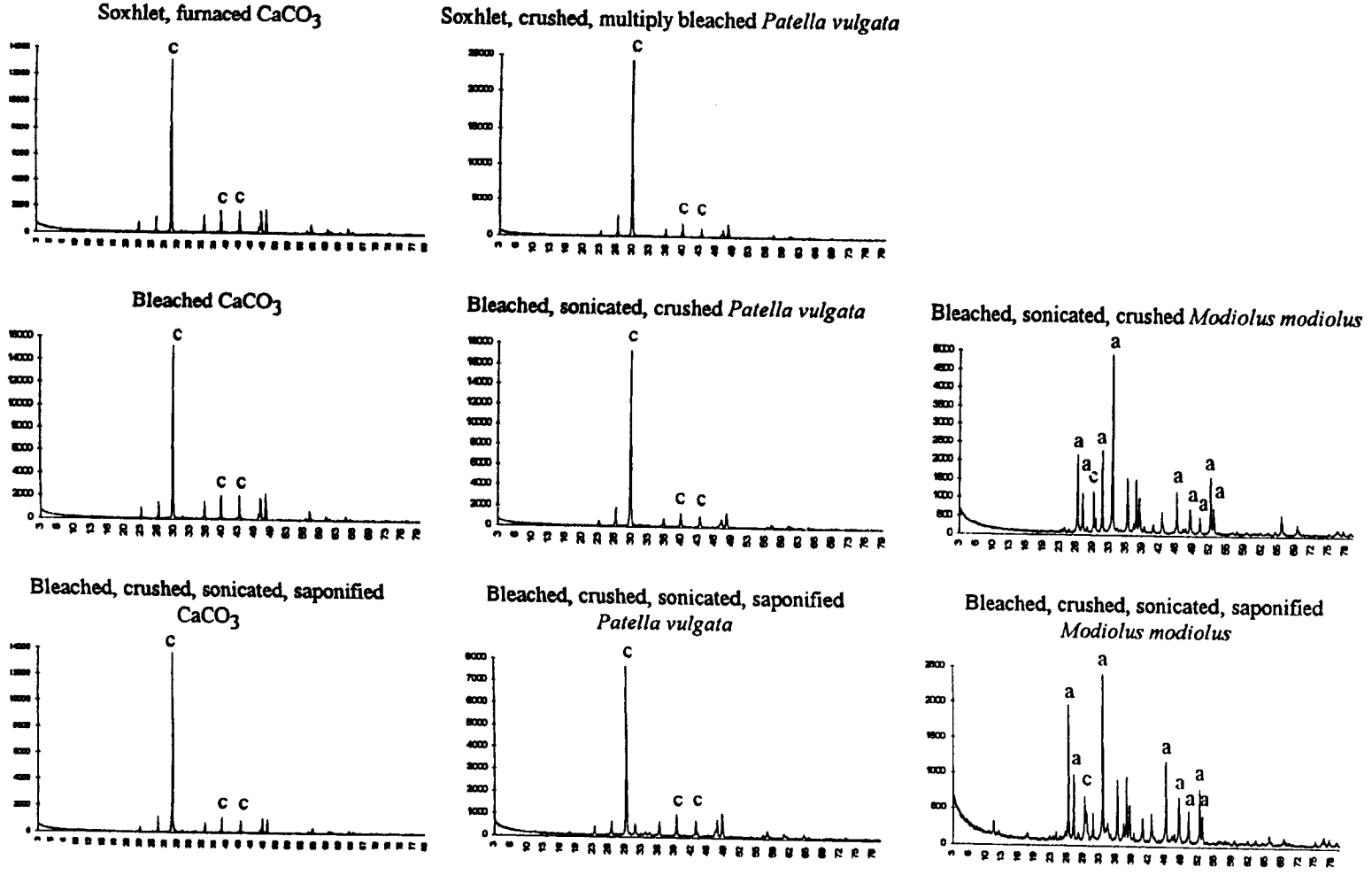


Figure 5. X-ray diffractograms (2-Theta angle in degrees versus counts) of selected shell and calcium carbonate blank subsamples after the treatments indicated. Peaks are identified by mineralogy, c = calcite, a = aragonite.

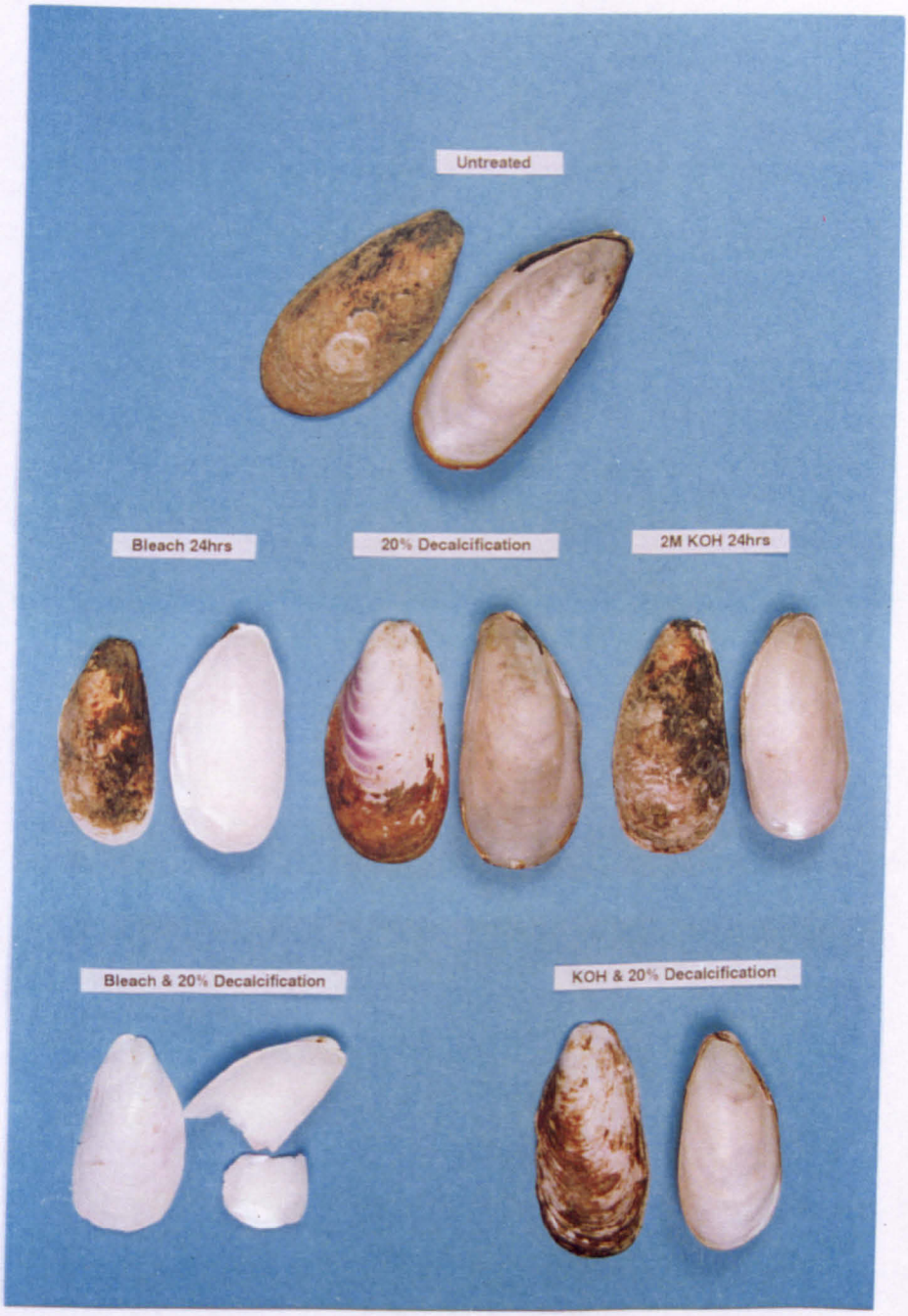


Figure 6. Photograph illustrating the visual effects of individual and combined cleaning treatments on the exterior and interior surfaces of *Modiolus modiolus* shells.

The results from GC analysis of KOH and hypochlorite extracts taken after the cleaning procedures are shown in Fig. 7. The gas chromatograph of the extract after KOH treatment is shown in Fig. 7a. This shows a dominant cholesterol peak and subsidiary sterol peaks (identified by characteristic m/z 129 fragmentation). Hypochlorite treatment (Fig. 7b) generated smaller unidentified and coeluting fragments. These differences in the breakdown products are due to the different ways in which the organic material is broken down. KOH is specific in breaking bonds (base catalysed hydrolysis of ester and peptide linkages) leaving soluble, GC amenable fragments. Hypochlorite oxidation breaks the organic material into smaller fragments which have poor chromatographic separation. This oxidation stage may cause analytical artefacts by oxidising, but not removing lipids. To remove these components, the shell surface is acid etched to chemically remove the surface layer and solvent washed (Fig. 2, surficial extract).

Saponification has been preferentially used after the initial cleaning treatments in protocols A and B. This releases bound lipids and destroys other organic material, this technique thus allows the direct comparison of intercrystalline and intracrystalline bound material.

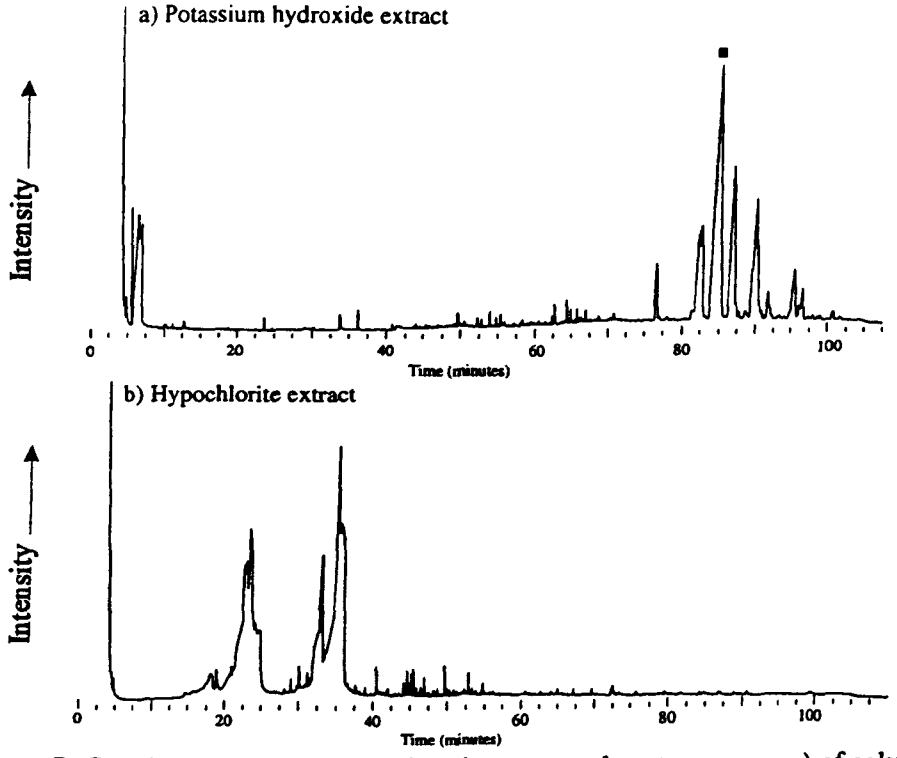


Figure 7. Gas chromatograms (retention time verses detector response) of solvent extracts of a) potassium hydroxide and b) hypochlorite solutions after cleaning of *Modiolus modiolus* shells. Cholesterol is labelled ■.

2) Solvent extraction of carbonate surfaces

The ratios of the standards C_{19} *n*-alkane, cholesterol and lupeol to the C_{27} FAME extracted from the surface of the inorganic calcium carbonate are shown as unfilled columns in Fig. 8. The ratios for the untreated standard mixture are shown as filled columns. The ratios of the standards recovered from the carbonate surface are similar to the untreated standard mixture which indicates that extraction was complete and that reproducible and reliable data can be obtained using sonic extraction of the carbonate surfaces with DCM. A statistical analysis is carried out in section 4. A solvent mixture of DCM / methanol (80 : 20 v/v) was used for the surficial extracts of extraction protocols B and C as this was observed to extract more chlorophyll pigment from the surfaces of untreated shells. However, the calcium carbonate powder used in the blanks formed a suspension when sonicated in this solvent mixture, so this was used for extraction of the surfaces of whole shells only.

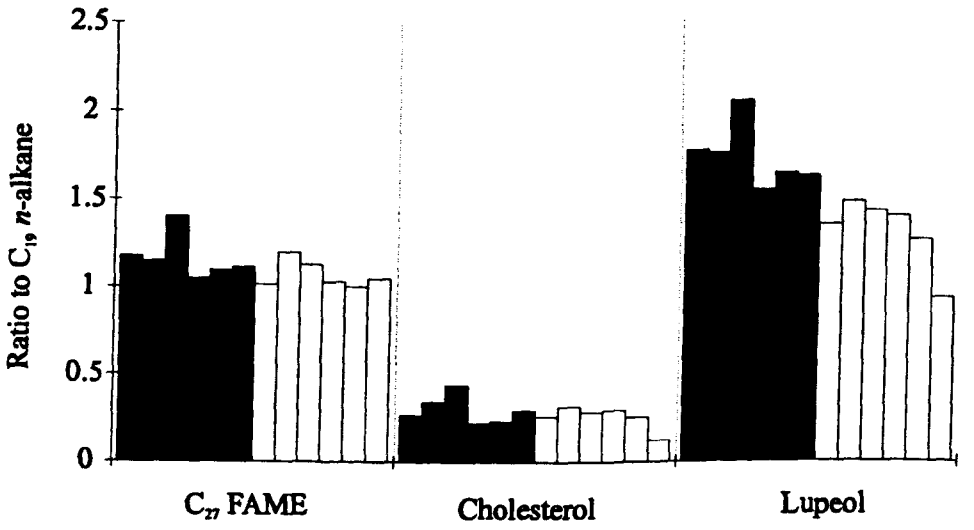


Figure 8. Ratios of the standard mixture of C_{27} FAME, cholesterol and lupeol to C_{19} *n*-alkane standard. Filled columns represent six untreated standard mixtures and the unfilled columns represent six solvent extracts from inorganic calcium carbonate.

3) Effects of alumina and cotton wool

The ratios of C_{27} FAME to C_{19} *n*-alkane, cholesterol and lupeol in a standard mixture after passing through cotton wool (grey columns) and alumina with cotton wool plugs (unfilled columns) is shown in Fig. 9. The ratios for the untreated standard mixture are shown as filled columns. The ratios of the standards recovered after passing through cotton wool are similar to the untreated standard mixture, therefore the cotton wool plug appears to have no effect on the solutions. However passing the standard mixture through the alumina and cotton wool plug removes most of the

lupeol standard and all of the cholesterol. Cholesterol was however found in some shell extracts using this methodology (extraction protocol A), suggesting the capacity of the alumina plug might be exceeded for some samples. Therefore the yields of cholesterol resulting from protocol A experiments should be treated with caution. The extracted *n*-alkanes and FAMEs from extraction protocol A are reproducible but have not been quantified.

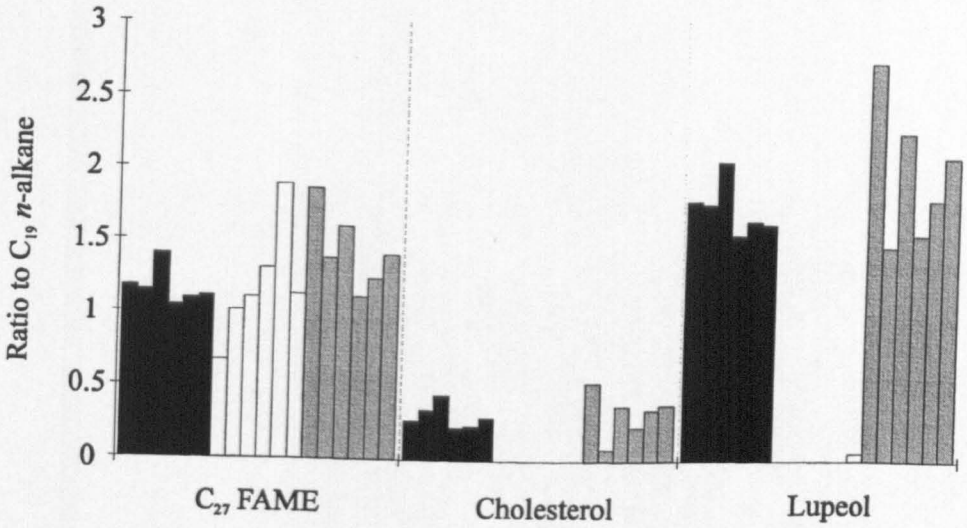


Figure 9. Ratios of the standard mixture of C_{27} FAME, cholesterol and lupeol to C_{19} *n*-alkane standard. Filled columns represent six untreated standard mixtures, unfilled columns six standard mixtures passed through alumina and grey columns six standard mixtures passed through cotton wool only. Zero values were obtained for the extraction of cholesterol and lupeol after passing through alumina.

4) Statistical analysis of solvent extraction of carbonate surfaces and the effects of alumina and cotton wool

A one-sample t-test of the ratios of the standard compounds to the C_{19} *n*-alkane after no treatment (the standard solution), extraction from $CaCO_3$, transfer through alumina and transfer through cotton wool has been carried out using the equation below;

$$\text{test statistic} = \frac{|\text{sample mean} - \text{hypothesized population mean}|}{\text{sample standard deviation} / \sqrt{\text{sample size}}}$$

The hypothesized population mean used the mean value of the standard solutions. The sample size was six. Results are shown in Table 1.

	Mean	Standard deviation	T-test result
Standard solutions			
C_{27} FAME	1.16	0.12	-
Cholesterol	0.30	0.08	-
Lupeol	1.75	0.18	-
Extracted from $CaCO_3$			
C_{27} FAME	1.07	0.08	2.76
Cholesterol	0.25	0.07	1.75
Lupeol	1.32	0.20	5.27
Transferees through Al_2O_3			
C_{27} FAME	1.18	0.40	0.12
Cholesterol	0	-	-
Lupeol	0.01	0.02	213
Transferred through cotton wool			
C_{27} FAME	1.43	0.27	2.45
Cholesterol	0.33	0.15	0.50
Lupeol	2.00	0.48	1.28

Table 1. Mean, standard deviation and t-test result of the ratios of the standard compounds to the C_{19} *n*-alkane after no treatment (= standard solution), extraction from $CaCO_3$, transfer through alumina and transfer through cotton wool.

For five degrees of freedom (sample size - 1) the critical value at a significance level of 1% is 3.36, therefore t-test results higher than this indicate that the results are biased (i.e. not similar).

With the exception of the cholesterol and lupeol extracts transferred through alumina which were previously shown to have been removed (Fig. 9), the lupeol extracted from the calcium carbonate is the only result shown to be biased. Lupeol was used to calculate the yield of cholesterol from extraction protocol C. However, when used as a surrogate standard the lupeol was added in DCM solvent which unlike these test experiments was not evaporated, therefore the lupeol surrogate standard should be unaffected by binding to the carbonate surface. Significantly the C_{27} FAME transferred through the alumina is not biased.

Comparison of different extraction protocols

1) *n*-Alkanes

The *n*-alkane carbon number distributions from each fraction of *Patella vulgata* shells and inorganic carbonate blanks are shown in Fig. 10. Each fraction has been normalised by dividing the peak area of each *n*-alkane by the total peak areas and multiplying to 100%. Shell extracts from protocols A (filled) and B (unfilled) are shown as columns on the same axes as the calcium carbonate blanks for each experimental batch which are plotted as open or closed boxes, this enables a direct comparison of the different protocols and the carbonate blanks. *n*-Alkanes extracted from protocol C are shown in Fig. 11. Only three *n*-alkanes were extracted from the surficial extract of protocol C and no *n*-alkanes were extracted from the intercrystalline fraction.

The surficial extracts from extraction protocols A and B show that *n*-alkanes remain on the shell surface after the hypochlorite, and partial decalcification treatments. So although the initial cleaning treatments are effective at removal of macroscopic organic material, they do not remove free lipids. The surficial extracts from protocols A and B are also the least similar, this suggests that contamination originating on the surface of the shells is extracted in this initial solvent extract. The *n*-alkane distributions become more similar as the extraction protocol continues, this may indicate that exogenous contamination is being removed to reveal the same indigenous *n*-alkanes. However, the *n*-alkane distributions between shells extracted using protocols A and B and the blanks are similar. This indicates that laboratory contamination is the source of the *n*-alkanes despite thorough extraction of the reagents and apparatus.

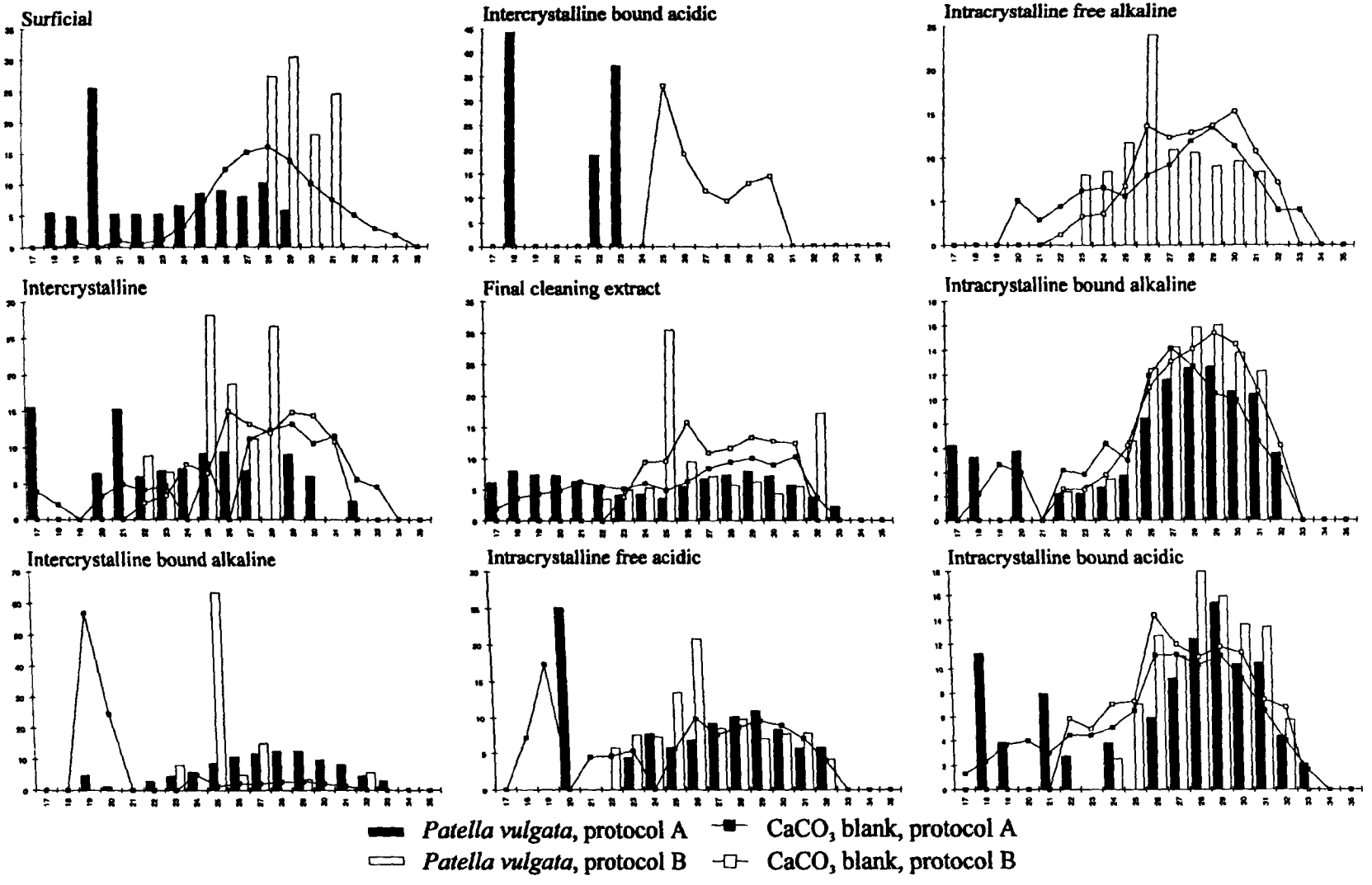


Figure 10. Plots of normalised *n*-alkane carbon number distributions from *Patella vulgata* shells and carbonate controls for each fraction. Shell extracts are shown as columns for protocol A (filled) and B (open). Calcium carbonate blanks are shown on the same graph as -■-, either filled (protocol A) or closed (protocol B).

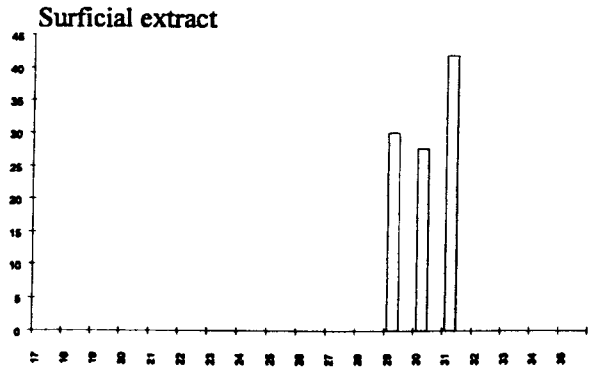


Figure 11. *n*-Alkanes from protocol C, no *n*-alkanes were extracted from the intercrystalline fraction and no blank was used.

Quantification of the intercrystalline bound and all the intracrystalline *n*-alkane extracts from *Patella vulgata* shells using extraction protocol B are shown in Fig. 12. Higher yields are observed in the shells than the calcium carbonate blanks. However, where the yields from the shells are low, as for the intracrystalline bound alkaline and acidic extracts, the contribution from laboratory contamination is significant.

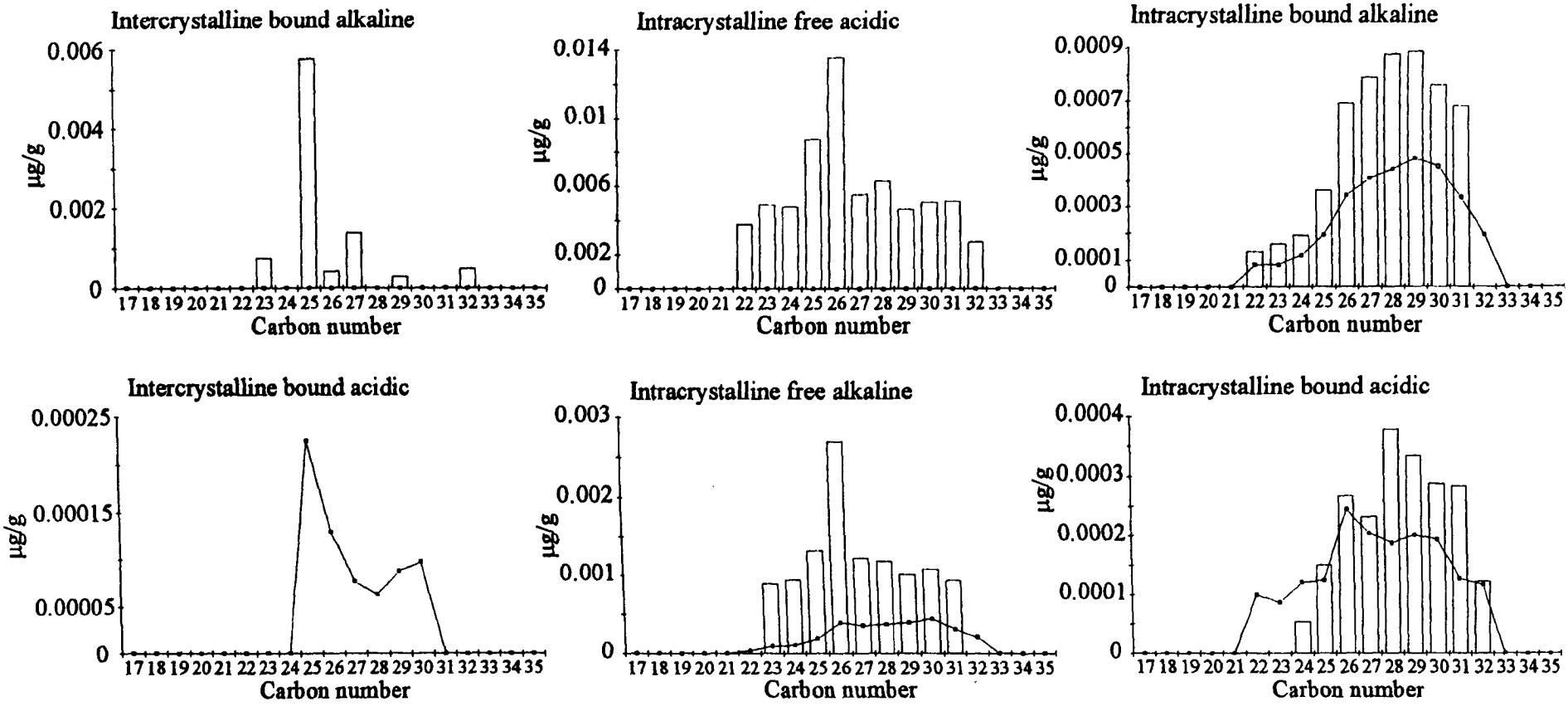


Figure 12. Quantified *n*-alkane yields (µg per g of sample) of intercrystalline bound and all intracrystalline fractions for *Patella vulgata* (columns) and the calcium carbonate blank (-■-) using protocol B.

2) Bound fatty acids

Bound fatty acids are extracted as fatty acid methyl esters (FAMEs). Fig. 13 shows the FAME carbon number distributions for *Patella vulgata* shell extracts from extraction protocols A, B and C. The data from extraction protocol A are unquantified.

These data show that all the shell FAME extracts have an even over odd carbon number distribution. A carbon number maximum at C_{16} is observed for the shells with the one exception of the C_{18} maximum for the intracrystalline bound alkaline extract from protocol B. Shell extracts from protocol A have similar distributions to the other protocols, but have a larger range of carbon numbers. Interestingly the calcium carbonate blank contains FAMEs which are not found in the shells. The intercrystalline bound acidic blank from protocol A contains an unusual distribution of C_{17} and C_{19} carbon number FAMEs whilst the intracrystalline bound alkaline and acidic blank fractions from protocol B contains a C_{18} FAME. No C_{16} FAME occurred in the blanks. The FAMEs in the blanks are therefore likely to be from a different but unknown source to those in the shells, laboratory contamination being the most likely source. The yields of FAME contamination are also lower than the FAMEs extracted from the shells (Fig. 13, protocol B). The limited quantification of FAMEs in fractions from protocol B shows that the intercrystalline bound extract dominates the intracrystalline bound extracts.

The similarity between the intercrystalline protocol C extract and intercrystalline bound extracts from protocols A and B suggests that Soxhlet extraction is similarly causing transesterification of bound fatty acids at intercrystalline sites, and so esterification and methylation of the bound fatty acids is occurring.

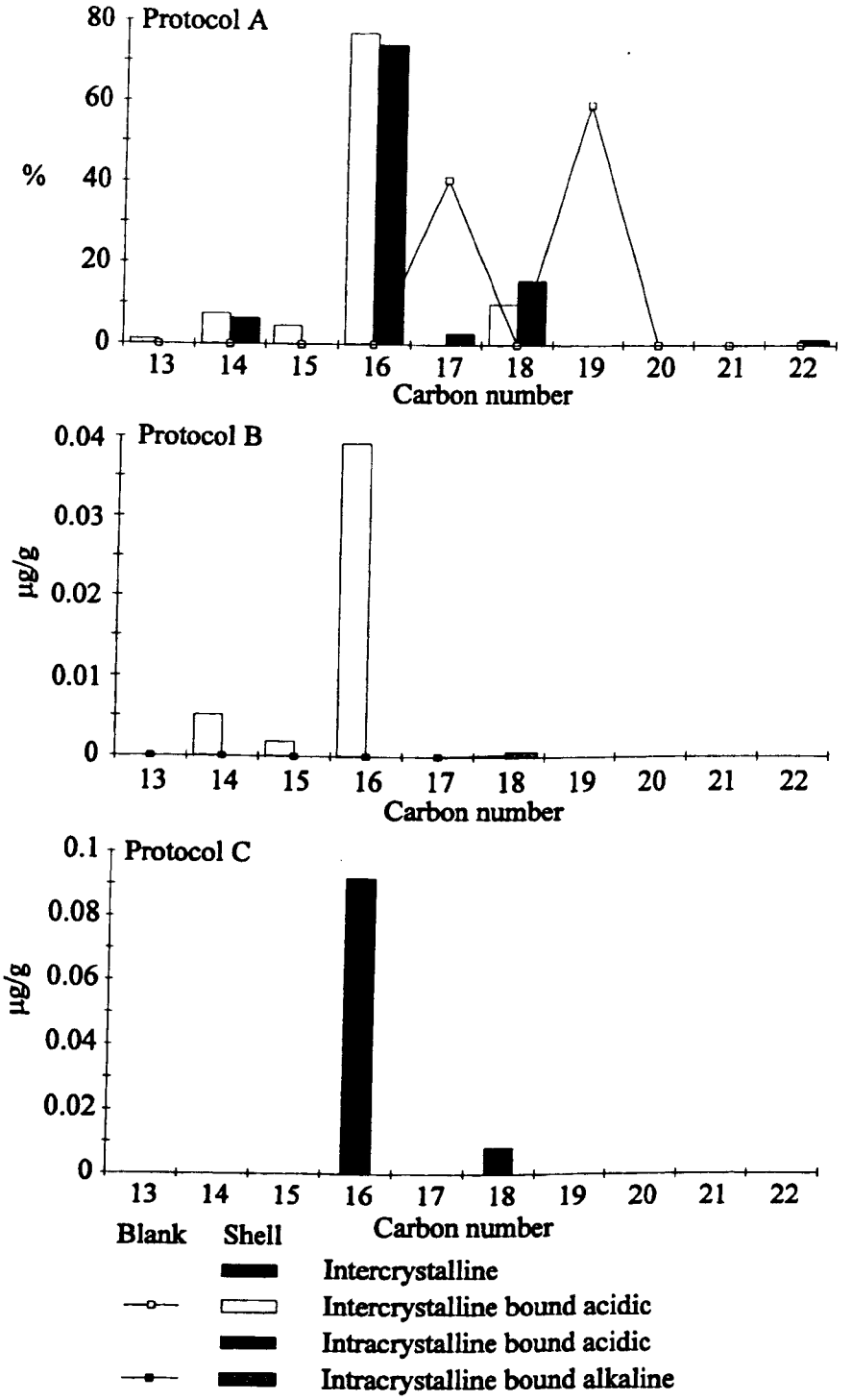


Figure 13. FAME carbon number distributions in *Patella vulgata* shells and the calcium carbonate blanks from extraction protocols A, B and C. Extracts from protocols A are shown as the relative abundance (%). Those from protocols B and C are quantified as µg per g of sample extracted. Shell extracts are shown as columns, calcium carbonate blanks are shown as -□-. The extract fraction is indicated by the shading.

3) Cholesterol

Cholesterol data are only shown for the quantified extraction protocols B and C (Fig. 14). A comparison of the results from these protocols shows cholesterol is completely removed from the surfaces of the shell by the hypochlorite and partial decalcification treatments of protocol B. Cholesterol was obtained in the surficial extract after the abrasion treatment of protocol C, probably due to handling during the cleaning process. Soxhlet extraction also revealed higher yields of cholesterol (protocol C, Fig. 2, intercrystalline) than sonic extraction of the crushed powder (protocol B, Fig. 2, intercrystalline). The higher yields in protocol C data compared to protocol B, could be due to relic surficial contamination after inefficient cleaning of protocol C, or to the partial destruction of intercrystalline cholesterol by hypochlorite treatment during protocol B. Extraction protocol C was not examined with a calcium carbonate blank to quantify contamination.

Bound intercrystalline cholesterol is extracted from the intercrystalline alkaline fraction (protocol B, Fig. 14) after saponification of the shell powder. This is indicative of a more resistant intercrystalline bound fraction. For the intracrystalline free acidic fraction the yield of cholesterol is similar to the blank, illustrating that laboratory contamination becomes significant when examining low yields of shell extract. The extraction of cholesterol from the intracrystalline free and bound fractions shows that cholesterol is present within the biomineral, these shell locations are inaccessible using extraction protocol C.

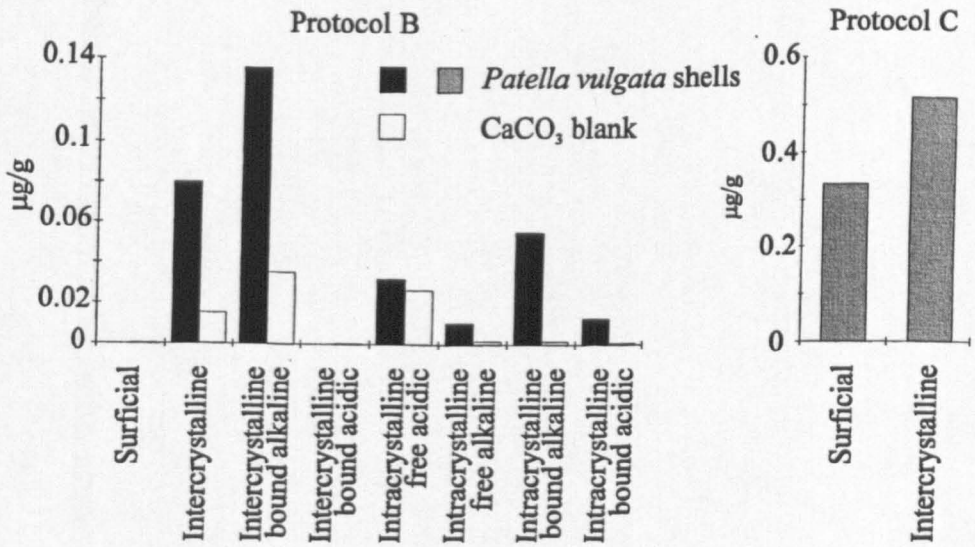


Figure 14. Cholesterol yields (µg per g of sample) from *Patella vulgata* shells (filled columns) and calcium carbonate blank (open columns) for protocol B and for shells (grey columns) in protocol C.

Depletion of intercrystalline lipids by partitioning into water

The intercrystalline lipids extracted in all these experimental protocols may be depleted by the solubility of intercrystalline lipids in water present in the intercrystalline spaces which is accessible to the external environment.

The partition coefficients of selected fatty acids between water and *n*-heptane are shown in Table 2 (data from Leo *et al.*, 1971). The partition coefficient (P) is expressed as the concentration present in the organic solvent (Cs) divided by the concentration in the aqueous phase (Cw) when at equilibrium (equation 1). Partitioning of these free fatty acids between the organic material in the shell and water in the intercrystalline spaces may also occur using the same principles.

$$P = \frac{Cs}{Cw} \qquad (1)$$

As the concentration is the amount divided by the volume, equation 2 is derived.

$$P = \frac{\text{(Amount of free fatty acid in shell) x (Volume of water)}}{\text{(Volume of organic material within shell) x (Amount of fatty acid in water)}} \qquad (2)$$

If the amounts of fatty acid present in the water and the shell are equal at equilibrium, then using equation 3 it is possible to estimate the volume of water required to achieve this equilibrium.

$$\text{Volume of water } (cm^3) = P \times \text{Volume of organic material in shell } (cm^3) \qquad (3)$$

Using a mean of 4.3% dry weight organic material present in shells (Price *et al.*, 1976), and assuming a density of 1 g/cm^3 for the organic material, the volume of the organic material within the shell is 0.043 cm^3 organic material per cubic centimetre of shell. Therefore the volume of water required to be associated with each cubic centimetre of shell to reach equilibrium is calculated in Table 2.

Fatty acid	Log P (*n*-heptane / water)	Volume of water (approximate) cm^3
$C_{18}H_{32}O_2$ (Linoleic)	5.08	5100
$C_{18}H_{34}O_2$ (Oleic)	5.36	9850
$C_{16}H_{32}O_2$ (Palmitic)	5.31	8800
$C_{12}H_{24}O_2$ (Lauric)	3.03	50

Table 2. Fatty acid partition coefficients (P) between *n*-heptane and water, and the calculated volume of water to achieve equal distributions between the shell organic material and water.

The results of these calculations indicate that partitioning between the organic material of the shell and the water in intercrystalline locations does not require much water. The total (intercrystalline and intracrystalline voids) water present in skeletal carbonates has been calculated as 3% (Gaffey, 1990), therefore in the cubic centimetre of shell 0.03 cm^3 is water. The C_{18} saturated fatty acid would require 300,000 volume changes, whilst the C_{12} saturated fatty acid would require 1,500 volume changes. These results can be compared with the possible 10,000 years shells can lie on a beach post-mortem (Kidwell & Bosence, 1991), which could contribute 7 million tidal washings if all the water within the shell was exchanged.

The shells examined by Price *et al.* (1976) were only lightly scrubbed, which indicates that external organic material such as periostracum and soft tissues may remain. The organic material within the shells of different species also exhibited a range of 1.4 to 21.4 % dry weight organic material. Different values for the organic content of the shell will affect the volume of water required to achieve equilibrium, but not the different behaviour of the fatty acid constituents. Different partition coefficients between the shell organic material and *n*-heptane, the time taken to achieve equilibrium, the restricted flow of water through the pores in the shell and the effect of the biomineral on binding lipids would further complicate this simplified model.

Water washing of crude oils, depletes the alkanes with carbon numbers below C_{15}, but does not affect the alkanes with a higher carbon number (LaFargue & Barker, 1988). Similarly, the different partitioning behaviours of the fatty acid components could affect the fatty acid distributions of the shells. This would result in the preferential loss of short chain fatty acids and the loss of unsaturated rather than saturated fatty acids. The intercrystalline FAMEs extracted in this study are bound into other organic material and therefore are less soluble and should not be affected.

However, the same fatty acids present in the ground water may be deposited into the shell organic material by the same partitioning process.

Comparison of protocols B and C

Extraction protocol C does not fully extract the samples as only the surficial and intercrystalline shell locations are exposed for solvent extraction. Additionally abrasion of the shell surfaces may not fully remove the surficial contamination. The intracrystalline shell locations, extracted using extraction protocols A and B, may be the only shell locations unaffected by losses of lipids to the external environment or by post-depositional ingress. The disadvantage of examining the intracrystalline shell locations using extraction protocols A and B, is primarily the time taken for each analysis (each experimental batch of six samples takes six to eight weeks). This limits the number of samples which it is possible to analyse and increases the likelihood of laboratory contamination.

CONCLUSIONS

Previous reported methods for the extraction of lipids from biominerals are inadequate as they pay little attention to proven contamination. Post-depositional ingress and losses of lipids by water washing may occur in all but intracrystalline sites, and laboratory contamination is present throughout the extraction protocols. The extremely low levels of shell lipids and the impossibility of totally excluding lipid contamination, under normal laboratory conditions, requires rigorous laboratory procedures and calcium carbonate blanks are essential. These need to be quantitative to assess the significance of contamination. Although the sources of contamination are unknown, a knowledge of the yields and variability of each contaminant type enables a single blank to be representative of the background contamination within an experimental batch.

The new protocol allows indigenous lipids and contamination to be characterized from different sites within the shell. Shells are cleaned by a combination of sodium hypochlorite solution followed by partial decalcification, which destroys the bulk of the surficial macroscopic organic material. The hypochlorite solution is able to penetrate into the intercrystalline sites of the biomineral and attack intercrystalline organic materials. Solvent extraction removes the remaining surficial lipids. Intercrystalline free lipids are released from the shell after crushing and further solvent extraction. Saponification non-destructively releases bound lipids from any insoluble organic material and crystal surfaces. Final sonication ensures the removal of intercrystalline lipids prior to full decalcification. Free intracrystalline fractions are

released after full decalcification. Insoluble intracrystalline lipids are released following further saponification.

Of the identified lipid components in *Patella vulgata* shells, surficial extracts contain *n*-alkanes. Intercrystalline spaces contain *n*-alkanes, bound fatty acids with an even over odd distribution, dominated by C_{16} maximum carbon number and cholesterol. Intracrystalline sites also contain *n*-alkanes and lower yields of cholesterol.

The method developed and described in this chapter is applicable to the analysis of a wide range of organisms with carbonate based skeletons.

CHAPTER 2. CHARACTERIZATION OF INDIGENOUS LIPIDS IN RECENT MOLLUSCAN SHELLS.

ABSTRACT

An experimental protocol for sequentially extracting protected lipids from the shells of Recent molluscs has been tested to distinguish the indigenous shell lipids from laboratory contamination and post-depositional ingress. The use of a calcium carbonate blank reveals the phthalate plasticisers extracted from the shells are due to laboratory contamination. Pristane, phytane and free fatty acids were rarely extracted which limits their use for interpretation. The *n*-alcohols, bound fatty acids, β-hydroxy fatty acids, cholesterol and other steroids are extracted from the shells in higher yields than the calcium carbonate blanks and are considered indigenous to the shells. Multivariate statistical analysis is used to compare the distributions of bound fatty acids and steroids extracted from different shell locations with the reported fatty acids and steroids for the soft tissues of the same species. The reported values for the soft tissues were used to indicate the original shell lipid composition. The shells lack the unsaturated bound fatty acids reported in the soft tissues. The saturated bound fatty acids of *Littorina littorea* shells also differ in the carbon number distributions to the reported saturated fatty acids of the soft tissues. Surficial shell extracts are characterized by steroidal ketones, representing sterols which have been oxidised by the cleaning treatments used. The steroids from both intercrystalline and intracrystalline shell locations in *Littorina littorea* are most similar to the soft tissues. However, the intercrystalline steroids are different to the intracrystalline steroids which may indicate a different original composition.

INTRODUCTION

Lipids (Thompson & Creath, 1966; Isa & Okazaki, 1987; Curry *et al.*, 1991; CoBabe & Pratt, 1995), proteins and free amino acids (Serban *et al.*, 1988), carbohydrates, chitin and even DNA (Hagelberg *et al.*, 1994) have all been found associated with ancient biominerals. In gastropod and bivalve shells the total organic content (ash free dry weight) ranges from 1.4 to 21.4%, with a mean of 4.3% (n=21) (Price *et al.*, 1976). The composition is 0.84 to 2.88% lipidic, but dominated by proteins (66.7 to 89.7%) with (0.15 to 0.29%) carbohydrates (Goulletquer & Wolowicz, 1989). The majority of this organic material is intercrystalline, surrounding the crystallites and ultimately accessible to the external environment (Watabe, 1963; Lowenstam & Weiner, 1989 page 103). These intercrystalline locations make the shells susceptible to contamination and there is also the potential for loss of indigenous shell lipids. Smaller amounts of organic material are occluded either within the crystals (Towe &

Thompson, 1972; Crenshaw, 1972; Towe, 1980 page 71), or at the crystal boundaries and imperfection sites (Berman *et al.*, 1993) - both of these possibilities are defined here as intracrystalline. It is the protective rôle of the shell, and the location of the organic material within the biomineral which leads to the enhanced preservation of the labile and fragile biomolecules (Towe, 1980). Shell lipids from Recent molluscs are capable of surviving rigorous cleaning treatments due to their incorporation into the protecting inorganic structure (Chapter 1). The hydrophobic nature of lipids also reduces their interactions with water and enhances their preservation potential over geological time scales.

Members of the phylum Mollusca use genetically controlled mineralization to form a variety of biominerals with a large number of shell microstructures. The mechanisms of this biomineralization have yet to be elucidated in anything but the most general terms (Lowenstam & Weiner, 1989; Mann *et al.*, 1989; Simkiss & Wilbur, 1989). The mechanism of incorporation of lipidic material into the shells is unknown, but could originate from a variety of different mechanisms. The extrapallial fluid, secreted by the mantle, provides the supersaturated solution for mineral precipitation and the macromolecules involved during biomineralization. Entrapment of this fluid during biomineralization is thought to produce the large number of small fluid inclusions found in mollusc shells (Lécuyer & O'Neil, 1994), and could provide a route for lipid incorporation into the aqueous inclusions. Lipids may also be carried into the biomineral associated with the hydrophobic matrix proteins, thought to be intimately associated in the biomineralization process, which become occluded within the biomineral (Addadi *et al.*, 1991). Deliberate transportation of lipids through the mantle tissue to the shell could also occur. Support for this active rather than passive lipid incorporation arises from the binding affinity for Ca^{2+} of the phospholipids (Isa & Okazaki, 1987) and therefore a suggested rôle in the biomineralization process (Wuthier, 1973; Abolinš-Krogis, 1979).

Pigmentation caused by organic compounds is also found in the shells of molluscs (Comfort, 1950). This colour remains in well preserved fossil shells (e.g. Kelley and Swann, 1988). These compounds may be extracted by the experimental protocol used here, and may provide further molecular information.

The presence of bound fatty acids, cholesterol and other sterols found in the soft tissues of molluscs, and in the shells, indicates that the soft tissues are a likely source for the shell lipids. The lipid class composition of the soft tissues consists of polar lipids (phospholipids), triacylglycerols, sterol esters, free sterols and free fatty acids

(Klingensmith & Stillway, 1982). The lipidic composition of the soft tissues is thought to be related to a variety of factors such as diet, temperature, water depth, sexual cycle, tissue type and phylogeny (Pollero *et al.*, 1979; Joseph, 1982; Piretti *et al.*, 1987).

Fatty acids in molluscan soft tissues have been extensively studied (see review by Joseph, 1982). Triglycerides but not phospholipids exhibit seasonal variations (Gardner & Riley, 1972; Pollero *et al.*, 1979). Fatty acids have been characterized in extracts from brachiopod shells. C_{16} and C_{18} saturated fatty acids dominate the unsaturated $C_{16:1}$, $C_{18:1}$, $C_{20:1}$ and $C_{20:2}$ components (Curry *et al.*, 1991). Saturated fatty acids are also dominant in molluscan shell extracts but with a wider range and higher yields of unsaturated fatty acids in bivalve shells (CoBabe & Pratt, 1995). β-hydroxy fatty acids have been extracted from live, Recent and fossil brachiopod shells (Clegg, 1993), but they have not been reported in the soft tissues or shells of molluscs. β-hydroxy fatty acids have been extracted from bacteria (Boon *et al.*, 1977), algae (Matsumoto & Nagashima, 1984), higher plants and sediments (Cardoso & Eglinton, 1983). Although they are intermediates in the degradation of fatty acids, the β-hydroxy fatty acids are not released by the enzyme complex (Ratledge, 1984).

Sterols from the soft tissues of molluscs and the marine environment have been extensively studied by other workers (for reviews see Idler & Wiseman, 1972; Morris & Culkin, 1977; Goad, 1978; Volkman, 1986; Kerr & Baker, 1991). Both free sterols and sterol esters are present in molluscan soft tissues in the same distributions (Stoilov *et al.*, 1984), the distribution of sterols between different tissue types is also uniform (Jarzebski *et al.*, 1986); this suggests no organ-specific rôle for these compounds.

Recent molluscan soft tissues are not always readily available for analysis. However, their shells are often accumulated in large numbers and are also the only remnants of molluscs in the geological record. The presence of indigenous lipids in Recent shells and a comparison of the lipid composition with the soft tissues from live specimens may allow the extension of lipid comparison back through an extensive geological record.

From the data presented in Chapter 1, it can be seen that the yields of lipids from skeletal carbonates are very low. Such low yields increase analytical difficulty through distortion of results caused by contamination. This contamination is divided into two

separate sources, i) laboratory contamination during the extraction and analysis of the samples, and ii) post-depositional ingress, which is defined here as non-indigenous contamination from the environment after the death of the animal. Therefore the aims of this Chapter are to examine the shells of Recent molluscs for the presence of indigenous lipidic material. The application of the methodology developed in Chapter 1 is tested to i) distinguish the extracted shell lipids from laboratory contamination, ii) distinguish the shell lipids from post-depositional ingress by the sequential removal of successively more inaccessible lipids, and as the source of the shell lipids is likely to be the soft tissues, iii) to compare the reported bound fatty acid and steroidal compositions of the soft tissues of *Littorina littorea* with those extracted from different locations within the shell. The original lipid composition of the shells is unknown; but given the uniform distribution of sterols in the soft tissues (Jarzebski *et al.*, 1986), it is reasonable to assume that if the sterol composition from within the shell becomes similar to the soft tissues, then that shell location is representative original shell steroidal composition.

MATERIALS AND METHODS

Sampling

The live and Recent samples with sampling location, experimental batch and method protocols used for their analysis are shown in Table 1. Species were identified by their shell morphology using Tebble (1966), Campbell (1976) and Graham (1988).

Preparation of reagents

All solvent was distilled on a 30 plate Oldershaw column, 100 ml from each 2.5 l distillate was evaporated under reduced pressure and analysed by gas chromatography (GC). Hydrochloric acid (HCl) (BDH, AnlaR 35.4% HCl), 10M potassium hydroxide (KOH) solution (BDH, General Purpose Reagent, pellets) and laboratory distilled water were liquid-liquid extracted four times in a separating funnel with an equivalent volume of dichloromethane (DCM). The fourth solvent extract was analysed by GC. Cotton wool and alumina (aluminium oxide, BDH) were Soxhlet extracted with DCM / methanol (93 : 7, v/v) for 24 h. Glassware was cleaned by immersion in chromic acid (30 g $Na_2Cr_2O_7$ per litre of concentrated H_2SO_4) for a minimum of 4 h., rinsed in tap and distilled water, dried and rinsed with the appropriate solvent three times prior to use. To avoid laboratory cross contamination, glassware was kept aside for the use with this project alone.

Sample (Vernacular name)	Extraction protocol	Age (Ma)	Location / Formation
Batch 1			
Patella vulgata 1 (Common limpet)	A	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713
Littorina littorea 1 (Edible winkle)	A	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713
Modiolus modiolus (Horse mussel)	A	live	Firth of Lorne, Scotland.
$CaCO_3$ 1	A	-	-
Batch 2			
Nucella lapillus 1 (Dog whelk)	A	Recent	Bamburgh beach, Northumberland, England. Sheet 75, 181356
Littorina littorea 2	A	Recent	Rockcliffe, Scotland. Sheet 84, 848536
Sepia officinalis (Cuttlefish)	A	Recent	South Coast, England.
Artica islandica 1 (Ocean quahog)	A	Recent	Ynys-las beach, Wales. Sheet 135, 909608
Artica islandica 2	A	Recent	Unknown, Durham University.
$CaCO_3$ 2	A		-
Batch 3			
Crepidula fornicata (Slipper limpet)	A	Recent	Walton on the Naze, Essex, England. Sheet 169, 267235
Tiostrea chiliensis	A	0.5	Kupe Formation, pecten layer. S22, 028365
Pecten benedictus marwicki	A	0.5	Kupe Formation, pecten layer S22, 028365
Tiostrea chiliensis	A	1.75	Waipuru Shellbed. S22, 166446
Crepidula "radiata"	A	1.75	Waipuru Shellbed. S22, 166446
$CaCO_3$ 3	A	-	-
Batch 4			
Patella vulgata 2	B	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713
Nucella lapillus 2	B	Recent	Bamburgh beach, Northumberland, England. Sheet 75, 181356
Littorina littorea 3	B	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713
$CaCO_3$ 4	B	-	-
Batch 5			
Patella vulgata 3	B	Live	Duncan's Hole, Shetland.
Tiostrea chiliensis	B	0.34	Landguard Sand. R22, 831368
Pecten tainui	B	0.34	Landguard Sand. R22, 831368
Tiostrea chiliensis	B	2.60	Middle Waipipi. Q22, 478521
Neothyris lenticularis	B	2.60	Middle Waipipi. Q22, 478521
$CaCO_3$ 5	B	-	-

Table 1. Live, Recent and fossil shells analysed, grouped by experimental batches examined concurrently with calcium carbonate blank. Numbers after species name indicate repeated samples of the same species. Also shown is the extraction protocol used and the sampling location. Map co-ordinates for Recent and live shells are from the Ordnance survey 1:50000 Landranger series. Live *Modiolus modiolus* were dredged at approximately 200 m water depth from the Firth of Lorne and held in tanks with fresh sea water for five days until killed. S. Rowland (Department of Environmental Sciences, University of Plymouth) provided *Patella vulgata* shells collected Live from Duncan's Hole, Shetland. *Artica islandica* 2 shells were dated by amino acid racemization as Recent (G. Sykes personal communication). Fossil shells were collected from the Wanganui Basin, North Island, New Zealand. Map co-ordinates for the fossil shells are from the New Zealand Topographical Map Edition 1, 1985.

Extraction protocols for the extraction of shell lipids

Extraction protocols A and B have been used here as shown in Fig. 1. In protocol A all concentrated samples were passed through a small volume of activated alumina, in protocols B the samples were passed through a cotton wool plug only. The use of alumina removed the more polar lipid components such as cholesterol and free fatty acids, although the fatty acid methyl esters (FAMEs) and alkanes were unaffected (Chapter 1). Quantification was carried out for samples analysed using protocol B only.

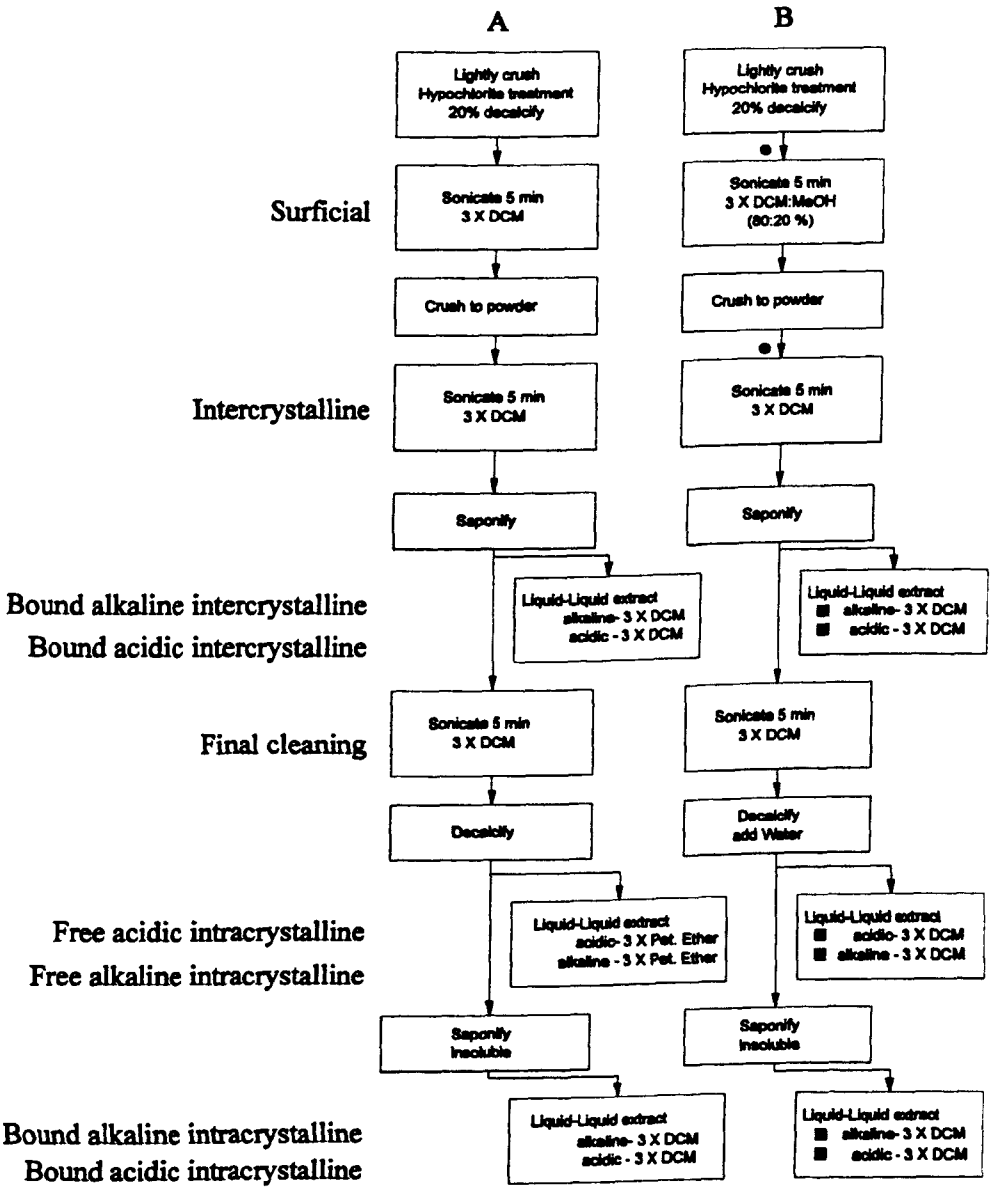


Figure 1. Flow diagram of extraction protocols A and B. Extract names apply throughout the text as on this figure. The addition of surrogate standards before solvent extraction is indicated by the following symbols: ● = lupeol, ■ = C_{36} *n*-alkane.

Inorganic calcium carbonate samples were extracted alongside the shell samples with each experimental batch. These samples are used as an experimental blank. The calcium carbonate (BDH, General Purpose Reagent, heavy precipitate) was previously Soxhlet extracted with DCM / methanol (93 : 7, v/v) for 24 h. and then heated at 450°C for 24 h.

To prevent oxidation of vulnerable lipids, such as polyunsaturated fatty acids and $\Delta^{5,7}$ sterols which had previously been reported in molluscan soft tissues (Khan & Goad, 1983), saponification was carried out under a nitrogen atmosphere and extracts were stored in a fridge.

Initial cleaning treatments

The shells were scraped to remove any encrusting organisms, lightly crushed to approximately 1 cm^2 fragments, then covered in hypochlorite (Clorox brand, >8% available chlorine) with two changes over three days and occasional sonication. The shells were rinsed, soaked in distilled water for 24 h. and decalcified by 20% of the shell weight with the slow addition of a stoichiometric amount of cold dilute HCl.

Solvent extraction of free lipids

Solvent extraction of the shells by the use of 5 min. sonication with three times 100 ml of DCM (protocol A) and three times 100 ml of DCM / methanol (80 : 20 v/v) (protocol B) with 20 ml solvent rinses, combined to yield the surficial extracts. To release intercrystalline lipids the shells were crushed in a ball mill, to pass a 500 µm sieve. Shell powders in protocols A and B were solvent extracted as above with DCM only, to provide the intercrystalline extract.

Release of bound lipids and solvent extraction

To release the bound lipids, samples were refluxed for 1.5 h. in a 2M KOH 80% (v/v) methanolic solution under a nitrogen atmosphere with constant stirring. Following this saponification, 50 ml of solvent extracted water was added to the aqueous supernatant and shaken with three times 100 ml DCM. Centrifugation (3000 rpm; 5 min.) was required to aid separation of the phases.

After full decalcification by the slow addition of concentrated HCl, two extraction techniques were used (Fig. 1). i) For protocol A, liquid-liquid extraction with three times 100 ml petroleum ether and centrifugation. This solvent is less dense than the decalcified solution and further aided phase separation. ii) For protocol B, liquid-liquid extraction with three times 100 ml DCM, with the addition of 100 to 150 ml of solvent extracted water was used to decrease the density of the decalcified solution. This only required centrifugation of the lower DCM layer to separate the insoluble

material. For all aqueous extractions, samples were extracted under both strong acidic and strong alkaline conditions, by the addition of HCl or KOH as required.

The extraction protocol uses liquid-liquid extraction of the bound fractions under firstly alkaline and then acidic conditions. The aim of the second extract was to ensure complete extraction of any protonated components (such as fatty acids) which had become dissociated under the initial alkaline conditions and therefore less soluble in the organic solvent used for extraction. Therefore the acidic and alkaline fractions are a result of the extraction technique and not the shell location. For some data analyses the alkaline and acidic fractions are combined.

Extract preparation

All extracts were prepared by removing solvent with a rotary evaporator at 30°C, and further concentrated under a stream of nitrogen at room temperature. Total extracts were derivatised with excess N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Fluka) overnight, with excess reagent removed under a stream of nitrogen prior to analysis.

Gas chromatography / gas chromatography - mass spectrometry

A Carlo-Erba 5160 fitted with an OV-1 coated (0.4 μm thickness) glass column (25 m x 0.32 mm i.d.) was used for GC analysis of reagents. This was equipped with an on-column injector and a flame ionization detector (FID). Hydrogen was used as the carrier gas, cold on-column injection was used and the FID maintained at 310°C. The temperature of the oven was programme from 50°C (2 min.) to 300°C (20 min.) at 4°C / min. For sample analysis a Carlo-Erba HRGC Mega series was employed, fitted with a HP-5 coated (0.25 μm film thickness) fused silica column (60 m x 0.25 mm i.d.). This was equipped with an on-column injector and a FID. Hydrogen was the carrier gas, cold on-column injection was used and the FID maintained at 310°C. The temperature of the oven was programmed from 50°C (2 min.) to 300°C (40 min.) at 4°C / min. Data were collected using Multichrom (VG Data Systems, Micro Vax 3100).

Gas chromatography combined with mass spectrometry (GC-MS) was carried out using a Fisons 8060 GC connected to a Fisons Trio1000 mass spectrometer. The GC was equipped with an on-column injector and fitted with a HP-5 coated (0.25 μm film thickness) fused silica column (30 m x 0.25 mm i.d.) with 2 m deactivated fused silica 0.32 mm i.d. retention gap. Cold on-column injection was used and helium was the carrier gas. The temperature of the oven was programmed from 50°C (2 min.) to

150°C at 10°C / min. then to 300°C (25 min.) at 4°C / min. The column was directly inserted into the ion source. Electron impact (EI) spectra were obtained at 70 eV with full scan from 50 to 650 m/z, cycle time 1 s. Positive chemical ionisation (+ CI) was also carried out, using methane as the collision gas with identical chromatographic conditions as described for EI GCMS.

Quantification

Quantification of surficial and intercrystalline cholesterol was carried out relative to the lupeol surrogate standard added prior to solvent extraction (Fig. 1). The remaining extracts were quantified relative to the C_{36} *n*-alkane surrogate standard. A relative response factor (RRF) of 1.563 was used for lupeol to cholesterol. RRFs for C_{19} *n*-alkane (0.762), C_{18} FAME (0.924) and cholesterol (1.242) to the C_{36} *n*-alkane surrogate standard were used for the extracted alkanes, FAMEs and cholesterol respectively. A RRF of one was used for the phthalate plasticisers and alcohols to the C_{36} *n*-alkane surrogate standard. See Appendix 3 for calculations.

Multivariate statistical analysis

A data set of samples with multiple variables are difficult to interpret even in a graphical form. For this reason statistical techniques have been developed to simplify the data and present the results in forms which are easier to interpret. Hierarchical cluster analysis and principal component analysis have been used in this study. These are discussed below. A simplified example is also shown. These statistical methods are reviewed elsewhere (e.g. Afifi & Clark, 1984; Manly, 1986).

For the samples examined in this study missing data with no or trace lipid components were assigned a zero value. This results in a loss of potential information, and assigns a specific value (zero) for an unknown. For these samples this was shown not to have a significant effect. Each data set was normalised to 100%. SPSS for Windows release 5.0 (June 20 1992) and release 6.0 (June 17 1993) were used for multivariate statistical analysis. For principal component analysis, the same number of factors as variables were used and a regression method was used to calculate the factor scores. Hierarchical cluster analysis using an agglomeration schedule with a between-group linkage cluster method and intervals measured by squared Euclidean distances was used to produce the dendrograms. A variety of cluster methods and interval measurements were tested and gave similar results to those presented here.

Example data set

A simple data set is shown in Table 2. This is to be used as an example of these statistical techniques. Each sample contains a number of measured variables. The variables used in this study are either the percentage compositions of each carbon chain length for a lipid class, or the percentage steroidal composition for each extract.

	Variables				
Samples	Mollusca	Brachiopoda	Gastropoda	Bivalvia	Cephalopoda
Patella vulgata	1	0	1	0	0
Littorina littorea	1	0	1	0	0
Artica islandica	1	0	0	1	0
Sepia officinalis	1	0	0	0	1
Neothyris lenticularis	0	1	0	0	0

Table 2. Shell species are the samples, variables are the Phylum (Mollusca or Brachiopoda) and the molluscan Class (Gastropoda, Bivalvia or Cephalopoda). 1 Indicates membership, 0 indicates the absence of membership.

Hierarchical cluster analysis

Hierarchical methods result in a dendrogram. The agglomeration method used here starts with groups each containing one sample, and combines the "closest" groups until all samples are within one group. The definition of "closest" depends on the method used. Squared Euclidean distances used here, measures the distances between the data points by extending Pythagorean theory to multiple dimensions.

As different methods for calculating the "closest" groups can produce different dendrograms different methods should also be tested.

Interpreting dendrograms

The relatedness between the samples is measured by the horizontal distance between the samples using only the lines on the dendrogram. Samples plotted with the least distance between samples have most similarity and samples at greatest distance having the least similarity. The vertical distances only separate the samples. As the distances are normalised, so that the greatest distance between samples is that horizontally across the page, it is impossible to compare different dendrograms. The use of outgroups, which are samples known to be unrelated to the samples being analysed, allows a comparison of the relative distances within the sample set.

Fig. 2 shows the dendrogram produced using the data set above. The horizontal distances between the *Patella vulgata* and *Littorina littorea* samples are the smallest, and Table 2 shows that their properties (variables) are identical. The horizontal distances between *Artica islandica, Sepia officinalis, Patella vulgata (sic)* and *Littorina littorea (sic)* are also exactly the same indicating a common property; that of being a mollusc and either being a bivalve, cephalopod or gastropod. The depth of the branching indicates that *Patella vulgata* and *Littorina littorea* are more closely related than *Artica islandica* and *Sepia officinalis*. The brachiopod is at the greatest distance from all the other samples and is acting as an outgroup. Deciding how many groups of samples are formed by the dendrogram is arbitrary and depends on the usage of the data. Generally the cutoff line is drawn where the horizontal distances between the branches are largest. Two cutoff values, shown by the dashed line, are plotted in Fig. 2. Cutoff 1 separates the molluscs at the Class level and the Brachiopoda Phylum, and cutoff 2 separates the Brachiopoda and Molluscan Phylum.

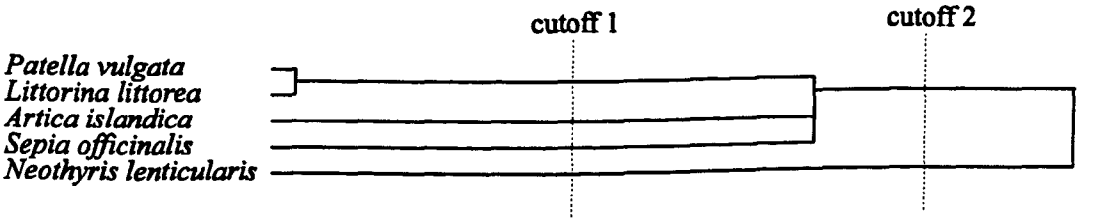


Figure 2. Dendrogram of shell species. Using an agglomeration schedule with a between-group linkage cluster method and intervals measured by squared Euclidean distances. Two cutoff values are indicated by the vertical dashed lines.

Principal component analysis

The object of principal component analysis is to take the original variables and find combinations of these (called factors) which are uncorrelated, and therefore independent to one another. These new factors are selected so that the greatest variance in the data set will be in the first factor, with the following factors having decreasing variance. In general the first few factors will contain most of the variation exhibited by the data set, this commonly allows a scatter plot with only two axes.

Interpreting principal component analysis

Table 3 shows the eigenvalues for each factor. The eigenvalues specify the variation in the factors. The variation decreases with each factor, and for this example all the variation is contained within the first three factors.

Factor	Eigenvalue	Percent of variation	Cumulative percent
1	2.30196	46	46
2	1.44804	29	75
3	1.25	25	100
4	0	0	100
5	0	0	100

Table 3. Eigenvalues, percentage variation and cumulative percent of the variation for each eigenvalue.

Table 4 shows the factor matrix containing the eigenvectors. The eigenvectors have values (loadings) between -1 and 1 and show the contribution of each of the original variables to the factors. Zero values show no contribution of that factor to the variables, Table 4 showing that factor 3 does not affect the brachiopods, gastropods or mollusc variables and factors 4 and 5 contain no variation (as shown in Table 3). A high positive or negative eigenvalue indicates that the variable is influential in causing the variation in the data set. Fig. 3 plots the first three factors from Table 4, it can be seen that factor 1 is influential for the Brachiopoda and Mollusca. The analysis of the contribution of variables to the variation of the data set is a major advantage of principal component analysis over hierarchical analysis.

Variables	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Bivalvia	0.18712	0.58308	-0.79057	0	0
Brachiopoda	-0.99396	-0.10977	0	0	0
Cephalopoda	0.18712	0.58308	0.79057	0	0
Gastropoda	0.50599	-0.86254	0	0	0
Mollusca	0.99396	0.10977	0	0	0

Table 4. Factor matrix of eigenvectors

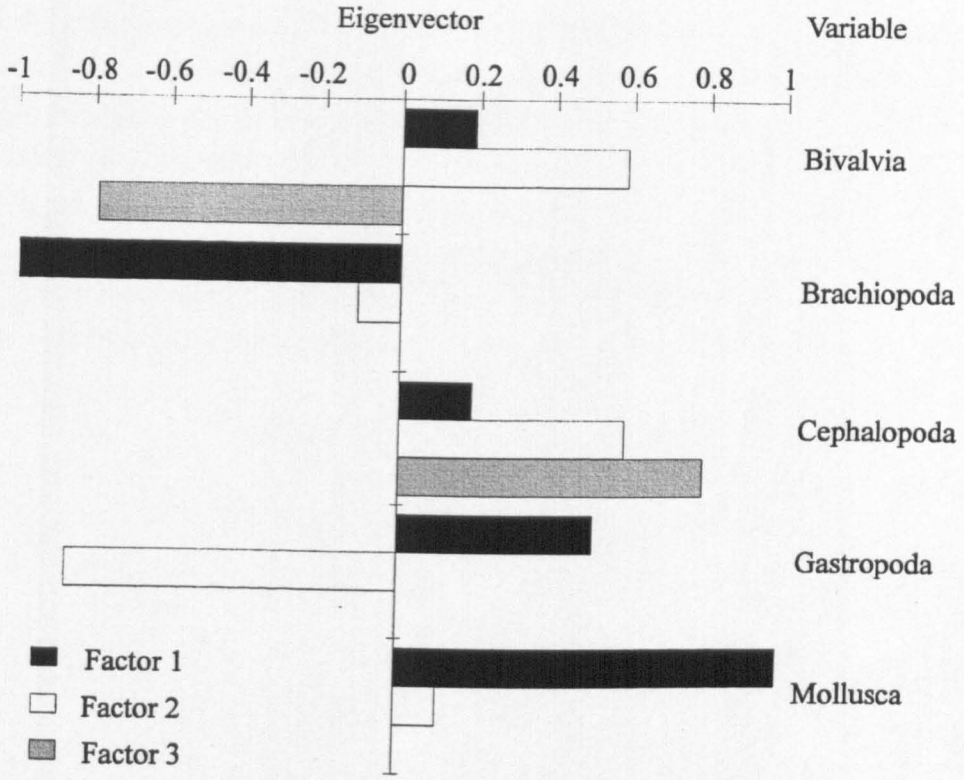


Figure 3. Loading plot of eigenvectors for factors 1, 2 and 3 for each variable.

Multiplying the eigenvectors and the variables of the original data set gives the factor scores for the samples. (e.g. For *Patella vulgata* the factor score 1 is = (0 x 0.18712) + (0 x -0.99396) + (0 x 0.18712) + (1 x 0.50599) + (1 x 0.99396)). However a regression method is used, so that the factor scores are adjusted so that the mean is zero. The sample set now shows the greatest variance and as above, the factor scores are independent and decrease in variation. Table 5 shows the scores for the example data set.

Sample	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Patella vulgata	.55428	-.94487	0	0	0
Littorina littorea	.55428	-.94487	0	0	0
Artica islandica	.33474	1.04305	-1.41421	0	0
Sepia officinalis	.33474	1.04305	1.41421	0	0
Neothyris lenticularis	-1.77804	-.19637	0	0	0

Table 5. Factor scores for the samples.

These factor scores can now be plotted on perpendicular axes. Fig. 4 shows the scatter plots for the combinations of the first three factors. As for the dendrogram, the *Patella vulgata* and *Littorina littorea* samples are plotted together as the original variables are identical. As observed above, factor 1 is influential for the Brachiopoda (high negative) and Mollusca (high positive) and therefore factor 1 separates these samples and this is the greatest variation in the sample set. Similarly factor 2 separates the gastropods from the bivalves and cephalopods. Combinations of the factors and a larger data set often result in clear distinctions between subsets of samples.

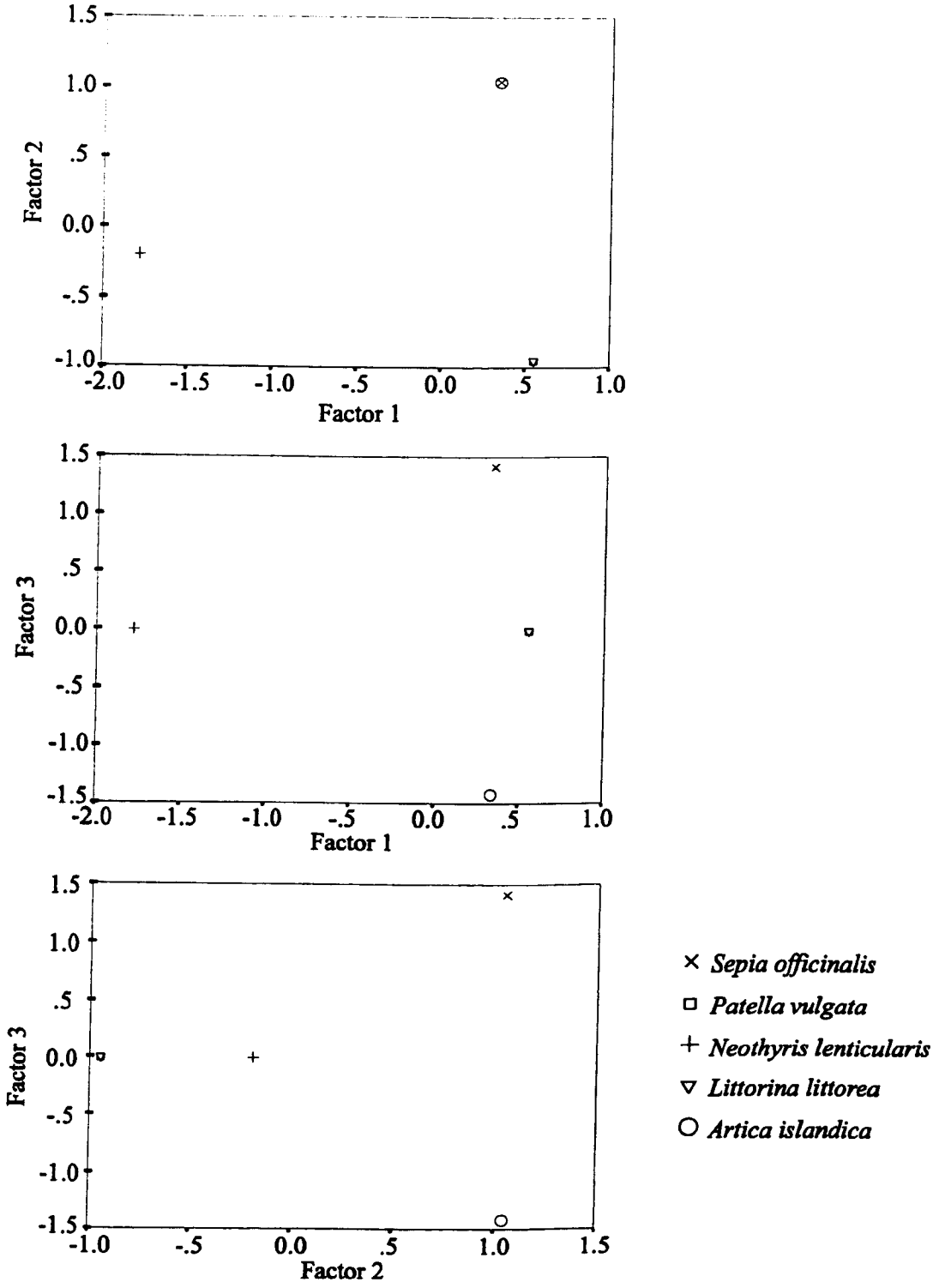


Figure 4. Scatter plot showing the combinations of factor scores extracted using principal component analysis.

RESULTS AND DISCUSSION

Pigments

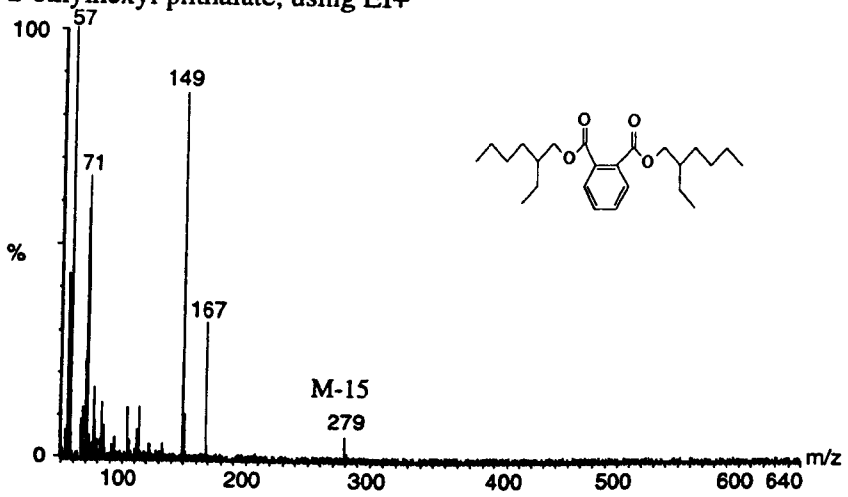
Untreated shells were observed to yield a green pigment from the surficial extracts, probably chlorophyll derived from algae. However, no coloured extracts were recovered from this fraction after hypochlorite and partial decalcification treatments. No coloured extracts were recovered from the other fractions. This indicates that the pigments were either not extracted by the extraction protocol, or they were destroyed by the cleaning methods and conditions used for lipid extraction, such as saponification and high or low pH values.

A) Distinguishing indigenous shell lipids from laboratory contamination

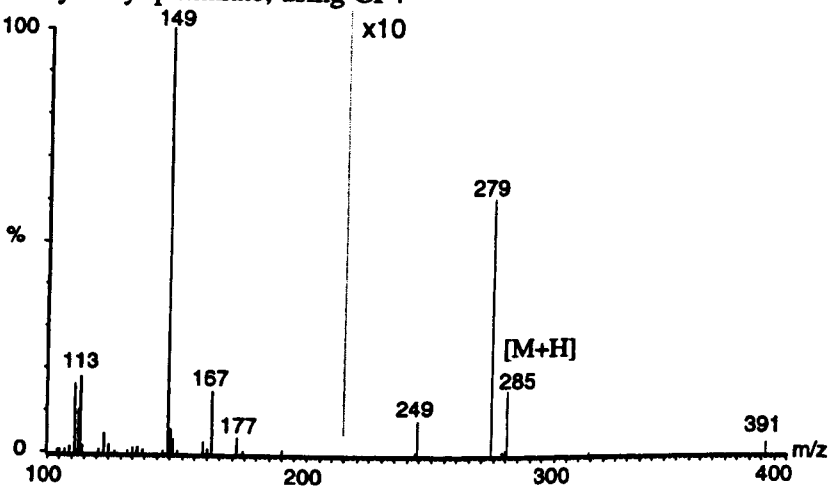
Phthalate plasticisers

Phthalate plasticisers are ubiquitous in the modern environment including the laboratory (Blumer, 1965; Middleditch, 1989). They are easily identified by their GC retention times and characteristic mass spectra (Safe & Hutzinger, 1973; Atlas & Giam, 1985; Middleditch, 1989). The mechanism of fragmentation producing the diagnostic 149 m/z is shown in Appendix 4. Six different phthalate plasticisers (labelled A to F) have been identified in the shell extracts, of these four (A, C, E and F) are dominant. Di-2-ethylhexyl phthalate (E) and di-*n*-butyl phthalate (F) have been identified from their mass spectra which are shown in Fig. 5.

E = Di-2-ethylhexyl phthalate, using EI+



E = Di-2-ethylhexyl phthalate, using CI +



F = Di-*n*-butyl phthalate

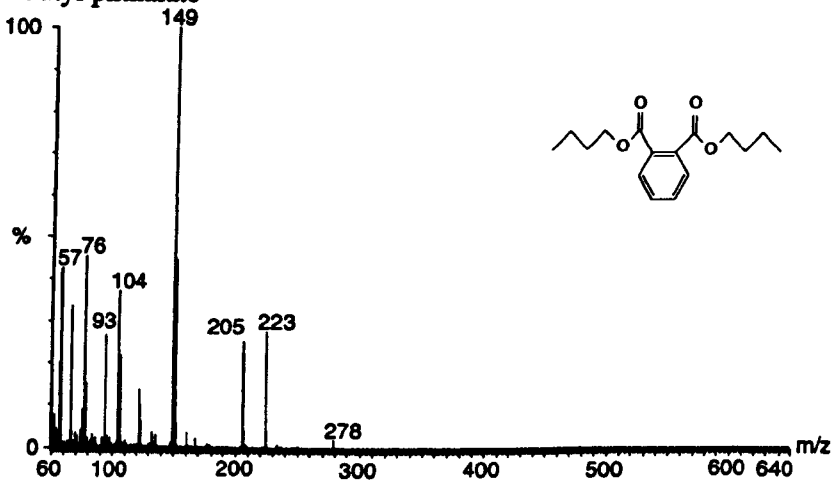


Figure 5. Mass spectra and structure of Di-2-ethylhexyl phthalate (E) using + EI and + CI, and the mass spectra and structure of Di-*n*-butyl phthalate (F) using + EI.

Fig. 6 shows the phthalate plasticisers extracted from Recent shells. Fig. 7 shows the phthalate plasticisers extracted from fossil shells. The yields (μg phthalate per g of extracted sample) of the six plasticisers (A to F) are shown for the intercrystalline bound and all intracrystalline fractions. The yields of phthalate plasticisers extracted from the calcium carbonate blanks are plotted as asterisks (*) on the same axes.

Fig. 6 shows that when extracted from Recent shells, the yields of phthalate plasticisers are greater in the shells than the calcium carbonate blank which indicates the phthalates are indigenous to the shell. However, the yields in the fossil samples are also higher than the calcium carbonate blank (Fig. 7), but the occurrence of indigenous phthalate plasticisers in fossil material, and particularly in the environmentally inaccessible intracrystalline sites can be excluded. Therefore the most likely source of these compounds is from laboratory contamination.

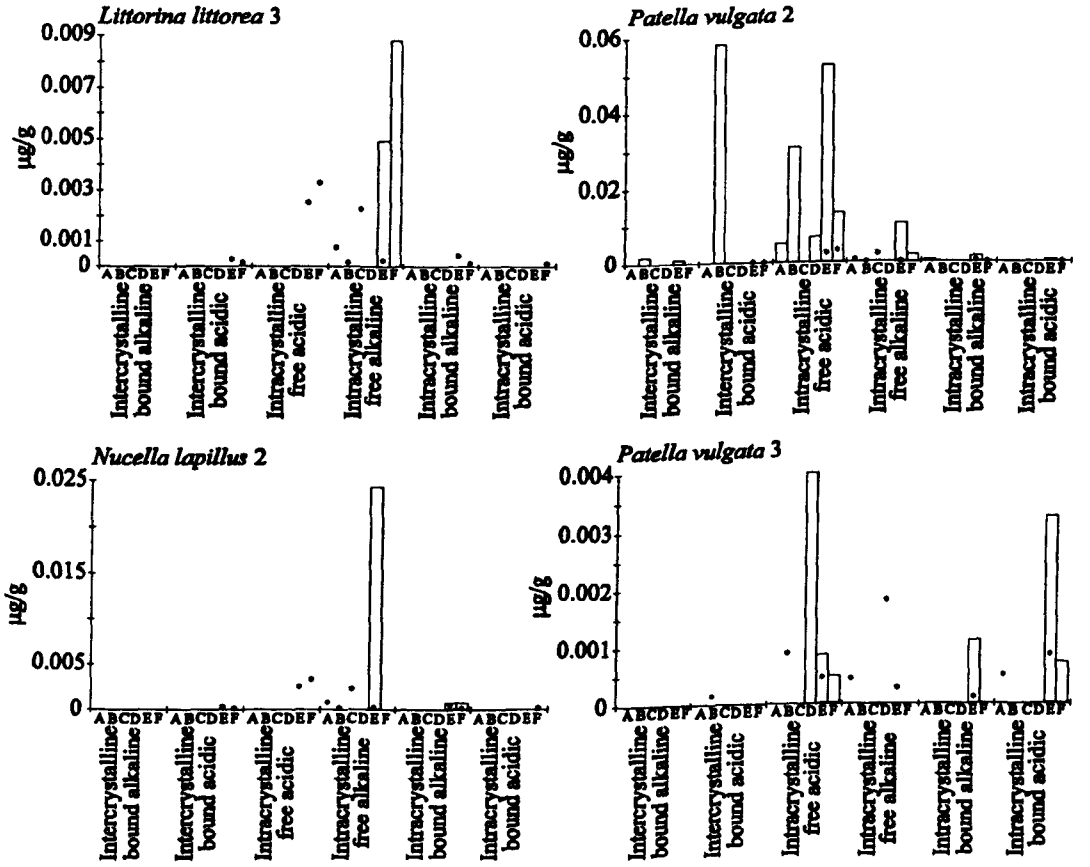


Figure 6. Quantified yields (μg) of phthalate plasticisers per g of extracted sample, for Recent shells. Phthalate plasticisers are labelled A to F for each shell fraction. The yields of phthalate plasticisers in the calcium carbonate blank are plotted as asterisks (*).

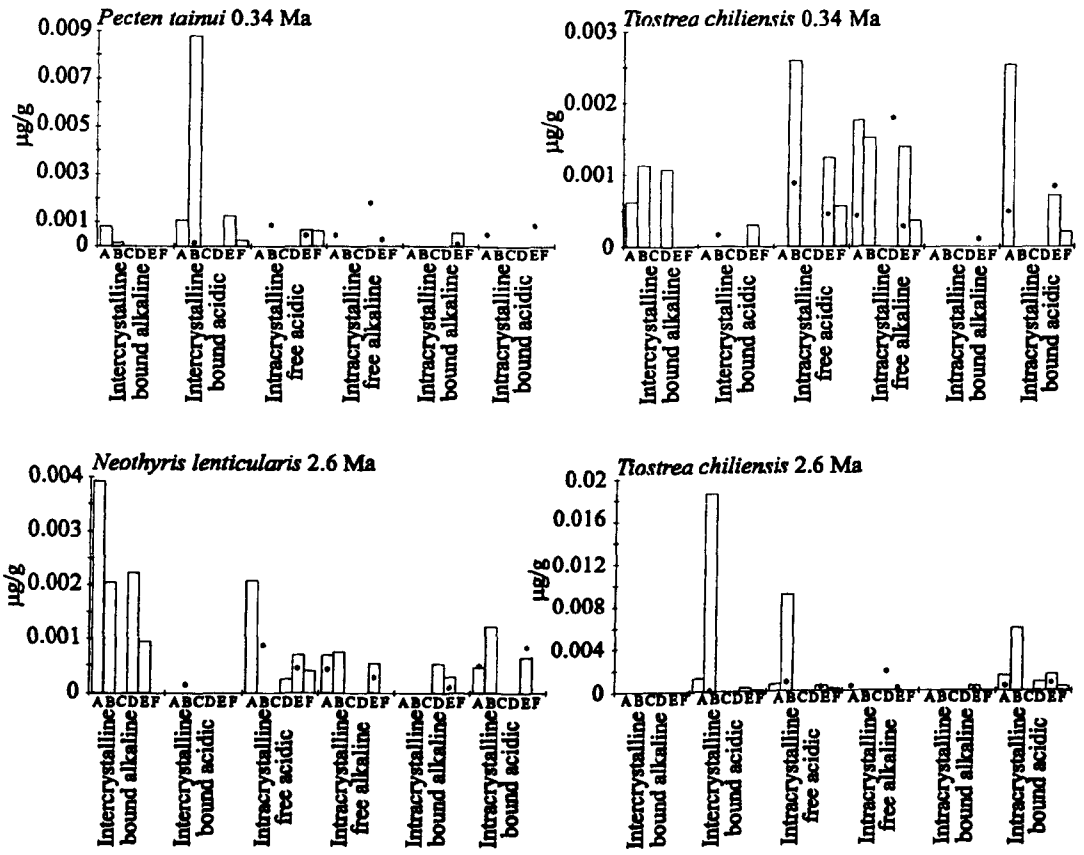


Figure 7. Quantified yields (µg) of phthalate plasticisers per g of extracted sample, for fossil shells. Phthalate plasticisers are labelled A to F for each shell fraction. The yields of phthalate plasticisers in the calcium carbonate blank are plotted as asterisks (*).

Higher yields of phthalates in the shells than the blanks indicates that phthalate contamination cannot be reliably revealed by the calcium carbonate blank. Table 6 shows the yields of phthalate plasticisers extracted from the calcium carbonate blanks 4 and 5. The extraction of phthalates in some fractions and not in others, shows that the distributions of the contamination is also extremely variable. The different distributions of the plasticisers A to F may also indicate a variety of different contaminant sources. Blumer (1965) suggests that these volatile compounds are present as a molecular dispersion in air, and are therefore absorbed onto any active surface with an increasing contaminant yield with increasing exposure times. Therefore these compounds which are undoubtedly present in the marine environment cannot be reliably examined due to their ubiquitous and irreproducible contamination of the laboratory.

$CaCO_3$ 5	Intercrystalline bound alkaline	Intercrystalline bound acidic	Intracrystalline free acidic	Intracrystalline free alkaline	Intracrystalline bound alkaline	Intracrystalline bound acidic	Mean	Standard deviation
A	0	0	0	0.000445	0	0.000503	0.000158	0.000245
B	0	0.000163	0.000884	0	0	0	0.000175	0.000354
C	0	0	0	0	0	0	0	0
D	0	0	0	0.001804	0	0	0.000301	0.000736
E	0	0	0.000462	0.000287	0.000118	0.000857	0.000287	0.000331
F	0	0	0	0	0	0	0	0
$CaCO_3$ 4	Intercrystalline bound alkaline	Intercrystalline bound acidic	Intracrystalline free acidic	Intracrystalline free alkaline	Intracrystalline bound alkaline	Intracrystalline bound acidic		
A	0	0	0	0.000773	0	0	0.000129	0.000315
B	0	0	0	0.000194	0	0	3.23E-05	7.9E-05
C	0	0	0	0.00229	0	0	0.000382	0.000935
D	0	0	0	0	0	0	0	0
E	0	0.000299	0.002524	0.000263	0.000458	0	0.000591	0.000964
F	0	0.000161	0.00329	0	0.000149	0.000168	0.000628	0.001307

Table 6. Yields (µg/g of $CaCO_3$ extracted) of phthalate plasticisers, labelled A to F, extracted from the intercrystalline bound and all intracrystalline fractions from calcium carbonate blanks 4 and 5.

n-Alkanes

The sum of the quantified *n*-alkanes (μg of total *n*-alkanes per g of sample) from each of the intercrystalline bound and intracrystalline free and bound fractions are illustrated in Fig. 8. The shells were extracted together in experimental batch 4 and the calcium carbonate blank is also shown.

The single extraction of shell *n*-alkanes from intercrystalline fractions limits the interpretation although the yields in the calcium carbonate blank are negligible which indicates the *n*-alkanes are indigenous. For the intracrystalline free fractions the yields are also higher in shell extracts than the blank. However for the intracrystalline bound fractions the yields of *n*-alkanes are similar in the shells and the blank.

The *n*-alkanes are not bound to the shell matrix by covalent bonding. Instead they are physically retained either by adsorption within an inorganic or an organic structure. The *n*-alkanes released after decalcification are extracted in the intracrystalline free fractions (Fig. 8); these are *n*-alkanes trapped within the shell by the inorganic structure. Other *n*-alkanes are released after disruption of the organic matrix by saponification and are extracted in the intercrystalline bound and intracrystalline bound fractions. The release of physically and chemically entrapped *n*-alkanes indicates that post-depositional ingress is not their source. Fig. 8 also shows the extraction of *n*-alkanes from the second solvent extraction of the aqueous solutions (giving the intercrystalline bound acidic, intracrystalline free alkaline and intracrystalline bound acidic fractions, as described in Fig. 1).
This indicates inefficient extraction by the first aqueous extract. This is however a function of the extraction protocol and not the shell location.

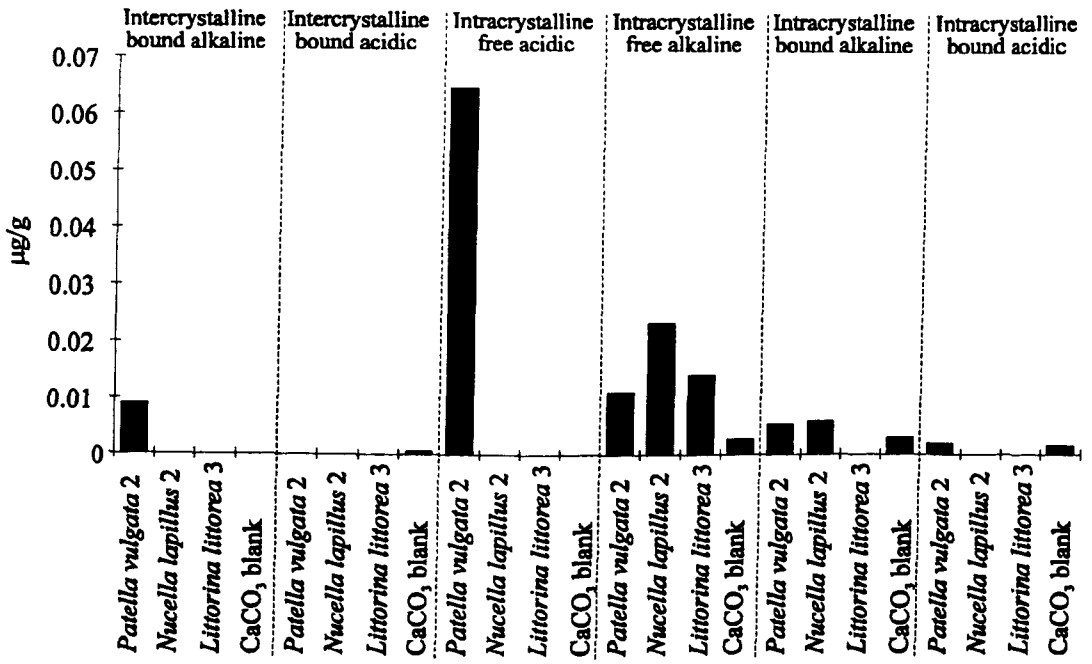


Figure 8. Quantified yields (µg) per g of extracted sample, for the total *n*-alkanes extracted from intercrystalline bound and all intracrystalline fractions.

The carbon number distributions of the quantified *n*-alkanes (µg of individual *n*-alkanes per g of sample) for *Littorina littorea* 3 shells are shown in Fig. 9. The only fraction which contained *n*-alkanes was the intracrystalline free alkaline extract. The yields and carbon number distributions of the blank are also shown. The yields of *n*-alkanes are higher in the shell than the blank for all the *n*-alkane carbon numbers, which strongly suggests that the *n*-alkanes are indigenous. Despite this, the contamination shown by the blank contributes significantly to the shell yields. As the *n*-alkanes are ubiquitous in the laboratory, useful information derived from the shell *n*-alkane extracts can only be obtained after a comparison of yields and carbon number distributions with the blanks. The *n*-alkane distributions for other species are discussed in Chapter 4.

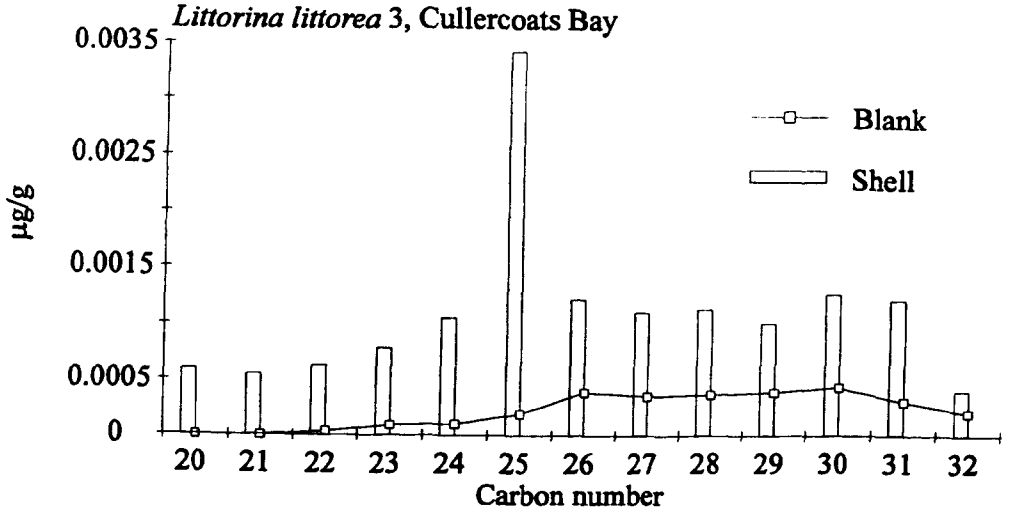


Figure 9. Quantified yields (µg) per g of extracted sample, for the *n*-alkane carbon number distributions. Columns indicate the intracrystalline free acidic extract of *Littorina littorea* 3. The calcium carbonate blank is shown as -□-.

Regular isoprenoids

Table 7 shows the ratio of pristane to phytane (where they are present) and also indicates where they occur singly. The extraction of pristane and phytane from the shells and not the calcium carbonate blank in some fractions, indicates these compounds could be indigenous to the shells. However these compounds were not quantified, although they were observed to be minor components of the total extracts. These data are therefore excluded from further analysis.

	Experimental batch	Surficial	Intercrystalline	Intercrystalline bound alkaline	Intercrystalline bound acidic	Final cleaning extract	Intracrystalline free acidic	Intracrystalline free alkaline	Intracrystalline bound alkaline	Intracrystalline bound acidic
$CaCO_3$ blank 1	1		Pr			0.69				
Littorina littorea 1	1	0.67	1.02			0.76			Pr	
Modiolus modiolus	1	Ph	Ph	0.42		0.42				1.40
Patella vulgata 1	1	Ph	Pr		Ph	0.80				Ph
Artica islandica 1	2		0.71			0.72		Ph		
Artica islandica 2	2	0.49	0.96	0.51						
Littorina littorea 2	2	Pr	2.16							
Nucella lapillus 1	2	1.20	Pr							
Sepia officinalis	2					0.90				
$CaCO_3$ blank 3	3				Ph				2.02	0.82
Crepidula fornicata	3					2.07	0.61	0.75		0.92

Table 7. Shells and calcium carbonate blanks where pristane and phytane were extracted. Shells are grouped by experimental batch with the calcium carbonate blank. Pristane to phytane ratios are shown and Pr or Ph indicate the extraction of only pristane or phytane respectively.

n-Alcohols

Fig. 10 shows the carbon number distributions of the intercrystalline bound alkaline (filled columns) and intracrystalline bound alkaline (open columns) *n*-alcohols from Recent shells. As no quantification was possible for extracts from protocol A, the data have been normalised by dividing the peak area of each compound by the total peak areas and multiplying to 100%. The *n*-alcohols extracted from the calcium carbonate blanks are shown as joined points (■ = intercrystalline bound alkaline, and □ = intracrystalline bound alkaline). *n*-Alcohol homologous series are predominantly extracted from the bound alkaline fractions and therefore only these extracts are discussed. All samples in Fig. 10 show an even over odd carbon number predominance, with a carbon number maximum at C_{16} or C_{18} for the shells. The calcium carbonate blanks similarly have an even over odd carbon number distribution and a carbon number maximum at C_{18}. However, the carbon number distributions for intercrystalline bound alkaline *Artica islandica* 2, *Littorina littorea* 2 and both fractions of *Nucella lapillus* 1 are different to the blank which suggests the *n*-alcohols are indigenous. With the exception of the *Artica islandica* 2, where both intercrystalline bound and intracrystalline bound fractions are extracted from the same

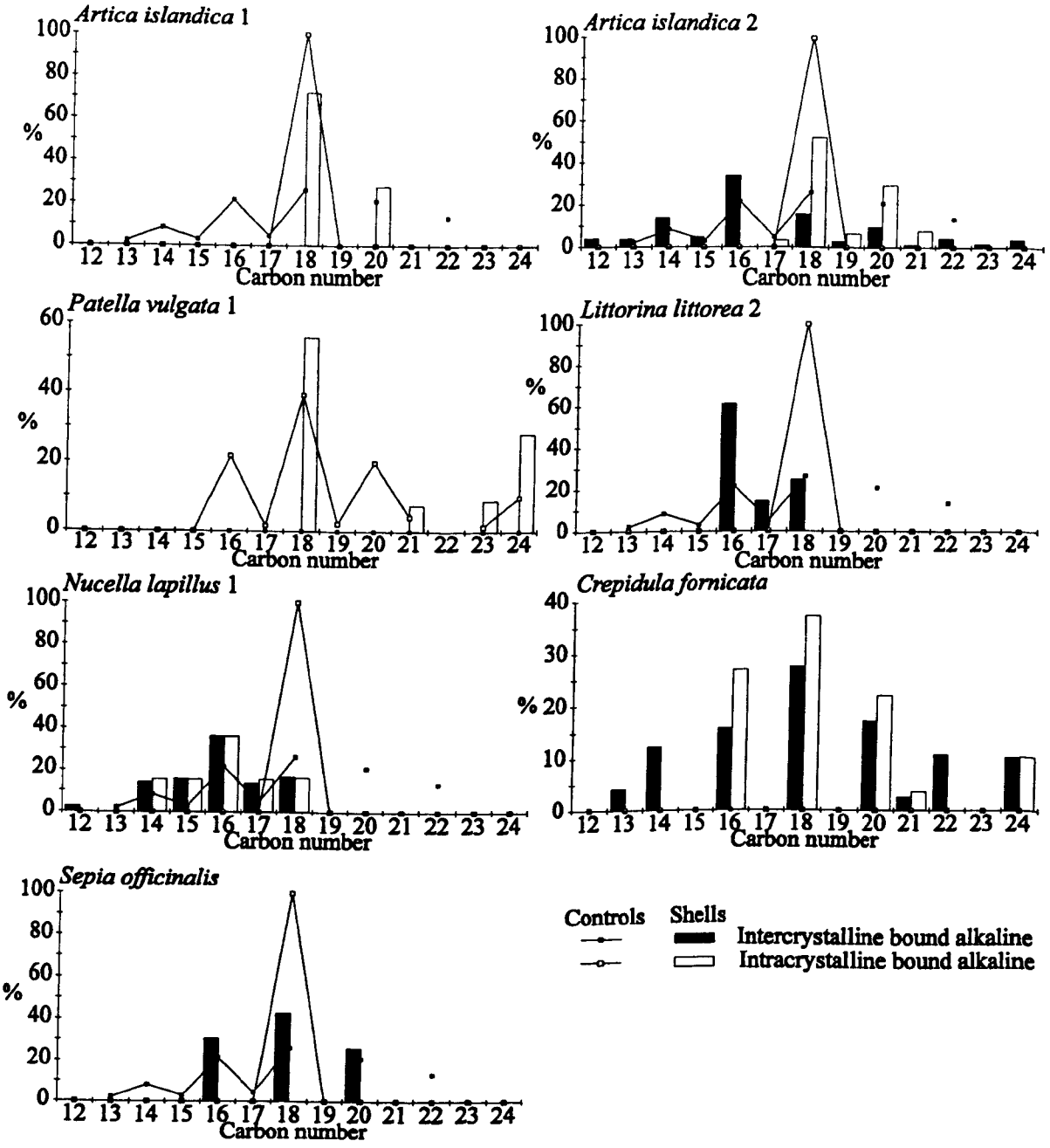


Figure 10. Relative abundance (%) of *n*-alcohol carbon number distributions for intercrystalline bound alkaline and intracrystalline bound alkaline shell fractions normalised to 100% of each extract. Shell extracts are illustrated by columns, shading representing each fraction. Calcium carbonate blanks are shown as -□- with the same shading as the fraction extracted.

shell (*Nucella lapillus* 1 and *Crepidula fornicata*) the carbon number distributions are similar. However without quantification it is difficult to ascertain the contribution of the laboratory contamination to the shells. The only quantified homologous series of *n*-alcohols from *Littorina littorea* 3 extracted using protocol B, is shown in Fig. 11. However no *n*-alcohols were detected in the calcium carbonate blank to compare the yields of potential contamination.

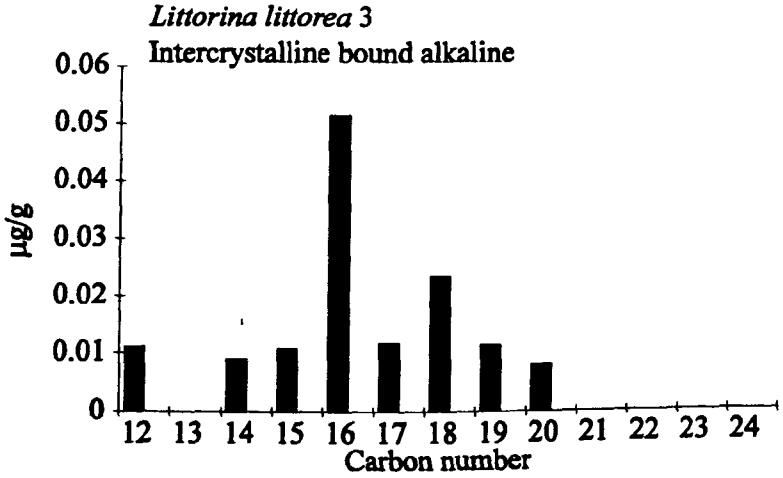


Figure 11. Quantified yields (µg) per g of shell extracted, for the *n*-alcohol carbon number distributions from the intercrystalline bound alkaline fraction of *Littorina littorea* 3

Free fatty acids

Free fatty acids were extracted from a limited number of shell samples (4 of 13 shells, each having nine extracted fractions), as shown in Table 8. It is also noteworthy that only a single homologue was extracted for three of the shells. No free fatty acids were extracted from the calcium carbonate blanks examined with these samples, which indicates that the free fatty acids are indigenous. However due to the limited data available, the free fatty acids were not subjected to any further interpretation.

Sample	Extract	15	16	17	18
Littorina littorea 2	Intracrystalline bound alkaline	0	0	0	100
Sepia officinalis	Intracrystalline bound alkaline	0	100	0	0
Littorina littorea 3	Intracrystalline free acidic	0	24	3	73
Nucella lapillus 1	Intercrystalline bound alkaline	0	0	100	0

Table 8. Carbon number distributions of the free fatty acids from Recent shells. The extract is shown and the carbon number distributions have been normalised to 100%.

Bound fatty acids

Bound fatty acids are extracted after saponification (Fig. 1), the resulting transesterification yields fatty acid methyl esters (FAMEs) due to the methanolic conditions used here. Therefore all FAMEs have been covalently bound by an ester bond into other organic material, possibly components such as phospholipids, triglycerides or steryl esters.

Fig. 12 shows the FAME yields (μg of FAMEs per g of extracted shell) from *Littorina littorea* 3 as a function of carbon number. The calcium carbonate blank shows no FAMEs were extracted from the intercrystalline bound acidic fraction and an insignificant amount of C_{18} FAME is extracted from the intracrystalline bound alkaline fraction. This indicates indigenous FAMEs are extracted from the shells. An even over odd carbon number distribution is maintained for the shell extracts. A carbon number maximum of either C_{16} or C_{18} is also observed. Intracrystalline fractions have lower yields than the intercrystalline extracts.

The shell FAMEs discussed here are exclusively saturated; unsaturated components were identified by GCMS but are not discussed further due to small yields and coelution.

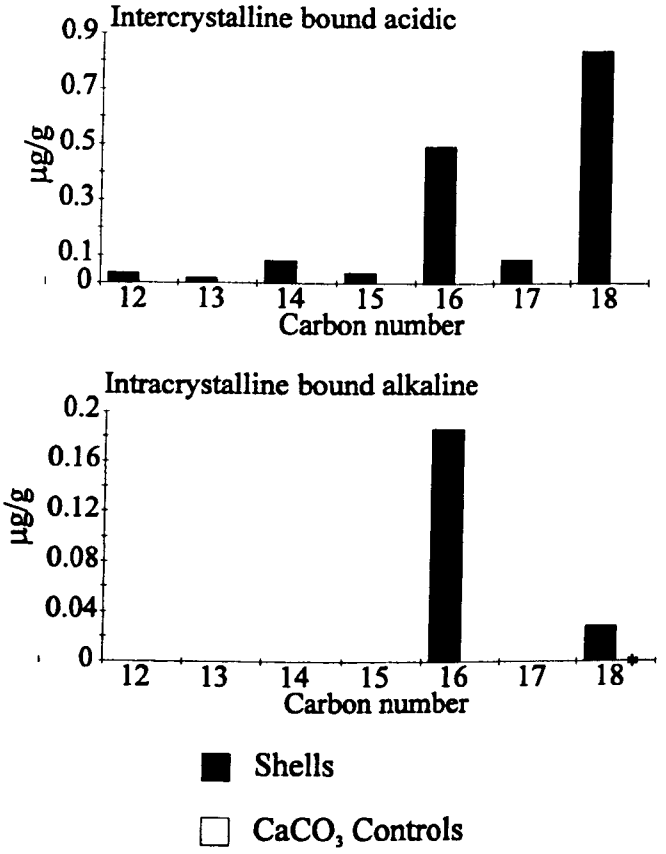
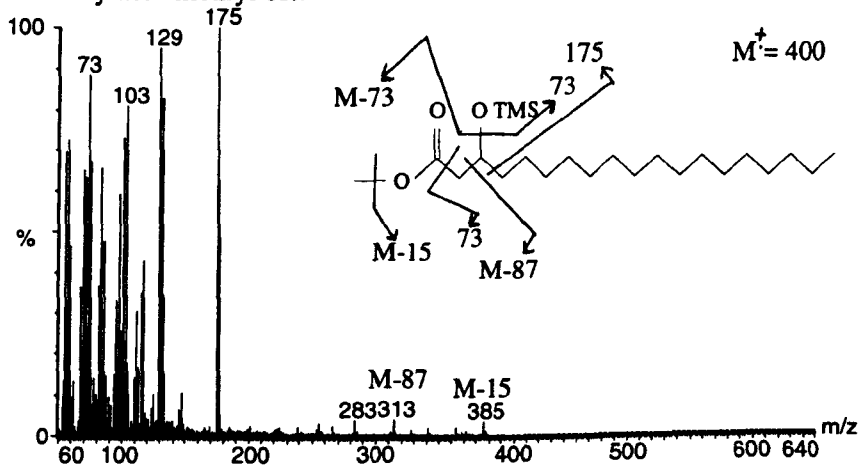


Figure 12. Quantified yields (µg per g of sample extracted) of the FAME carbon number distributions for *Littorina littorea* 3. Shell extracts are illustrated by filled columns and the calcium carbonate blanks by unfilled columns. Only the intracrystalline bound alkaline C_{18} FAME from the calcium carbonate blank (marked by *) was extracted.

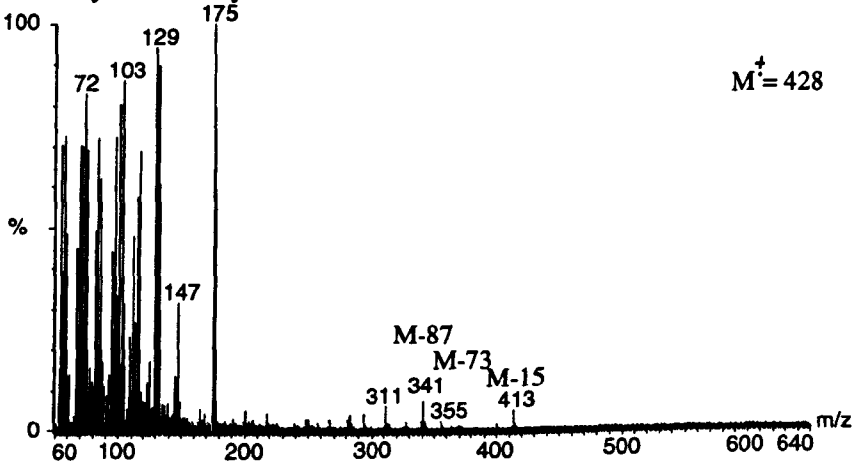
β-hydroxy fatty acids

A series of homologous peaks have been tentatively identified as β-hydroxy fatty acids. Mass spectra (Fig. 13) are comparable to those of Matsumoto and Nagashima (1984) and Mielniczuk *et al.* (1992) although with less abundant M^{+}-15 ions than reported.

C_{19} β-OH fatty acid methyl ester



C_{21} β-OH fatty acid methyl ester



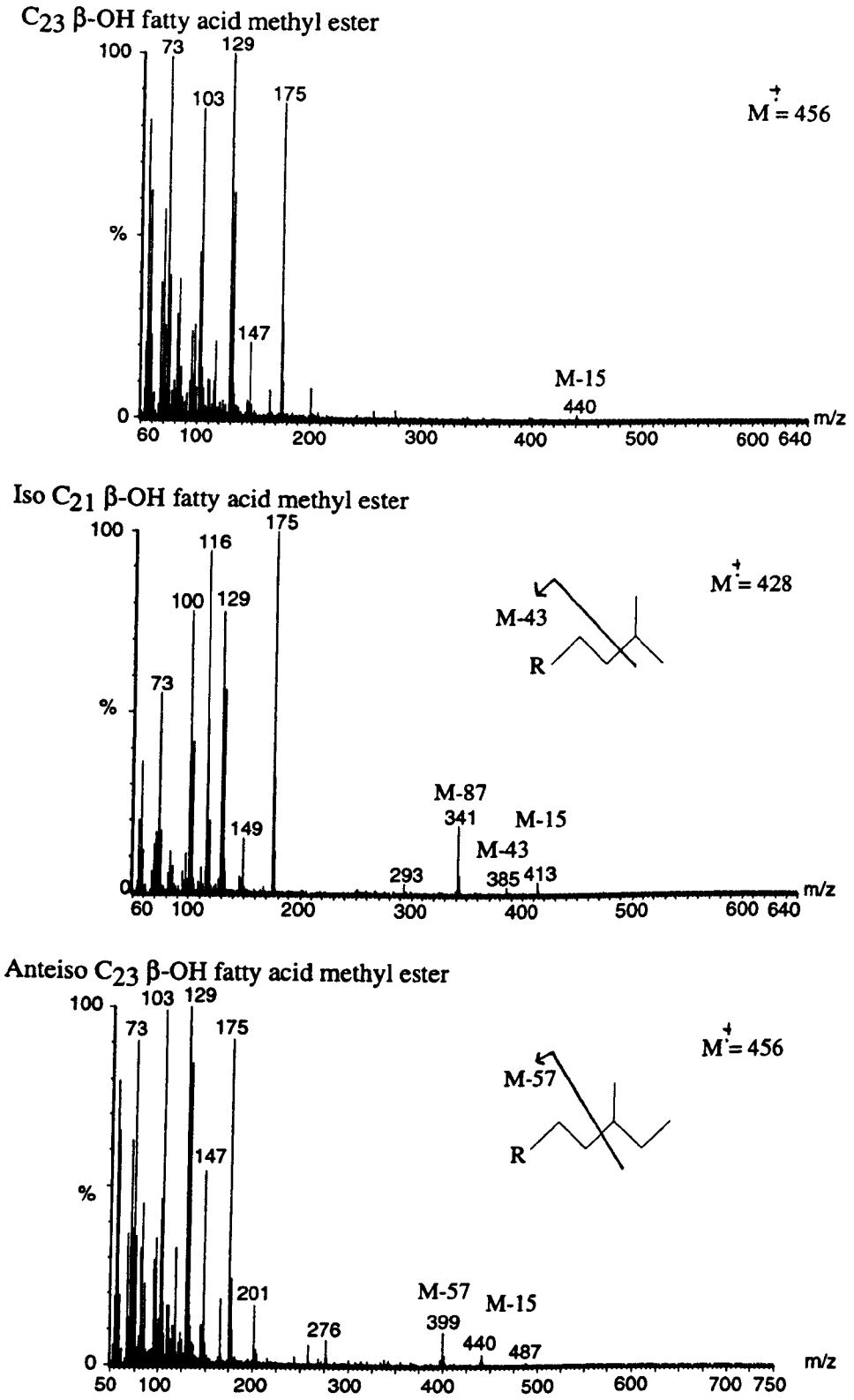
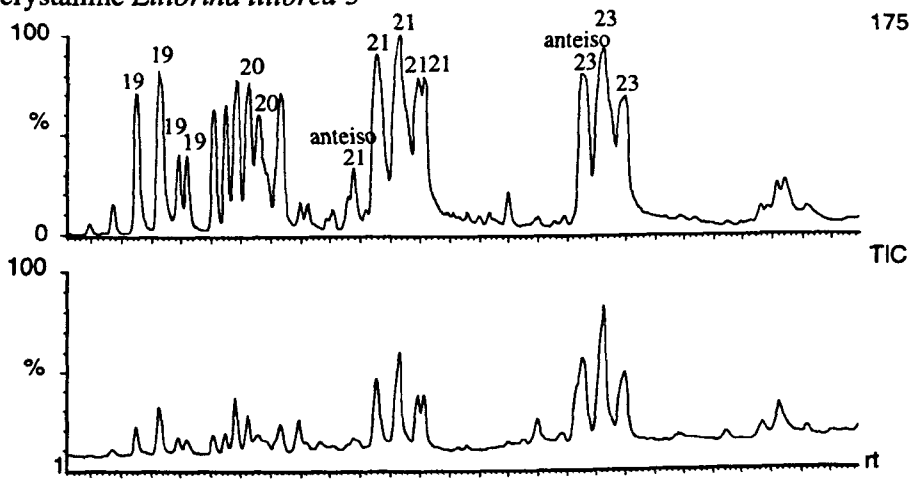


Figure 13. Selected mass spectra of TMS derivatised β-hydroxy methyl esters. The chemical structure and suggested fragmentation are illustrated.

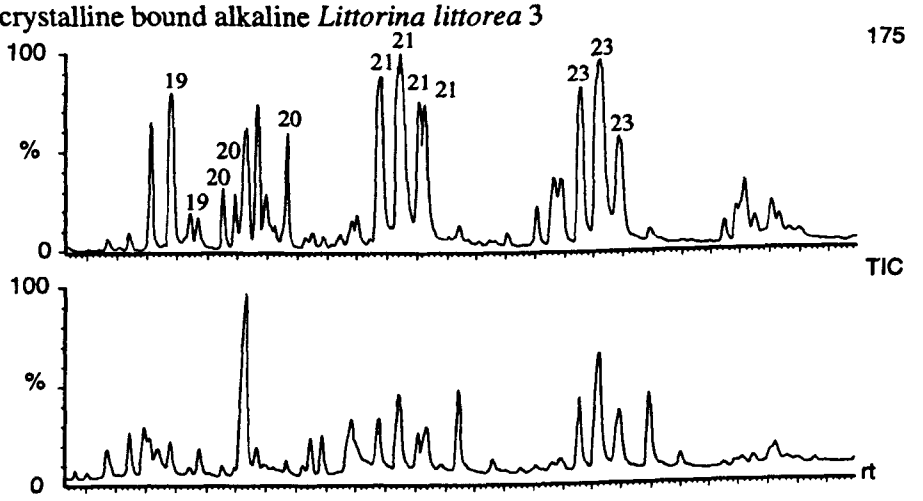
The extraction of the β-hydroxy fatty acids as the methyl ester suggests trans-methylation of an ester is occurring during saponification. However, these compounds were also extracted before saponification in the intercrystalline fraction. Intercrystalline β-hydroxy fatty acids were only extracted using extraction protocol B, and not from other earlier batches using extraction protocol A. Methylation in protocol B, may be occurring during the earlier surficial extract which used a solvent mixture of DCM / methanol (4 : 1 v/v). The methanolic solvent could penetrate into the intercrystalline sites, methylate, although not solvate these compounds until the inorganic structure is further disrupted by crushing to release the intercrystalline fraction during solvent extraction.

The total ion count and the diagnostic 175 m/z mass chromatograms are shown in Fig. 14. This shows a series of peaks with the same carbon number, forming a homologous series with an odd over even carbon number distribution, from C_{19} to C_{23}. This is unlike Cardoso and Eglinton (1983) who reported an even over odd carbon number predominance for sediments. The range of β-hydroxy fatty acids of the same carbon number is unlikely to be due to unsaturation as no evidence of M^+-15 ions of lower weight than the saturated M^+-15 ions was found. Instead, the mass spectra indicate iso and anteiso branching (Fig. 13). The identified anteiso branched components have shorter retention times than the normal compound of the same molecular weight, an observation in agreement with the results of Matsumoto and Nagashima (1984). However, an iso C_{21} β-hydroxy fatty acid extracted from intercrystalline *Nucella lapillus* 2 (Figs. 13 and 14) has a retention time after the normal C_{21} β-hydroxy fatty acid. No β-hydroxy fatty acids were found in the calcium carbonate blank which suggests they are indigenous to the shell. These components were only extracted from *Littorina littorea* and *Nucella lapillus* shells, and unlike the other shell lipids they are extracted only from intercrystalline and intercrystalline bound alkaline fractions and not in intracrystalline shell locations.

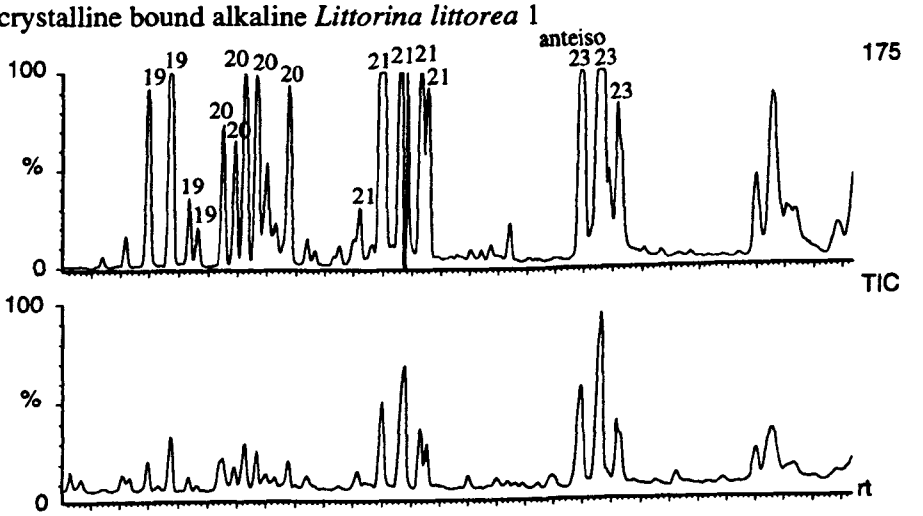
Intercrystalline *Littorina littorea* 3



Intercrystalline bound alkaline *Littorina littorea* 3



Intercrystalline bound alkaline *Littorina littorea* 1



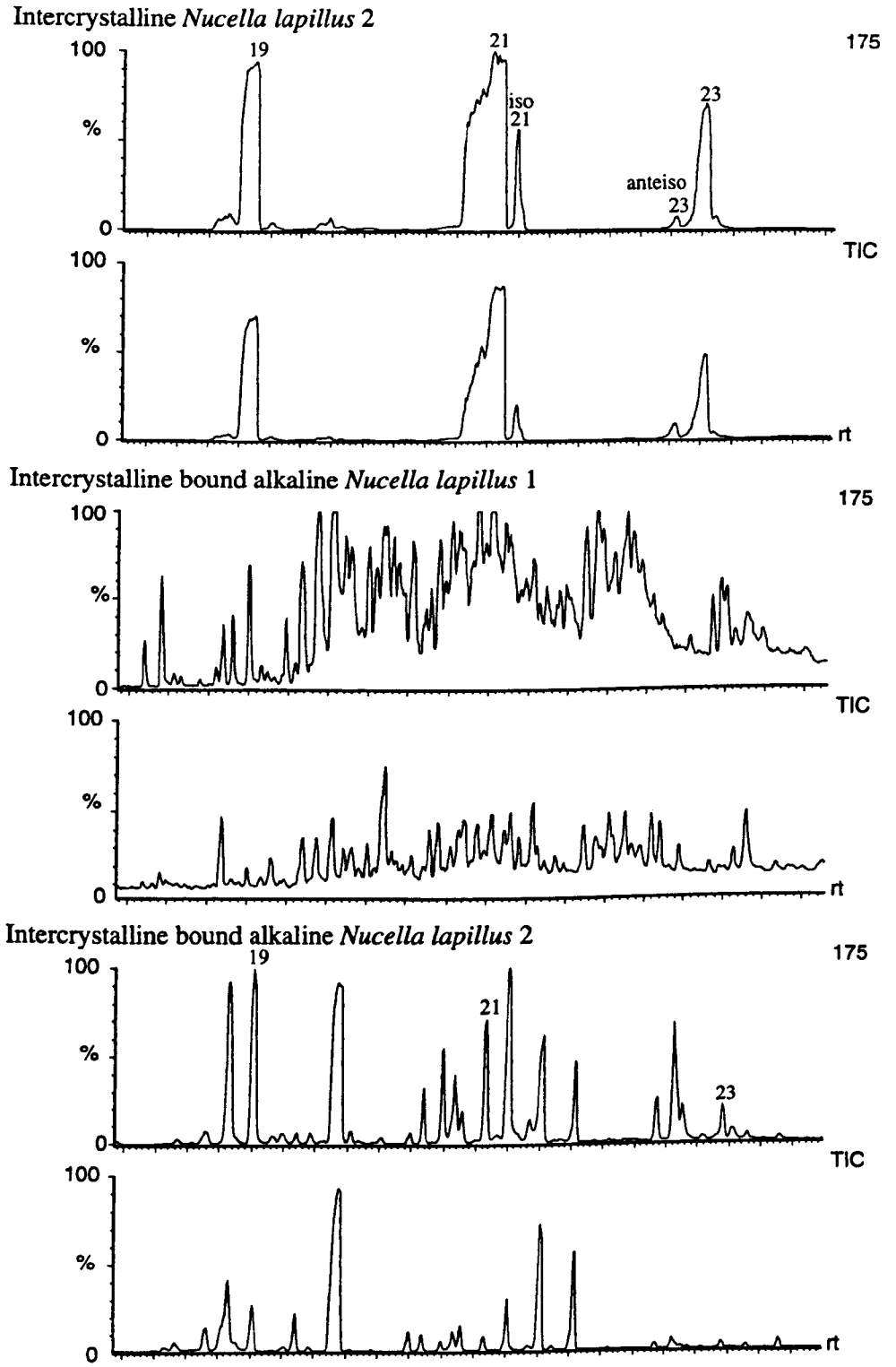


Figure 14. Partial mass chromatograms, 175 m/z (upper trace) and total ion count (lower trace) of β-hydroxy fatty acids. Carbon numbers and possible branching is indicated.

Cholesterol

The amounts of cholesterol extracted from the shells and calcium carbonate blanks plotted as a function of extract fraction are shown in Fig. 15. The yields of cholesterol (μg of cholesterol per g of sample) vary between the different species as shown by Figs. 15a to 15d which are all plotted with the same scale for the yields. Figs. 15e and 16f have the scale of Figs. 15c and 15d enlarged, this illustrates that for low yields of shell cholesterol the levels of background contamination may be significant. However with the exception of the intracrystalline free acidic (E) fraction of *Patella vulgata* 2 (Fig. 15e), the higher yields of cholesterol in the shells than the calcium carbonate blank indicates that the cholesterol is indigenous.

Despite the differences in yields, cholesterol is extracted from all the shell fractions and the distributions of cholesterol between the fractions of the shells are similar. In all the samples the presence of surficial (A) cholesterol is most likely due to contamination not fully removed by the hypochlorite and partial decalcification treatments. The highest cholesterol yields are from the intercrystalline (B) extract. This intercrystalline cholesterol is not chemically bonded to the shell matrix as it is solvent extracted after crushing the shells (see Fig. 1). Bound intercrystalline (C and D) cholesterol is solvent extracted from the first (alkaline) extract after saponification, the extraction of this fraction implies that this cholesterol is either physically retained by or chemically bound to other organic material within the shell as opposed to the intercrystalline cholesterol which is solely trapped by the inorganic phase. Intracrystalline free (E and F) cholesterol is predominantly extracted during the first (acidic) extraction, and bound intracrystalline cholesterol (G and H) from both the alkaline and acidic extracts. This indicates close association with other organic material within the crystallites as well as the presence of free cholesterol. The greater yields of cholesterol from the bound fractions as compared with the free fractions shows that covalent bonding as an ester is the most likely process controlling the total cholesterol within these Recent shells.

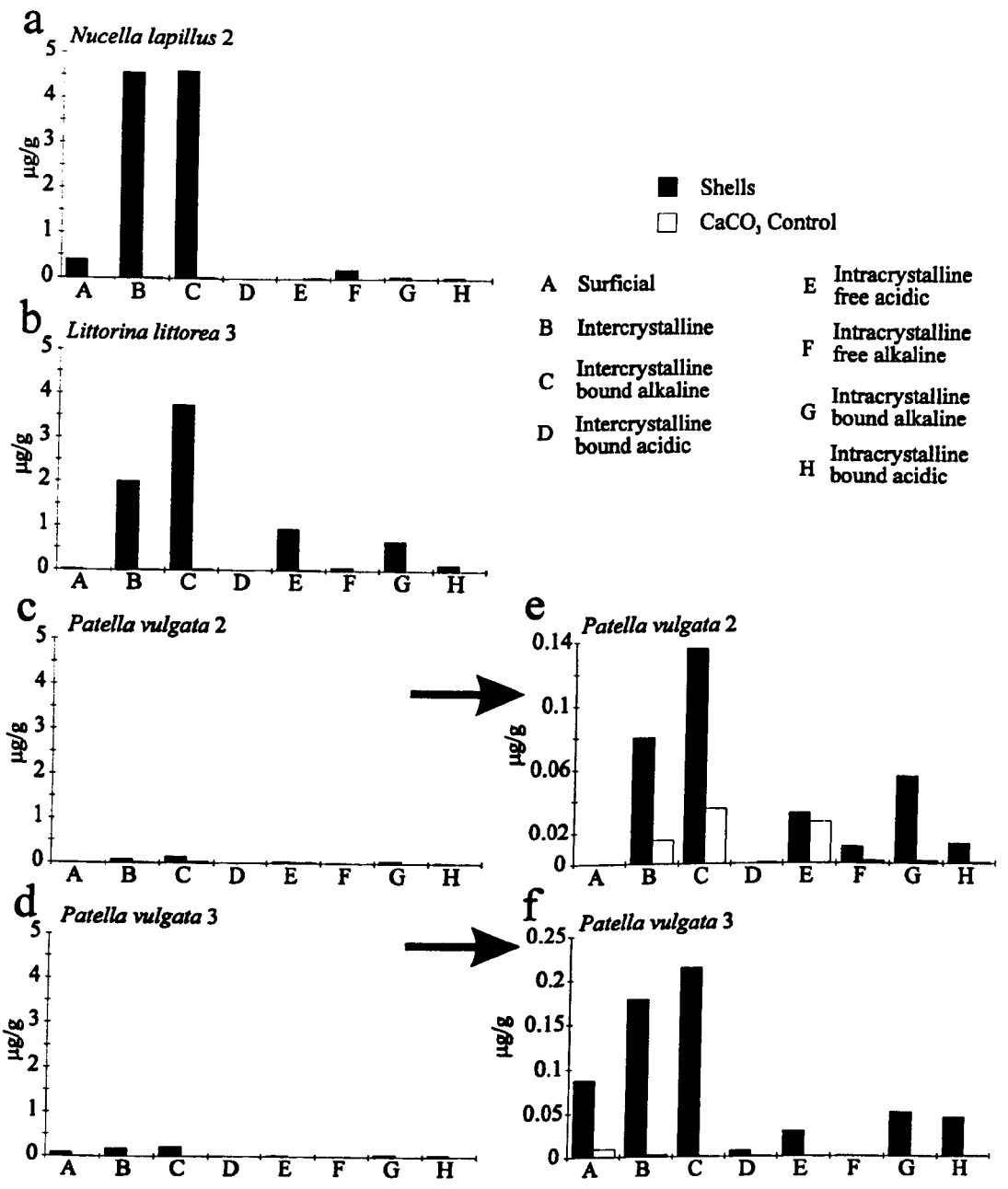
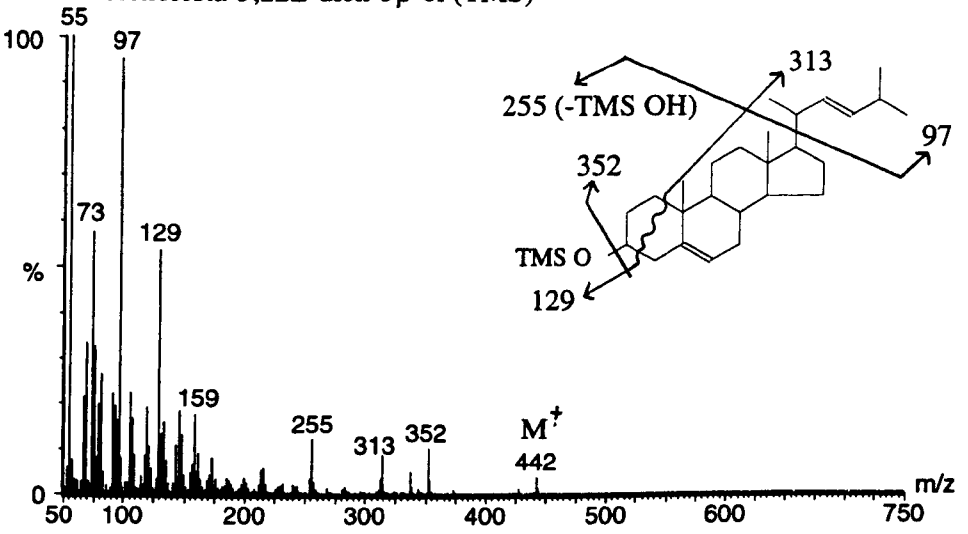


Figure 15. Quantified yields (µg/g of material extracted) of cholesterol for each extract labelled A to H. Shell extracts are shown as filled columns and calcium carbonate blanks unfilled columns. a to d are plotted on the same scale, e and f have the scale expanded for graphs c and d respectively.

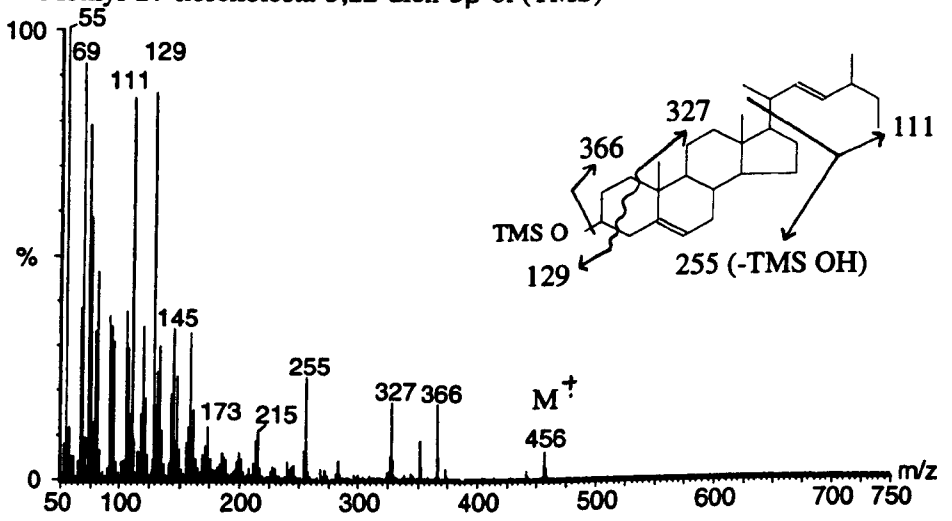
Steroids

Mass spectral assignment of steroids was carried out by comparison with published mass spectra (Ballantine *et al.*, 1975; Idler *et al.*, 1978; Romero & Seldes, 1986; Pizzoferrato *et al.*, 1993) and with electronic library mass spectral data. Identified structures with mass spectra and suggested fragmentations are shown in Fig. 16. The names, carbon numbers and relative retention times to cholesterol found in this study and those reported in the literature for the TMS derivatives of sterols are shown in Table 9. An example of a partial mass chromatogram showing the steroids is shown in Fig. 17. Steroids have been identified by their mass spectra only due to the variations in retention times, and the presence of non-sterol components in the total extracts. The values of the relative retention times found here are different to those reported by Voogt (1973), Idler *et al.* (1978) and Nichols *et al.* (1984) (Table 4). The variation of retention times, and even changes in the order of elution, due to changes in the phase of the GC column, has been examined by Ballantine *et al.* (1975). However, with two exceptions the order of retention times match those of the literature. There exceptions are: compound N (identified as 24-methylcholesta-5,24(28)-dien-3β-ol) has a longer retention time than is reported, and two components having different retention times (1.01-1.04 and 1.08 to 1.12) but sharing identical mass spectra were identified as 24-methylcholest-5-en-3β-ol (G). The component eluting earlier is more similar to the retention time for 24-methylcholest-5-en-3β-ol (G) and has been assigned as such. Those steroids not fully identified, but characterized by GCMS as 3-hydroxy Δ^5 sterols by characteristic 129 m/z and M†-129 m/z mass fragmentation (suggested mechanism from Brooks (1979) is shown in Appendix 5) are combined as unknowns (steroid X). The sole use of mass spectra without the use of co-injections of known standards results in some tentative identification assignments.

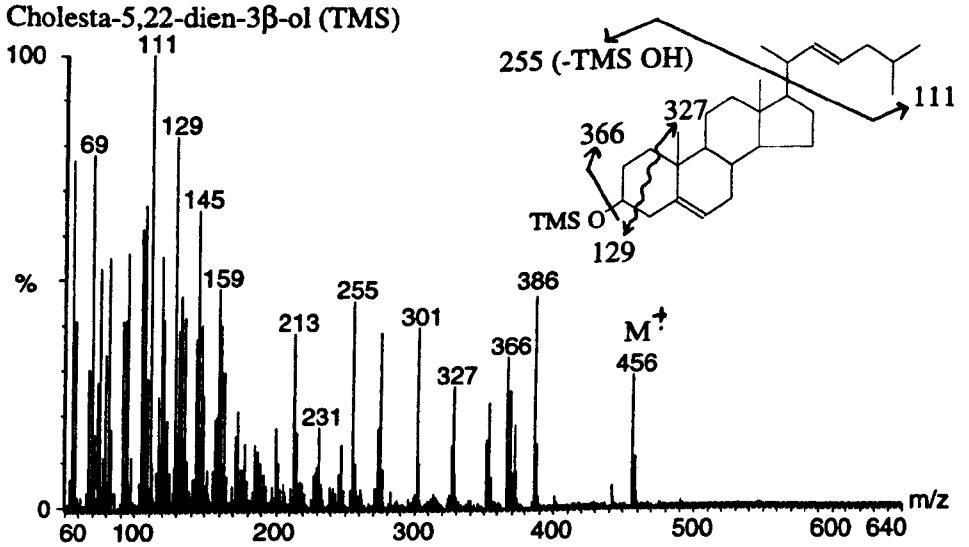
A) 25-Bisnorcholesta-5,22E-dien-3β-ol (TMS)



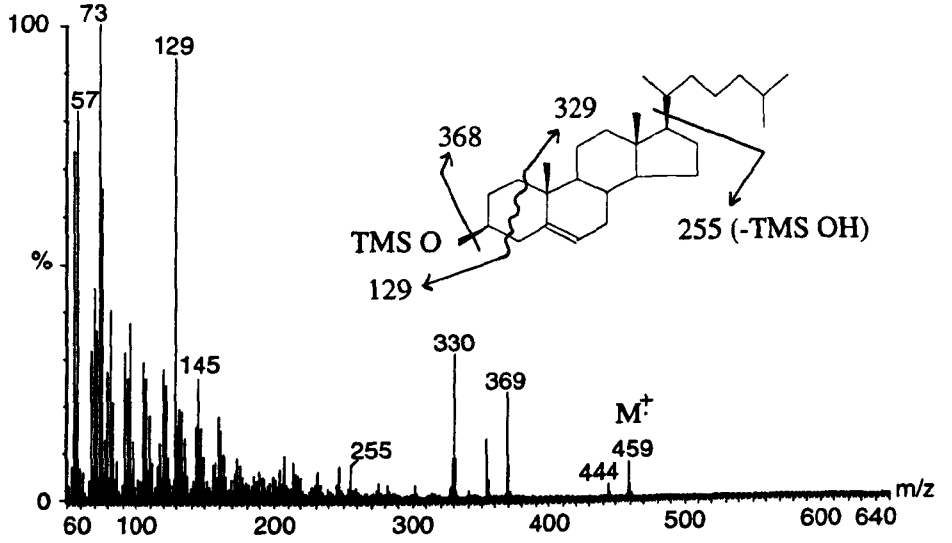
B) 24-Methyl-27-norcholesta-5,22-dien-3β-ol (TMS)



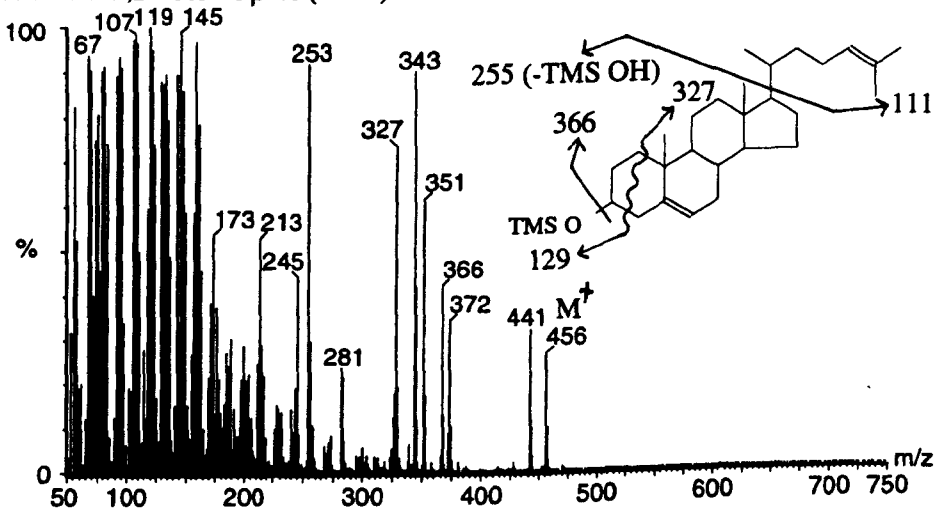
C) Cholesta-5,22-dien-3β-ol (TMS)



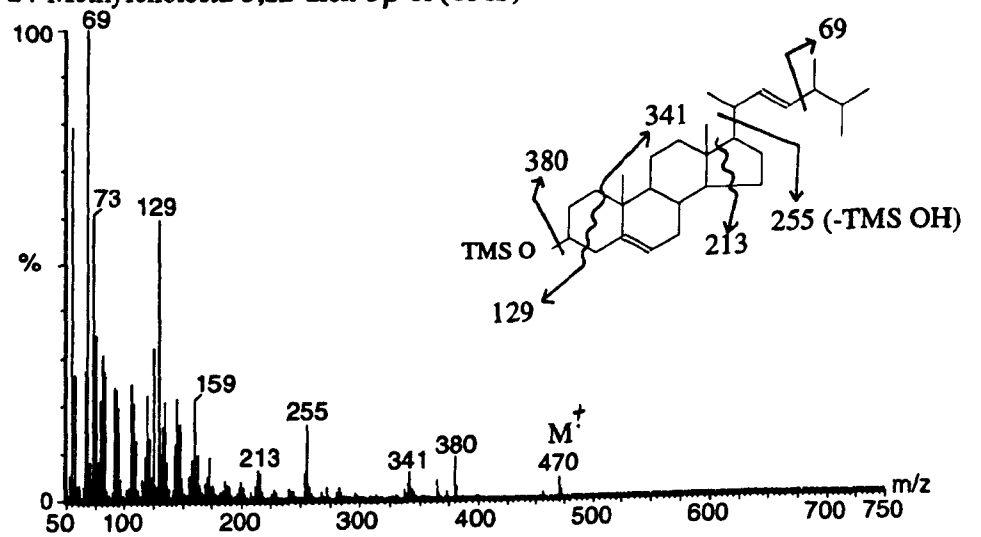
D) Cholesterol



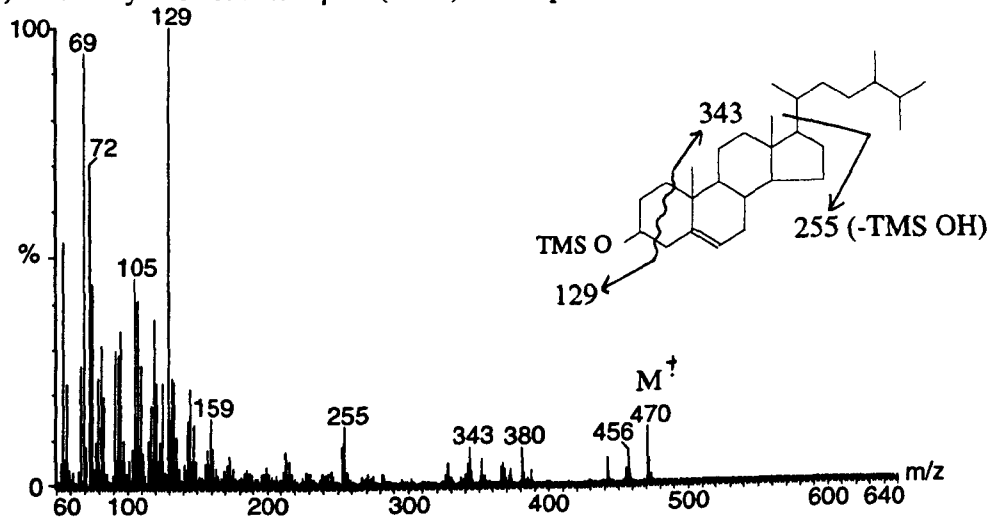
E) Cholesta-5,24-dien-3β-ol (TMS) = Desmosterol



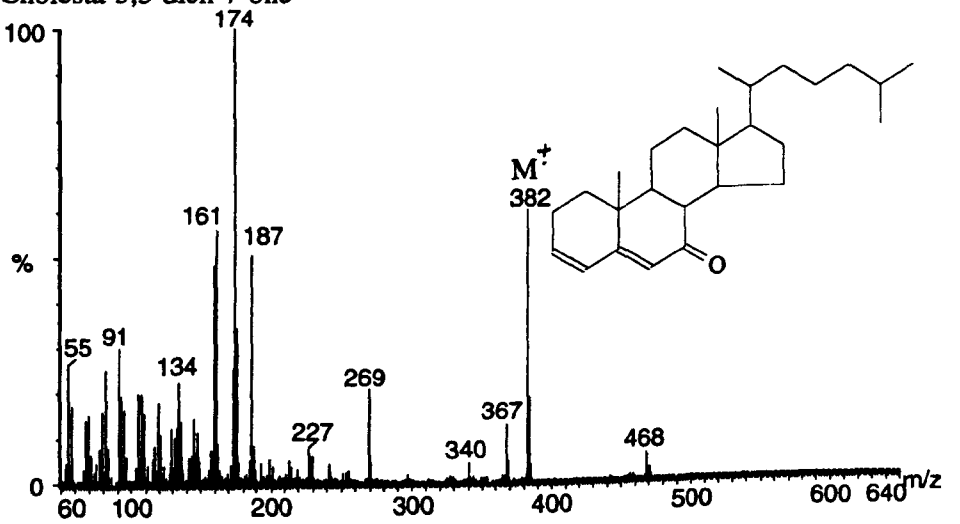
F) 24-Methylcholesta-5,22-dien-3β-ol (TMS) = Brassicasterol



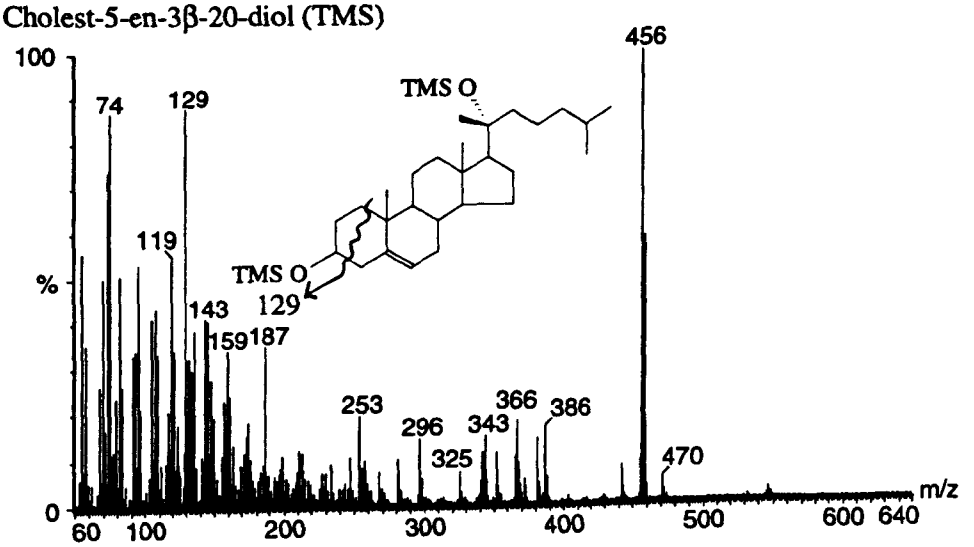
G) 24-Methylcholest-5-en-3β-ol (TMS) = Campesterol



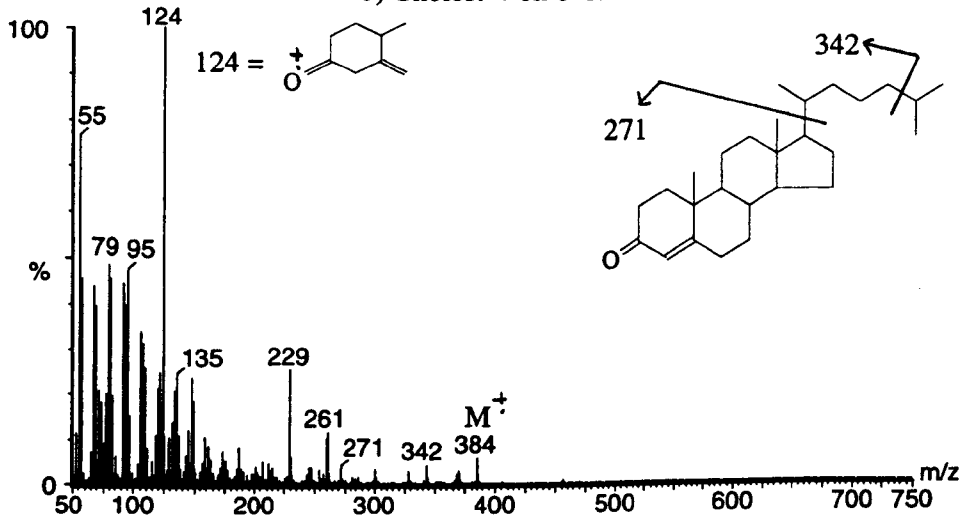
H) Cholesta-3,5-dien-7-one



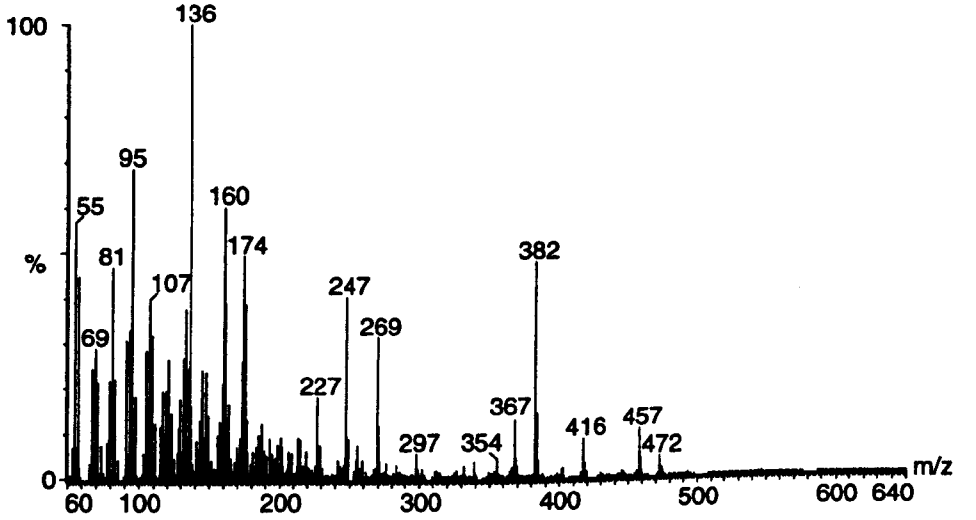
I) Cholest-5-en-3β-20-diol (TMS)



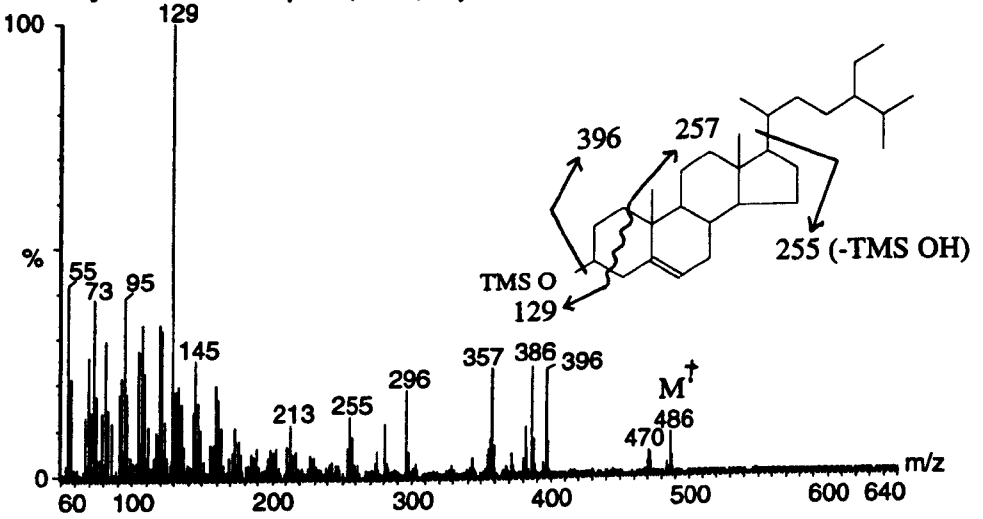
J) Cholest-4-en-3-one



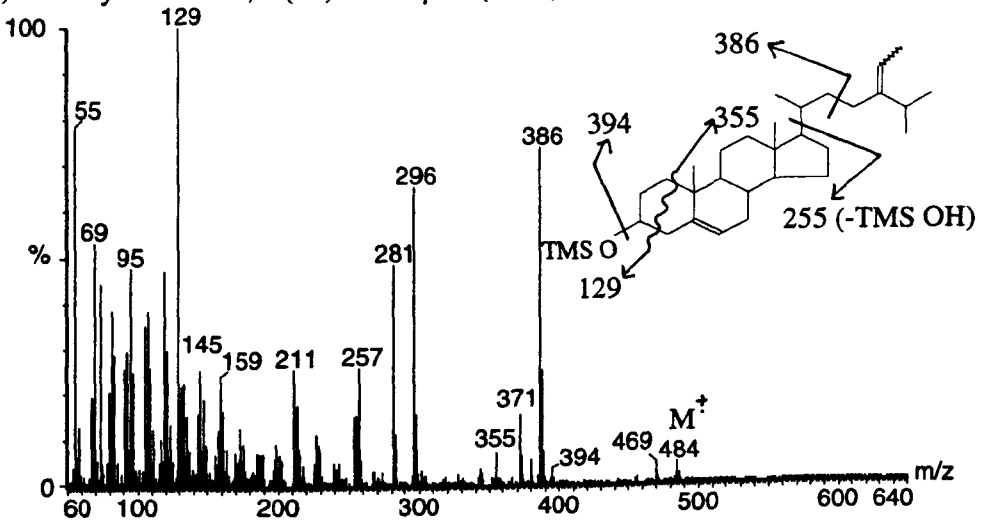
K) Unidentified steroidal ketone



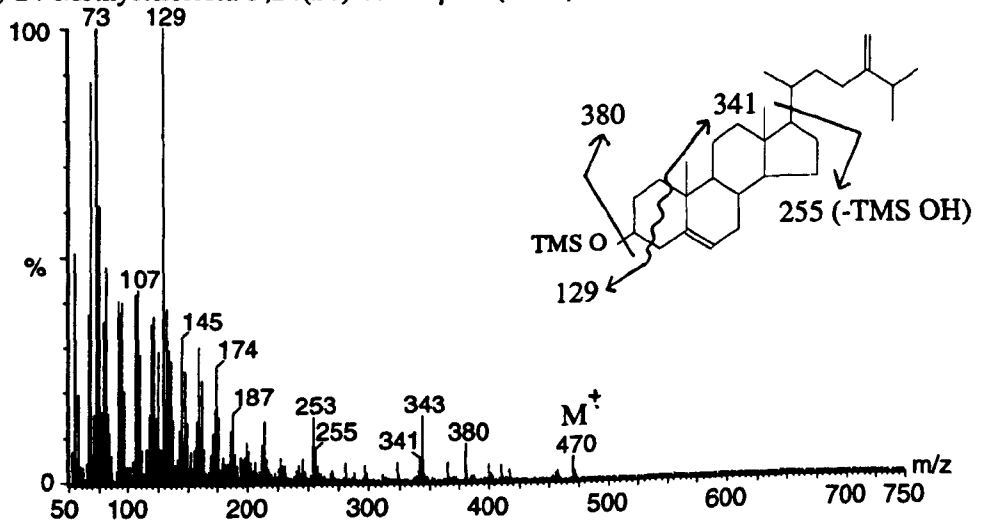
L) 24-Ethylcholest-5-en-3β-ol (TMS) = β Sitosterol



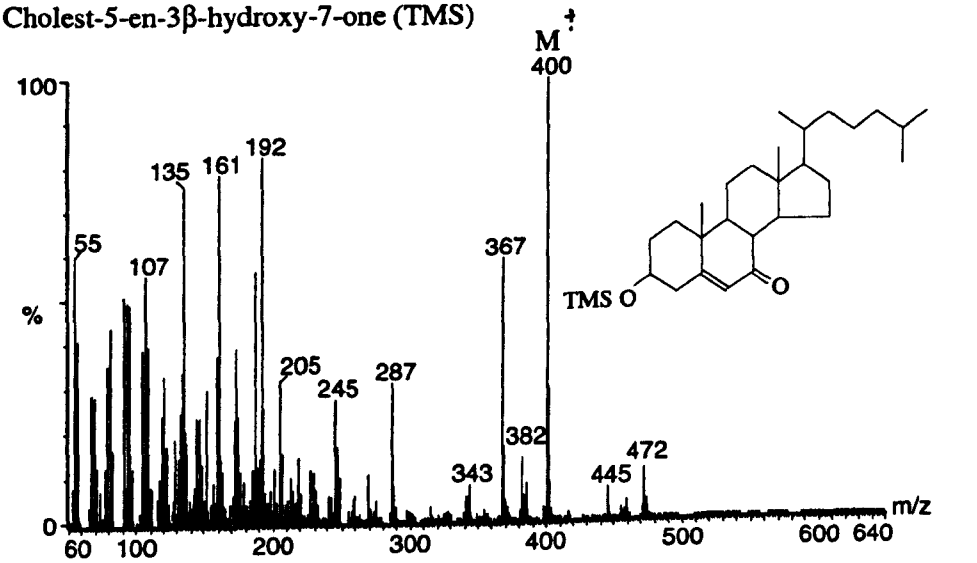
M) 24-Ethylcholesta-5,24(28)-dien-3β-ol (TMS)



N) 24-Methylcholesta-5,24(28)-dien-3β-ol (TMS)



O) Cholest-5-en-3β-hydroxy-7-one (TMS)



P) Sterane

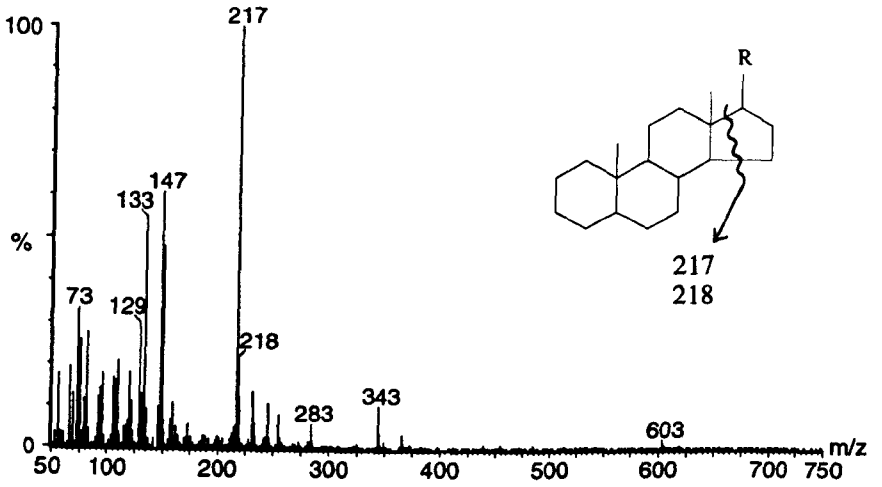


Figure 16. Mass spectra, chemical structure and suggested fragmentation of the steroids extracted in this study.

Code	RRT to cholesterol (HP-5)	Idler *et al.*, (1978) derived RRT to cholesterol (OV1)	Voogt, (1973) derived RRT to cholesterol (SE-30)	Voogt, (1973) derived RRT to cholesterol (NPGS)	Nichols *et al.*, (1984), RRT to cholesterol (SE-30)	Carbon number	Nomenclature used here	Alternative nomenclature
A	0.94	0.61				26	25-bisnorcholesta-5,22-dien-3β-ol	
B	0.98	0.85				27	24-methyl-27-norcholesta-5,22-dien-3β-ol	
C	0.98-0.99		0.89	0.86	0.89	27	Cholesta-5,22-dien-3β-ol	22-dehydrocholesterol
D	1 by definition	1.00	1.00	1.00	1.00	27	Cholesterol	Cholest-5-en-3β-ol
E	1-1.01	1.28				27	Cholesta-5,24-dien-3β-ol	Desmosterol
F	1.01	1.12	1.13	1.13	1.12	28	(R or S) 24-methylcholesta-5,22-dien-3β-ol	Brassicasterol
G	1.01-1.04	1.34	1.33	1.36		28	(R or S) 24-methylcholest-5-en-3β-ol	Campesterol
H	1.02					27	Cholesta-3,5-diene-7-one	
I	1.02-1.04					27	Cholest-5-en-3β-20-diol	
J	1.03-1.04					27	Cholest-4-en-3-one	
K	1.05					-	Unidentified steroidal ketone	
L	1.06-1.09	1.69	1.75	1.73	1.63	29	(R or S) 24-ethylcholest-5-en-3β-ol	β-sitosterol, Clionasterol;
M	1.08-1.09	1.83 / 1.93				29	24-ethylcholesta-5,24(28)-dien-3β-ol	Fucosterol (E) / Isofucosterol (Z)
N	1.1				1.28	28	24-Methylcholesta-5,24(28)-dien-3β-ol	
O	1.12					27	Cholest-5-en-3β-hydroxy-7-one	
P	1.13					-	Sterane	

Table 9. Steroid relative retention times (RRT) to cholesterol (retention time of steroid / retention time of cholesterol) from this study and RRT derived from the literature with GC column phase indicated. Carbon number and nomenclatures are also shown.

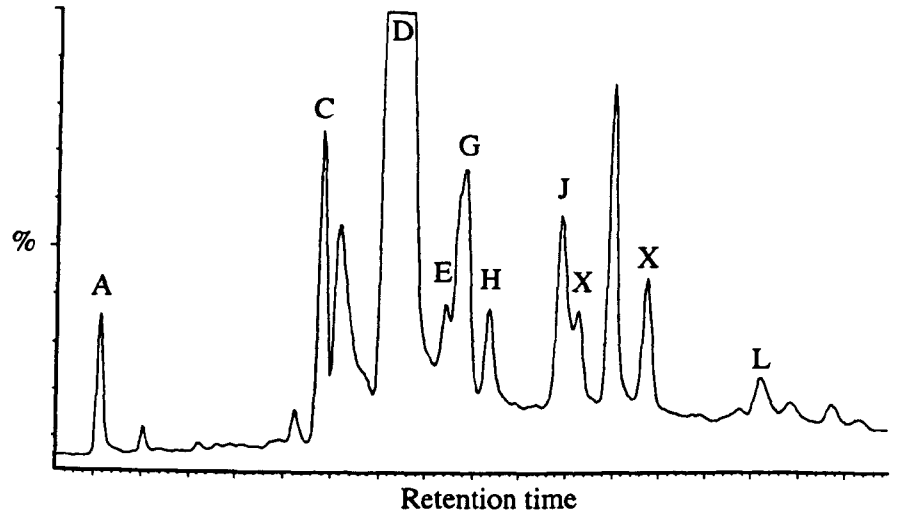


Figure 17. Partial mass chromatogram (total ion count) of steroids extracted from the intracrystalline bound alkaline fraction of *Nucella lapillus* 2. Identified steroids are labelled according to Table 9.

The percentage steroidal composition extracted from the shells is shown in Table 10. With the exception of the extraction of cholesterol (D) in the calcium carbonate blanks, no other steroidal contamination was found. The sterols extracted in this study, with the exclusion cholest-5-ene-3β-20-diol (I), have all been observed in the soft tissues of molluscs by other workers (Ballantine *et al.*, 1975; Idler *et al.*, 1978; Romero & Seldes, 1986; Pizzoferrato *et al.*, 1993).

Despite the attempts to prevent oxidation of the sterols as described in the methodology, the extraction of Δ^5 7-keto sterols (compounds H and O) indicates autoxidation from Δ^5 sterols, particularly during saponification (Smith, 1981). Cholesta-3,5-dien-7-one (H) also results from dehydration of the 3β-hydroxy-Δ^5-7-keto derivative during instrumental analysis (Romero & Seldes, 1986). Cholest-5-en-3β-hydroxy-7-one (O) and cholest-5-ene-3β-20-diol (J) are also typical cholesterol oxidation products (Pizzoferrato *et al.*, 1993). Although cholest-4-en-3-one (J) has biological origins, it too can be a product of the diagenesis of cholesterol (Mackenzie *et al.*, 1982). Where cholest-4-en-3-one (J) is extracted as the sole sterol, this is probably due to oxidation of the abundant cholesterol present in the *Sepia officinalis* soft tissues (Zandee, 1967). No further oxidation or diagenetic products were observed except for a single sterane (P) from *Littorina littorea.*

Extract	Species	25-Bisorcholesta-5,22-dien-3β-ol	24-Methyl-27-norcholesta-5,22-dien-3β-ol	Cholesta-5,22-dien-3β-ol	Cholesterol	Cholesta-5,24-dien-3β-ol	24-Methylcholesta-5,22-dien-3β-ol	(R or S) 24-Methylcholest-5-en-3β-ol	Cholesta-3,5-dien-7-one	Cholest-5-en-3β,20-diol	Cholest-4-en-3-one	Unidentified steroidal ketone	24-Ethylcholest-5-en-3β-ol	24-Ethylcholesta-5,24(28)-dien-3β-ol	24-Methylcholesta-5,24(28)-dien-3β-ol	Cholest-5-en-3β-hydroxy-7-one	Sterane	Sum of remaining unidentified 3-OH Δ5 sterols
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	X
Intracrystalline bound alkaline	*Artica islandica 1*	5.6		8.7	55.4		11.7						8.8					9.8
Intercrystalline bound alkaline	*Littorina littorea 1*				47.5	10.3				8.9			3.0				1.4	28.9
Intercrystalline bound alkaline	*Littorina littorea 2*				55.5	15.1			10.5	9.5					1.6			7.7
Intracrystalline bound alkaline	*Littorina littorea 2*				93.0	5.4												1.7
Intracrystalline bound acidic	*Littorina littorea 2*				72.4				4.1		10.4							13.1
Surficial	*Littorina littorea 3*				14.8				11.0		8.1	36.7				29.4		0.0
Intercrystalline	*Littorina littorea 3*			10.0	46.4	9.0								1.4				33.2
Intercrystalline bound alkaline	*Littorina littorea 3*				49.8	18.8			4.5	12.4			3.2	2.4				9.0
Intracrystalline free acidic	*Littorina littorea 3*				73.5	21.2												5.3
Intracrystalline bound alkaline	*Littorina littorea 3*				80.6													19.4
Intracrystalline bound acidic	*Littorina littorea 3*				88.8													11.2
Intercrystalline bound alkaline	*Nucella lapillus 1*				57.9			4.5	2.1	8.6								26.8
Intracrystalline bound alkaline	*Nucella lapillus 1*	2.1	4.5		73.5	1.1		6.3	1.6		4.9		1.5					4.4
Intracrystalline bound acidic	*Nucella lapillus 1*										100.0							0.0
Intercrystalline	*Nucella lapillus 2*				91.2			0.9										7.9
Intercrystalline bound alkaline	*Nucella lapillus 2*				94.9				1.8	0.0	2.7							0.6
Intracrystalline free acidic	*Patella vulgata 1*				96.1													3.9
Intracrystalline bound acidic	*Sepia officinalis*										100.0							0.0

Table 10. Steroids extracted from the shells, as a percentage of the total steroids. Steroids are labelled and arranged A to P by increasing retention times. Unidentified sterols have been combined and labelled X.

B) Bound fatty acids and steroidal distributions in *Littorina littorea* shells and reported soft tissues

Bound fatty acids

Fig. 18 is a dendrogram comparing for *Littorina littorea*, the distributions of the published bound saturated fatty acids from the soft tissues (■) and the bound fatty acids (extracted as FAMEs) recovered from the shells in this study (□). Included in this analysis are the saturated fatty acids from two dinoflagellates (●) and Human skin (○) which act as outgroups, and the calcium carbonate blanks (∗) examined with the shells.

The cutoff line plotted on Fig. 18 produces five groups. Two groups contain calcium carbonate blanks. These bound fatty acids are most distantly related to the other samples as they separate at the largest distance. With the exception of the intracrystalline bound alkaline 3, intercrystalline bound alkaline 1 and calcium carbonate blank 2 extracts, the remaining samples can be divided into three general groups; i) the soft tissues of *Littorina littorea* (■) and the dinoflagellates (●), ii) the hydrogenated soft tissue fatty acids and the free fatty acids from Human skin (○) and iii) the shell extracts (□).

This indicates that the saturated fatty acids in the shells, although dissimilar to the outgroups, are not similar to the saturated fatty acid component of the soft tissues. This may be due to the dominance of triglyceride derived fatty acids in this data set. Phospholipids have been suggested to have a rôle in the process of biomineralization (Wuthier, 1973; Abolinš-Krogis, 1979; Isa & Okazaki, 1987). The polar May (2) derived fatty acids were presumed to be mostly phospholipids (Ackman *et al.*, 1971), however this is the only example examined here. No grouping is observed with the shell extracts.

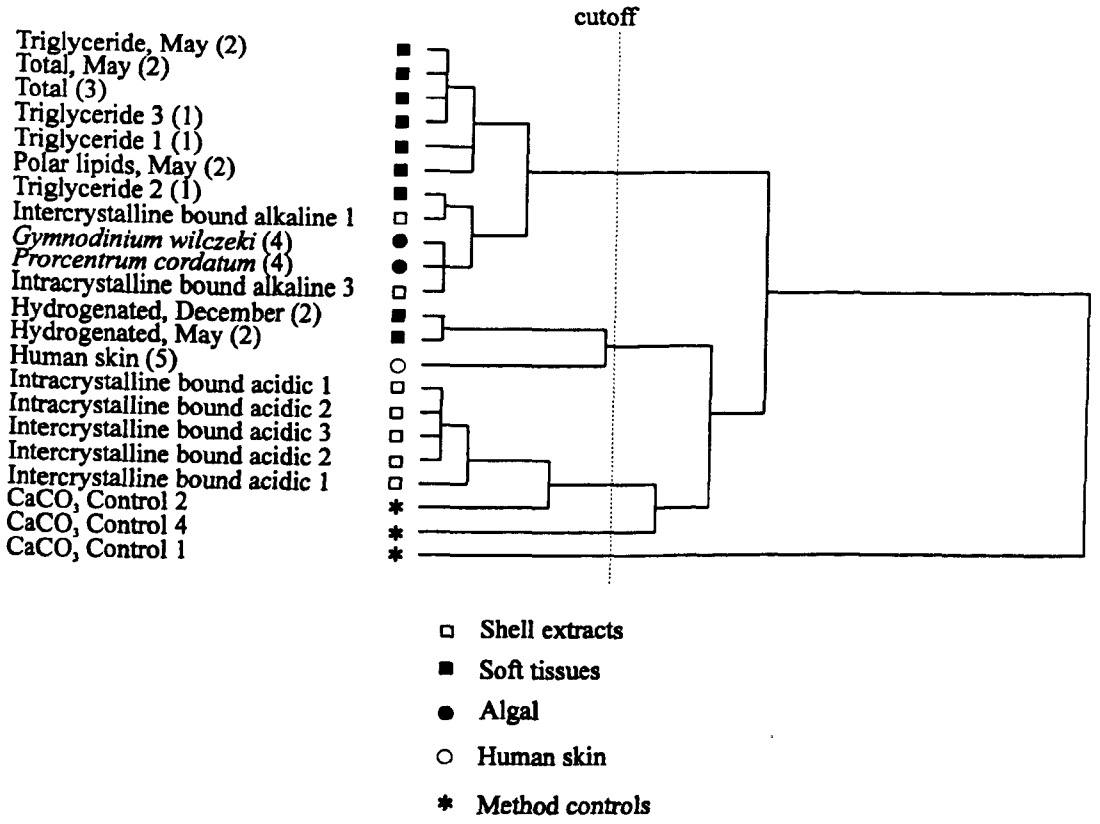


Figure 18. Dendrogram derived from the saturated bound fatty acid composition of *Littorina littorea* reported soft tissues and the shells examined here. The numbers after the shells and calcium carbonate blanks refers to the sample number as shown in Table 1. References are indicated by numbers enclosed in brackets 1 = Brockerhoff *et al.* (1967), 2 = Ackman *et al.* (1971), 3 = Paradis & Ackman (1976), 4 = Nichols *et al.* (1984), 5 = Wertz & Downing (1991). A cutoff value forming six groups is shown by the dashed line.

The data used to produce Fig. 18 are shown in Table 11 with additional data for the unsaturated fatty acids from the soft tissues of *Littorina littorea*. For the shell the saturated fatty acid distributions have a carbon number maximum of C_{16} or C_{18}, whilst the soft tissue saturated fatty acids have a C_{16} carbon number maximum. Higher carbon number maxima are observed for the unsaturated soft tissue fatty acids (C_{18} and C_{20}). Hydrogenation of the total soft tissue lipids gives a carbon number maximum of C_{20}. This indicates that the bound fatty acids in the shell do not originate simply from the saturated fraction of the soft tissues, nor are the saturated shell fatty acids a product of the hydrogenation of the total soft tissue fatty acids.

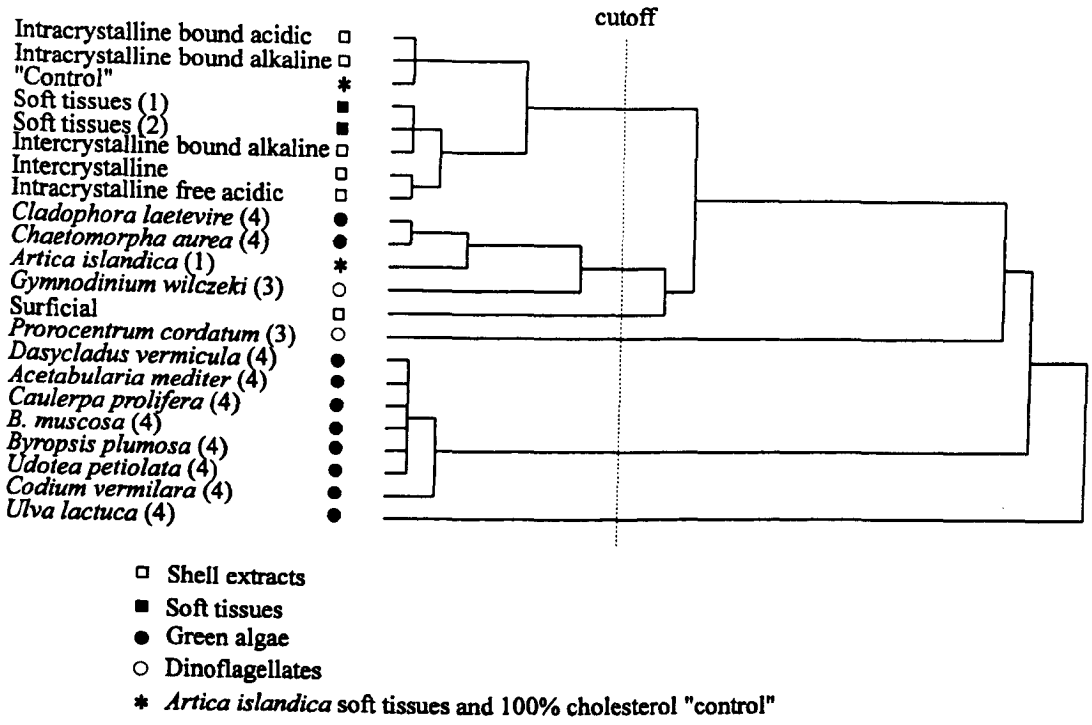
Tissue	Soft tissue	Soft tissue	Soft tissue	Soft tissue	Soft tissue	Soft tissue	Soft tissue	Soft tissue	Soft tissue	Marine dinoflagellates	Marine dinoflagellates	Human skin free fatty acids	Shell	Shell	Shell	Shell	Shell	Shell	Shell	CaCO3 1	CaCO3 2	CaCO3 4
Species	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Gymnodinium wilczeki*	*Prorocentrum cordatum*	*Homo sapiens*	*Littorina littorea 1*	*Littorina littorea 1*	*Littorina littorea 1*	*Littorina littorea 2*	*Littorina littorea 2*	*Littorina littorea 3*	*Littorina littorea 3*	CaCO3 blank 1	CaCO3 blank 2	CaCO3 blank 4
Lipid fraction	Triglyceride, position 1	Triglyceride, position 2	Triglyceride, position 3	Total lipids hydrogentaed, December	Total lipids hydrogentaed, May	Triglyceride, May	Polar lipids, May	Total lipids, May	Total lipids	Total lipids	Total lipids	Free fatty acids	Intercrystalline bound acidic	Intercrystalline bound alkaline	Intracrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound alkaline	Intercrystalline bound acidic	Intracrystalline bound acidic	Intracrystalline bound alkaline
Reference	1	1	1	2	2	2	2	2	3	4	4	5										
Total % (sic.)	95	94	88	100	98	99	100	99	91	100	100	100	100	100	100	100	100	100	100	100	100	100
Total saturated %	19	27	8	95	94	20	22	20	26	100	100	100	100	100	100	100	100	100	100	100	100	100
Total unsaturated %	76	67	80	0	0	79	79	79	65	0	0	0	0	0	0	0	0	0	0	0	0	0
Iso., Branched & others %				5	4	0			9													
Cmax saturated	16	16	16	20	20	16	16	16	16	16	16	24	18	16	18	18	18	18	16	18	16	18
Cmax unsaturated	18	20	18	-	-	20	20	20	20	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbon number, saturated																						
12				0.2		0.1	0.2	0.2		0.2			0.1		1.3	8.5	1.1	2.2				
13				0.1			0.1	0.1					0.1			0.8	0.4	1.2				
14	2.	9.	3.	6.7	2.8	5.1	4.2	4.6	6.	7.8	12.5		0.5		2.8	9.	2.9	5.1				
15				0.7	0.2	0.3	0.4	0.3	0.4	3.	1.3		0.4			1.7	1.6	2.3			23.2	
16	13.	17.	4.	17.8	15.3	10.2	9.2	10.	13.6	81.6	82.4	9.1	16.8	73.8	27.4	23.6	25.6	30.9	86.7		42.	
17				1.1	0.7	0.4	0.4	0.4	0.8	0.5		1.	3.9	5.5	4.1	5.5	6.1	5.4		40.6		
18	4.	1.	1.	26.	24.9	3.8	7.3	4.	4.8	6.	3.8	11.2	74.1	20.6	60.2	49.3	62.3	52.9	13.3		34.8	100.
19				0.5	0.4							1.4	0.6		4.2					59.4		
20				32.6	39.1				0.1	0.2		7.2				1.6						
21					0.2							2.3										
22				9.5	10.5					0.7		7.2	3.5									
23												7.6										
24												33.										
25												6.1										
26												10.4										
27																						
28												3.3										
Carbon number, unsaturated																						
14						0.5	0.3	0.4														
15																						
16	6.	9.	10.			11.4	5.7	8.5	6.1													
17						0.1	0.2	0.1														
18	32.	17.	26.			28.6	13.3	23.1	17.1													
19																						
20	24.	29.	24.			33.4	47.8	39.	37.3													
21						0.2		0.1														
22	14.	12.	20.			4.	11.	7.3	4.6													
23																						
24						0.3	0.3	0.4														

Table 11. Table of saturated and unsaturated fatty acids from *Littorina littorea* soft tissues, the saturated fatty acids from two dinoflagellates, the free saturated fatty acids from Human skin and the *Littorina littorea* shells extracted here. 1 = Brockerhoff *et al.* (1967), 2 = Ackman *et al.* (1971), 3 = Paradis & Ackman (1976), 4 = Nichols *et al.* (1984) and 5 = Wertz & Downing (1991). Where no extracts were recovered the tabulated values are blank.

Steroids

From the 13 shell samples analysed the *Littorina littorea* 3 sample gave the greatest number of extracts containing steroids and Fig. 19 shows a dendrogram produced by analysis of the percentage steroidal composition from the shells (□) and reported soft tissues (■). A "control" (∗) consisting of 100% cholesterol was also included to indicate potential laboratory contamination as cholesterol was the sole sterol observed in the calcium carbonate blanks and as cholesterol is the almost exclusive sterol found in Human skin (Wertz & Downing, 1991). To act as outgroups and represent potential contamination from exogenous organisms the steroidal compositions of green algae (●) and two marine dinoflagellates (○) are included. The soft tissue steroid composition of the bivalve *Artica islandica* (∗) have also been analysed. The data set is shown in Appendix 6.

The cutoff plotted on Fig. 19 separates the samples into six groups. This shows that; i) the steroids from the algae and dinoflagellates form 3 groups which are at the greatest distance to the shell and soft tissue derived steroids, ii) the *Artica islandica* soft tissues, two algae and one dinoflagellate form another group separated from the shells and soft tissues of *Littorina littorea*, iii) the surficial shell extract is distinct from the other shell extracts, and iv) the *Littorina littorea* intercrystalline and intracrystalline shell fractions group with the *Littorina littorea* soft tissues.



□ Shell extracts
■ Soft tissues
● Green algae
○ Dinoflagellates
* *Artica islandica* soft tissues and 100% cholesterol "control"

Figure 19. Dendrogram derived from the steroidal composition reported for *Littorina littorea* soft tissues and the shells of *Littorina littorea* 3 extracted here. References are indicated by numbers enclosed in brackets 1 = Idler & Wiseman (1971), 2 = Voogt (1969), 3 = Nichols *et al.* (1984), 4 = De Napoli *et al.* (1982). A cutoff value forming six groups is shown by the dashed line.

The first and second principal components produced from the same data as above (Fig. 9) are shown in Fig. 20a. (Factors shown in Appendix 7). The dashed line indicates the sequence of extraction of the shell fractions. Fig. 20b shows the eigenvectors of factors 1 and 2 for each steroid (steroids B, P had zero values and the unknown X (3-OH Δ^5 sterols) were not included in this analysis).

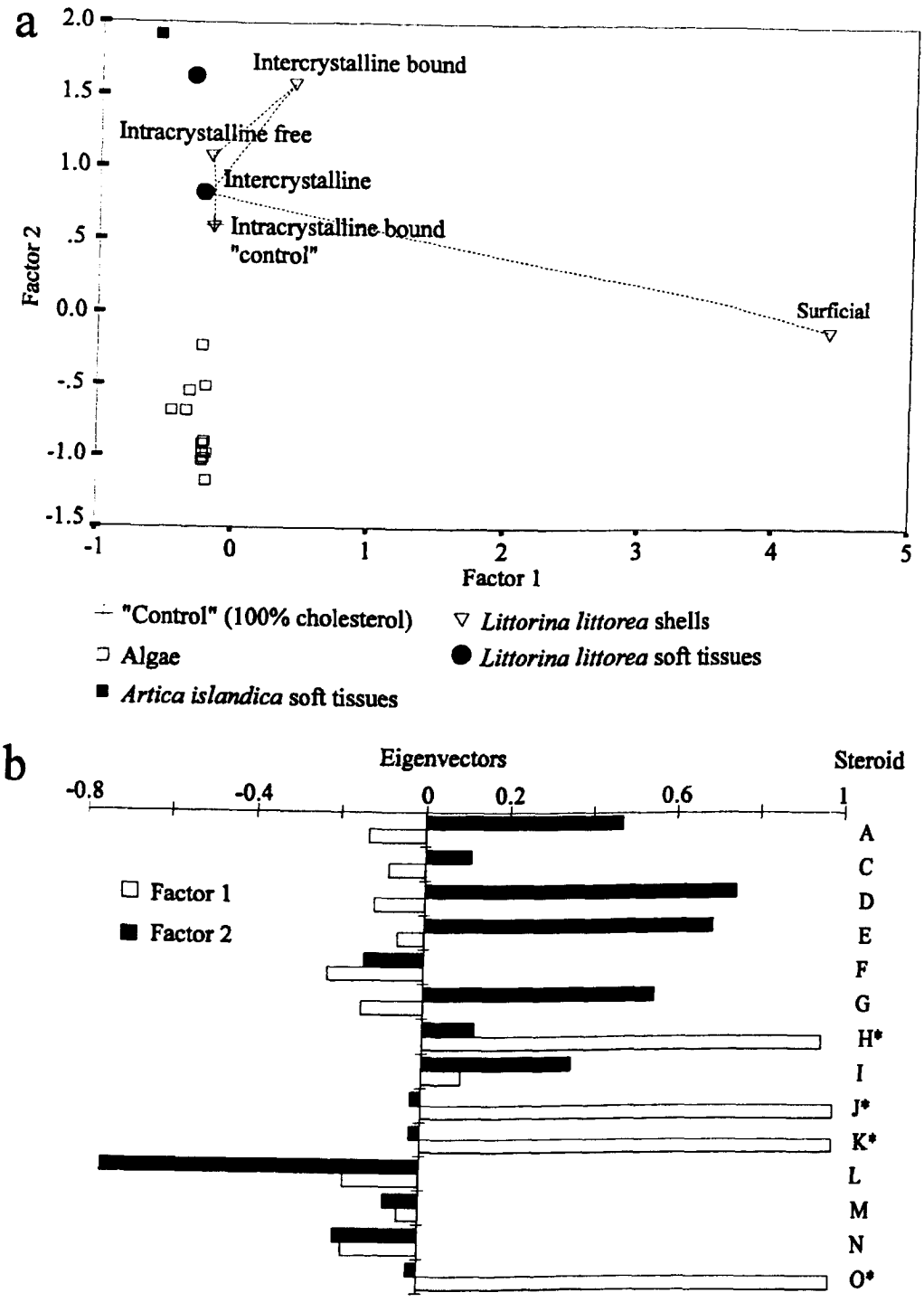


Figure 20. a) First and second principal components derived from the percentage steroids extracted from *Littorina littorea* 3 shells. Included is the sterol composition from algae (De Napoli *et al.*, 1982), the soft tissues of *Artica islandica* (Idler & Wiseman, 1971), *Littorina littorea* (Idler & Wiseman, 1971; Voogt. 1969) and a "control" of 100% cholesterol. The line indicates the sequence of shell extraction. b) Bar graph showing eigenvectors of the first and second factors for the steroid variables.

The surficial extract is distinctly separate from the other samples (Fig. 20a), which is due to the steroids H, J, K and O (marked by asterisks) having a strong influence on factor 1 as shown in the bar chart of eigenvectors in Fig. 20b. These steroidal ketones (H, J, K and O) are probably an artefact caused by sterol oxidation during the initial cleaning stages, particularly with the use of hypochlorite. Although Chapter 1 has shown that hypochlorite is capable of penetration into the intercrystalline spaces, the intercrystalline and intercrystalline bound fractions are not strongly affected by factor 1, and they may therefore be protected from the rigorous cleaning treatments.

Most closely grouping with the soft tissues (●) are the intercrystalline and intracrystalline extracts, these shell locations therefore represent the best sites for preservation of indigenous shell lipids. The "control" (+) also groups with these extracts and this is due to cholesterol which was the only identified sterol extracted from the intracrystalline bound fractions. However, Fig. 15 shows that the cholesterol yields from the calcium carbonate blank are not a significant contaminant for this sample.

The variation between the two soft tissue data from *Littorina littorea* (●) shows the variability of the steroidal content between the soft tissues from different samples. Although Jarzebski *et al.* (1986) reported the uniform distribution of sterols in the soft tissues from the same sample, the difference between the intercrystalline and intracrystalline steroids examined here may be due to differences in the original steroid composition of the shell locations.

CONCLUSIONS

A variety of different lipid classes have been extracted from Recent molluscan shells. Each lipid class has particular benefits and disadvantages for their analysis from the shell extracts. The use of quantification to ascertain yields and the comparison of lipid distributions between the calcium carbonate blank and the shell extracts makes it possible to distinguish between the shell lipids and laboratory contamination. The *n*-alkanes, bound fatty acids (extracted as FAMEs), β-hydroxy fatty acids, cholesterol and other steroids extracted from the shells have been shown to have higher yields than the laboratory contamination and are therefore indigenous to the shells. Analysis of other components extracted from the shells; phthalate plasticisers, pristane, phytane, *n*-alcohols and the free fatty acids are problematic either due to large variations in background contamination revealed by the calcium carbonate blank, the components not being quantified, or high yields of laboratory contamination and low yields from shells when quantified, or their limited extractions from the shells.

Therefore these lipids have been excluded from further analysis. A homologous series of β-hydroxy fatty acids with an odd over even distribution, from C_{19} to C_{23} were extracted from the intercrystalline and intercrystalline bound alkaline fractions only. No β-hydroxy fatty acids have been reported in the soft tissues of molluscs.

The reported fatty acids and steroidal composition of the soft tissues is used to represent the indigenous lipids. A comparison of the extracted lipids from various shell locations with the reported values from the soft tissues is used to distinguish indigenous shell lipids from post-depositional ingress.

Bound fatty acids are extracted after saponification from both intercrystalline bound and intracrystalline bound fractions. An even over odd distribution with a carbon number maximum of C_{16} or C_{18} was observed. The almost exclusive extraction of saturated fatty acids in the shells contrasts with reported composition of the soft tissues, although having a similar range of saturated fatty acids. The shell fatty acids are dissimilar to the soft tissue saturated fatty acids and the hydrogenated soft tissue derived bound fatty acids. This indicates that the shell lipids are not derived from hydrogenation of unsaturated fatty acids.

Cholesterol dominates the shell steroidal extracts as it does the soft tissues. A variety of other steroids are also extracted from the shells. Principal component analysis of the steroids extracted from *Littorina littorea* shells reveals that the surficial extract is least similar to the steroids reported for the soft tissues; probably due to the formation of oxidised steroid components by the use of hypochlorite in the initial cleaning treatments. The other shell locations are unaffected by this treatment. The intercrystalline and intracrystalline fractions are most similar to the reported soft tissue steroidal composition. However these intercrystalline and intracrystalline fractions are not identical. This may be due to differences in the original shell composition. This shows that the sterols extracted from the biomineral-protected locations within shells have the potential to be of use in chemo-taxonomy and biomarker studies.

CHAPTER 3. BOUND FATTY ACIDS AND STEROIDS FROM THE SHELLS OF RECENT MOLLUSCS IN PHYLOGENETIC AND DIETARY ANALYSIS.

ABSTRACT

Potential Class level phylogenetic differences between the shells of Recent molluscs are revealed by their steroidal and bound saturated fatty acid compositions. The bivalves (n=3) have bound saturated fatty acids with a carbon number maximum of C_{16} whilst the gastropods (n=8) have a maximum of C_{16} or C_{18} and higher yields. β-hydroxy fatty acids may indicate phylogenetic differences below the Class level for the Gastropoda. Principal component statistical analysis of the shell steroidal composition indicates differences at the Class level. Steroidal markers indicative of the dietary intake have been found in the shells.

INTRODUCTION

Traditional molecular phylogenetic analyses have been restricted to the studies of biomolecules from the soft tissues of extant species. Recent studies using similar techniques have been applied to fossil material (Robbins *et al.*, 1993). Proteins (e.g. Lowenstein & Scheuenstuhl, 1991), and DNA (e.g. Marshall, 1988) have been commonly used as sources of phylogenetic information. The occurrence of original, preserved lipids associated with biominerals may be equally useful. This would allow the extension of this technique both for readily accessible Recent shells and material throughout an extensive geological record.

Indigenous lipidic material is preserved within the shells of Recent molluscs. Of a variety of lipids extracted from the shells, the bound fatty acids and steroidal compositions of the shells show the best potential to be indigenous and provide a source of information relating to the phylogeny and diet (Chapter 2). The location of the lipids within the shell affects their indigeneity and preservation. The saturated fatty acids and steroidal content from intercrystalline and intracrystalline shell locations most closely resemble those of the soft tissues (Chapter 2). This study therefore examines the potential for the use these lipids in phylogenetic analysis and dietary studies.

The fatty acid content of molluscan soft tissues has been examined by several workers (e.g. Gardner & Riley, 1972; Joseph, 1982; Zhukova *et al.*, 1992) and is thought to be related to a variety of factors including sex, sexual cycle, temperature, diet, food availability, the tissue type, phylogeny and symbionts. These studies have primarily examined the unsaturated fatty acids. The almost exclusive extraction of saturated fatty acids from the shells is expected to limit this phylogenetic analysis.

In the soft tissues of molluscs it is claimed that there is a general Class level phylogenetic trend from complex sterol mixtures towards a sole cholesterol content with increasing evolutionary level (Morris & Culkin, 1977). Only the molluscan Class Polyplacophora contains a phylogenetically distinct Δ^7 cholesterol (Morris & Culkin, 1977). Volkman (1986) highlighted that few sterols have a restricted distribution enabling them to be used as markers for assigning specific marine and terrestrial steroidal sources into sediments. The sterol composition of molluscan soft tissues is further complicated by a number of factors. Dietary intake can be from a variety of sources and can be dependent on food selectivity and particle size. The dietary intake of benthic filter feeders also includes the reworking of the fine surficial sediment layer which contains a variety of sterols (Volkman, 1986). Molluscs are capable of alteration of dietary sterols as well as *de novo* synthesis. Symbiotic relationships with algae and bacteria (Fang *et al.*, 1992) also change the soft tissue steroidal composition. Differences in the ^{13}C isotopic composition of cholesterol has been observed in chemosymbiotic and heterotrophic bivalve shells (CoBabe & Pratt, 1995).

The inorganic microstructure of molluscan shells is under phylogenetic control (Carter, 1990a & 1990b). The microstructures of the Gastropoda and Bivalvia Classes are distinct. In the gastropods the nacreous microstructure is columnar; in bivalves this microstructure has a sheet structure. Generally the gastropods have also been observed to have a more intricate growing plane (Kobayashi, 1983). The composition of lipidic material in the shells of molluscs could be dependent on this shell microstructure, both in terms of the original lipidic composition of each structure as lipids may be involved in the process of biomineralization (Wuthier, 1973; Abolinš-Krogis, 1979; Isa & Okazaki, 1987), and to the protection afforded to the entombed lipids. This would give an apparent phylogeny for the lipid content of molluscan shells, where there is none present for the same compounds in the soft tissues.

This chapter reports the yields and distributions of indigenous bound fatty acids and steroids from the shells of different molluscan taxa with the aim of examining the phylogenetic and dietary controls on these lipid distributions. Both multivariate statistical analysis for comparison of distributions with those reported for the soft tissues, and the use of the sterols as biomarkers for specific dietary sources are used.

MATERIALS AND METHODS

The extraction protocols developed in Chapter 1, and described in Chapter 2, for the extraction of lipids from molluscan shells, are used here for the analysis of Recent molluscan shells listed in Table 1.

Fig. 1 illustrates the extraction protocols used. After initial cleaning treatments to remove surficial contamination, shells were sequentially extracted by the use of solvent extraction, saponification and full decalcification with further solvent extractions and saponification treatments. This results in the release of increasingly protected free and bound lipids from within the shell structure. Two extraction protocols (A and B) were employed, differing in the method used for some solvent extractions. Concentrated extracts from protocol A were also passed through a plug of alumina whilst those from protocol B were passed through a plug of cotton wool only. Shells were analysed in experimental batches, each with an inorganic calcium carbonate blank.

Gas chromatography (GC), gas chromatography - mass spectrometry (GCMS), the preparation of reagents and extracts, the use of surrogate standards for quantification, and multivariate statistical analysis was carried out according to Chapter 2.

Species	Extraction protocol	Age	Location
Batch 1			
Patella vulgata 1	A	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713
Littorina littorea 1	A	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713
Modiolus modiolus	A	live	Firth of Lorne, Scotland.
$CaCO_3$ 1	A		Inorganic control.
Batch 2			
Nucella lapillus 1	A	Recent	Bamburgh beach, Northumberland, England. Sheet 75, 181356
Littorina littorea 2	A	Recent	Rockcliffe, Scotland. Sheet 84, 848536
Sepia officinalis	A	Recent	South Coast, England.
Artica islandica 1	A	Recent	Ynys-las beach, Wales. Sheet 135, 909608
Artica islandica 2	A	Recent	Unknown, Durham University.
$CaCO_3$ 2	A		Inorganic control.
Batch 3			
Crepidula fornicata	A	Recent	Walton on the Naze, Essex, England. Sheet 169, 267235
$CaCO_3$ 3	A		Inorganic control.
Batch 4			
Patella vulgata 2	B	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713
Nucella lapillus 2	B	Recent	Bamburgh beach, Northumberland, England. Sheet 75, 181356
Littorina littorea 3	B	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713
$CaCO_3$ 4	B		Inorganic control.
Batch 5			
Patella vulgata 3	B	Live	Duncan's Hole, Shetland.
$CaCO_3$ 5	B		Inorganic control.

Table 1. Live and Recent shells analysed, grouped by experimental batches examined concurrently with calcium carbonate blank. Numbers after species name separate repeated samples of the same species. Also shown is the extraction protocol used and the sampling location. Map co-ordinates are from the Ordnance survey 1:50000 Landranger series. Live *Modiolus modiolus* were dredged at approximately 200m water depth from the Firth of Lorne and held in tanks with fresh sea water for five days until killed. S. Rowland (Department of Environmental Sciences, University of Plymouth) provided *Patella vulgata* shells collected Live from Duncan's Hole, Shetland. *Artica islandica* 2 shells were dated by amino acid racemisation as Recent (G. Sykes personal communication).

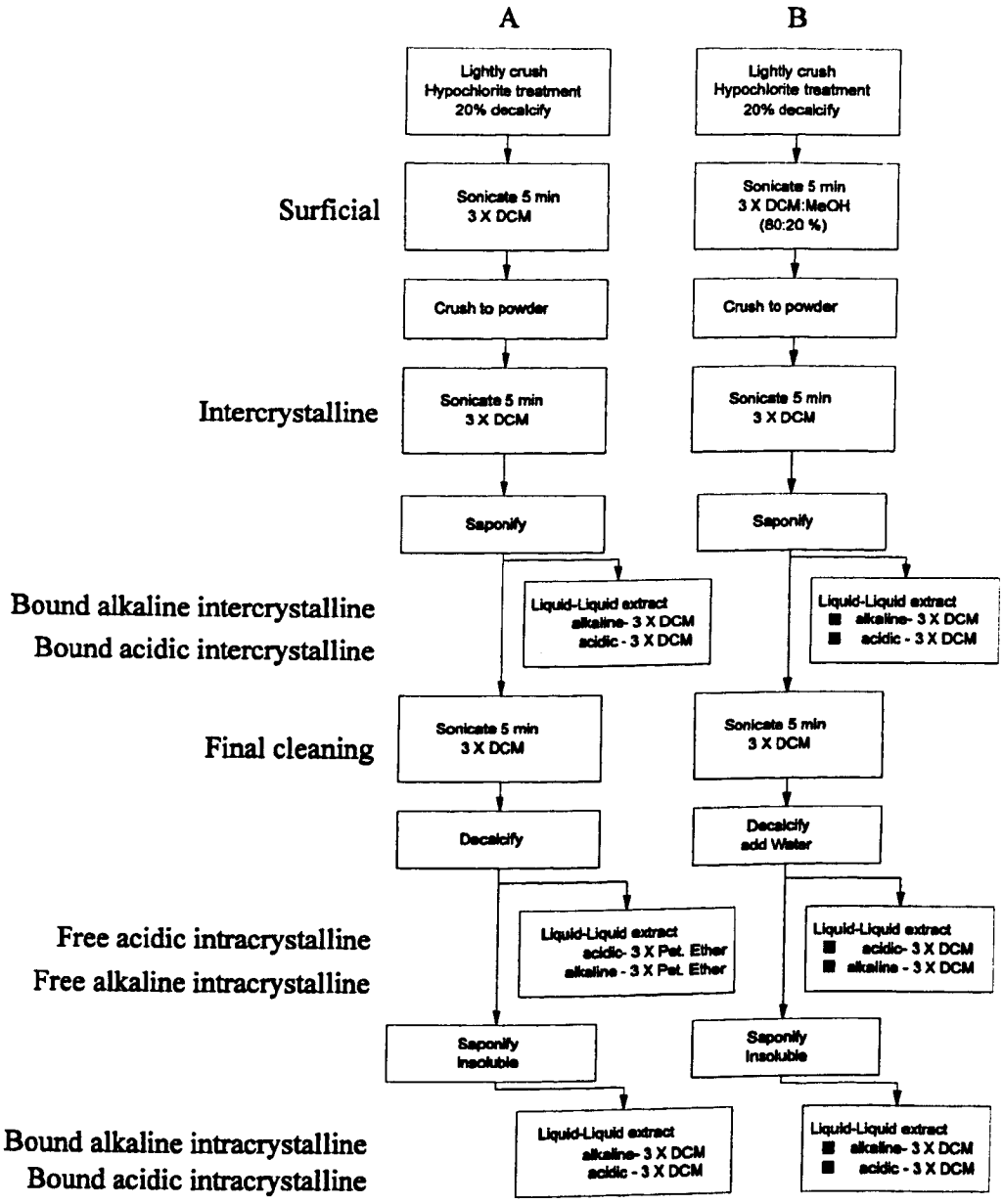


Figure 1. Flow diagram of extraction protocols A and B. Extract names apply throughout the text as on this figure. The addition of surrogate standards before solvent extraction is indicated by; ● = lupeol, ■ = C_{36} *n*-alkane.

RESULTS AND DISCUSSION

Shell microstructure

The effects of the biomineral on lipid phylogeny must be considered as the shell is deposited under phylogenetic control. Although there are no reported data to allow a detailed comparison of the shell microstructures of the species examined here, the more intricate microstructures of gastropods (Kobayashi, 1983) could lead to enhanced lipid preservation. This may explain the distinction between the gastropods and bivalves examined, particularly the higher FAME yields (Table 3), the more regular extraction of steroids (Table 5) and the extraction of β-hydroxy fatty acids from gastropod shells only (Chapter 2). The rate of penetration of hypochlorite into shells has been shown to be dependent on the microstructure (Chapter 1), which indicates that the preservation of shell lipids, particularly intercrystalline fractions which are accessible to the environment, may be similarly dependent on the shell microstructure.

1) Bound fatty acids

Carbon number maximum

The bound fatty acids are extracted as fatty acid methyl esters (FAMEs). The percentage distribution of saturated FAMEs from shells and calcium carbonate blanks are shown in Table 2. Both C_{16} and C_{18} carbon number maxima were observed in gastropods, whereas all the bivalves were observed to have a C_{16} maximum. This observation is intriguing, but as only 8 gastropods and 3 bivalves were analysed it is not statistically significant and interpretation will have to await a larger data set.

For the gastropod samples, *Littorina littorea*, *Patella vulgata* and *Nucella lapillus*, a change in the FAME carbon number maximum occurs between different extracts from the same shells and between duplicate analyses of the same species, as illustrated in Table 2. Sample heterogeneity can be excluded as the large sample size (~100 g) represents a large selection of pooled shell material. Where FAMEs are not present in duplicate shell extracts of the same species, the lack of FAME extraction is due to the analysis of small amounts of material at the limits of detection. For two of the shells (*Littorina littorea* 1 and *Nucella lapillus* 2), FAMEs are inefficiently extracted from the first liquid-liquid extraction, giving the intercrystalline bound alkaline fraction, the remaining FAMEs are extracted in the second intercrystalline bound acidic fraction. This is a function of the experimental protocol and not due to the location within the shell. A change in carbon number maximum is observed for the *Littorina littorea* 1 shells; as the yields are unknown this may be due to contamination.

Variation between the extracted FAMEs from different locations in the same shells, could be due to different original bound fatty acid compositions of these shell locations. This would result from different modes of fatty acid incorporation into the different shell fractions. These differences in lipid distributions from the same shell demonstrates the importance of examining all the shell fractions.

Yields of quantified FAMEs

It was observed during GC analysis that the yields of FAMEs from the gastropod samples were generally higher than the bivalve extracts. However, no bivalve FAMEs were quantified. To allow comparison between these Classes the flame ionisation detector (FID) response has been normalised by adjusting for the amount of shell extracted, GC dilution and injection volumes. Although of limited use due to varying analytical conditions this allows some quantitative comparison of yields for these data as shown in Table 3. The extracts have been separated into three groups by the horizontal lines in Table 3, those samples with similar values to the calcium carbonate blanks can be excluded due to laboratory contamination. It is the *Littorina littorea* and *Nucella lapillus* (Gastropoda) extracts which exhibit significantly higher FAME yields than the bivalves and cephalopoda.

Species	*Modiolus modiolus*	*Modiolus modiolus*	*Artica islandica 1*	*Artica islandica 2*	*Artica islandica 2*	*Crepidula fornicata*	*Crepidula fornicata*	*Littorina littorea 1*	*Littorina littorea 1*	*Littorina littorea 1*	*Littorina littorea 2*	*Littorina littorea 2*	*Littorina littorea 3*	*Littorina littorea 3*	*Nucella lapillus 1*	*Nucella lapillus 1*	*Nucella lapillus 2*	*Nucella lapillus 2*	*Patella vulgata 1*	*Patella vulgata 1*	*Patella vulgata 2*	*Sepia officinalis*	CaCO3 blank 1	CaCO3 blank 2	CaCO3 blank 2	CaCO3 blank 5
Extract	Intercrystalline bound acidic	Intracrystalline bound acidic	Intercrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound acidic	Intercrystalline bound alkaline	Intercrystalline bound acidic	Intracrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound alkaline	Intercrystalline bound acidic	Intracrystalline bound acidic	Intracrystalline bound alkaline	Intercrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound acidic	Intercrystalline bound acidic	Intercrystalline bound acidic	Intercrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound acidic	Intercrystalline bound acidic
Cmax	16	16	16	16	16	16	16	16	18	18	18	18	18	16	18	18	16	16	16	16	16	16	19	16	16	16
12						7			0	1	8	1	2		0			1								
13	2					2			0		1	0	1		1		3	1	1							
14	14		8	25		15			1	3	9	3	5		2	1	16	2	7	6	11	5				32
15	8		6	18		7			0		2	2	2		3	2	13	5	4		4	4			23	13
16	57	71	48	48	74	52	81	74	17	27	24	26	31	87	33	25	42	45	77	74	85	57		65	42	49
17	3		3		11	2		6	4	4	6	6	5		7	6	12	9		3		5	41			
18	15		35	5	15	16	19	21	74	60	49	62	53	13	43	50	14	38	10	16		29		35	35	6
19									1	4					0								59			
20				4							2				8	15										
21																										
22		3							3						4					1						
23																										
24		8																								
25		13																								
26		5																								

Table 2. Percentage composition of bound fatty acid shell extracts by carbon number, with species, fraction and carbon number maxima.

Species	Molluscan Class	Shell extract	Sum of the normalised FAME FID response (μV.s)
Artica islandica 1	Bivalvia	Intracrystalline bound acidic	25
Artica islandica 2	Bivalvia	Intracrystalline bound alkaline	30
Sepia officinalis	Cephalopoda	Intracrystalline bound acidic	39
Crepidula fornicata	Gastropoda	Intracrystalline bound acidic	139
Modiolus modiolus	Bivalvia	Intracrystalline bound acidic	221
$CaCO_3$ Control 2	-	Intracrystalline bound acidic	293
Artica islandica 2	Bivalvia	Intracrystalline bound acidic	507
Patella vulgata 1	Gastropoda	Intracrystalline bound acidic	839
$CaCO_3$ Control 1	-	Intercrystalline bound acidic	1958
$CaCO_3$ Control 2	-	Intercrystalline bound acidic	2624
Artica islandica 2	Bivalvia	Intercrystalline bound acidic	3498
Modiolus modiolus	Bivalvia	Intercrystalline bound acidic	6624
Patella vulgata 1	Gastropoda	Intercrystalline bound acidic	20840
Littorina littorea 1	Gastropoda	Intercrystalline bound alkaline	31097
Littorina littorea 1	Gastropoda	Intracrystalline bound acidic	33756
Artica islandica 1	Bivalvia	Intercrystalline bound acidic	36123
Crepidula fornicata	Gastropoda	Intercrystalline bound acidic	36279
Sepia officinalis	Cephalopoda	Intercrystalline bound acidic	40356
Littorina littorea 2	Gastropoda	Intracrystalline bound acidic	122253
Nucella lapillus 1	Gastropoda	Intercrystalline bound acidic	153291
Littorina littorea 2	Gastropoda	Intercrystalline bound acidic	203808
Nucella lapillus 1	Gastropoda	Intracrystalline bound acidic	216788
Littorina littorea 1	Gastropoda	Intercrystalline bound acidic	352901

Table 3. Sum of individual FID response to FAMEs for experimental batches 1 to 3, normalised for GC dilution, injection volumes and material extracted. Listed by species and extract and sorted by increasing yield. Horizontal lines separate the extracts into three groups.

Those gastropod samples which have yielded quantified FAMEs (μg/g of material extracted) are shown in Fig. 2. The alkaline and acidic fractions have been combined to give the intercrystalline bound and intracrystalline bound fractions which are dependent on the location within the shell and not the extraction procedure. The calcium carbonate blank is also shown and reveals low yields of laboratory contamination. The even over odd carbon number distributions and changes in carbon number maximum with shell location are seen.

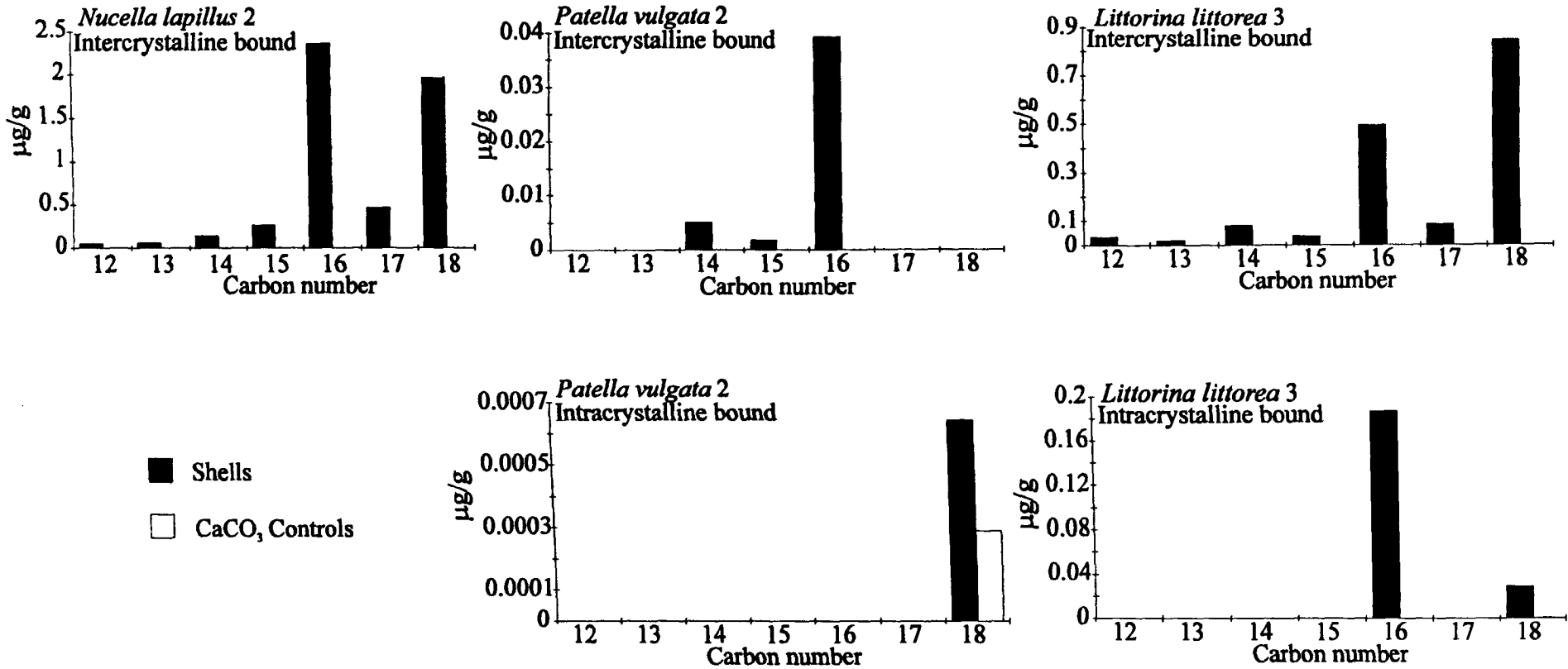


Figure 2. Yields (µg/g of material extracted) of FAMEs by carbon number for intercrystalline bound and intracrystalline bound shell locations. Also showing yields from calcium carbonate blank.

Multivariate statistical analysis

The dendrogram shown in Fig. 3 was produced using the percentage distributions of only the saturated fatty acids reported by CoBabe and Pratt (1995). Included in this analysis as outgroups are the reported saturated fatty acids from two marine dinoflagellates and the free fatty acids from Human skin. The fatty acids from Human skin are most distantly related to all the samples. However, there is no grouping of the saturated fatty acids from the shells with those from the soft tissues of the same species. The algae, sediment and fossil shells are also at similar distances to the soft tissues and shells of the same species, and therefore the phylogenies are not distinguished.

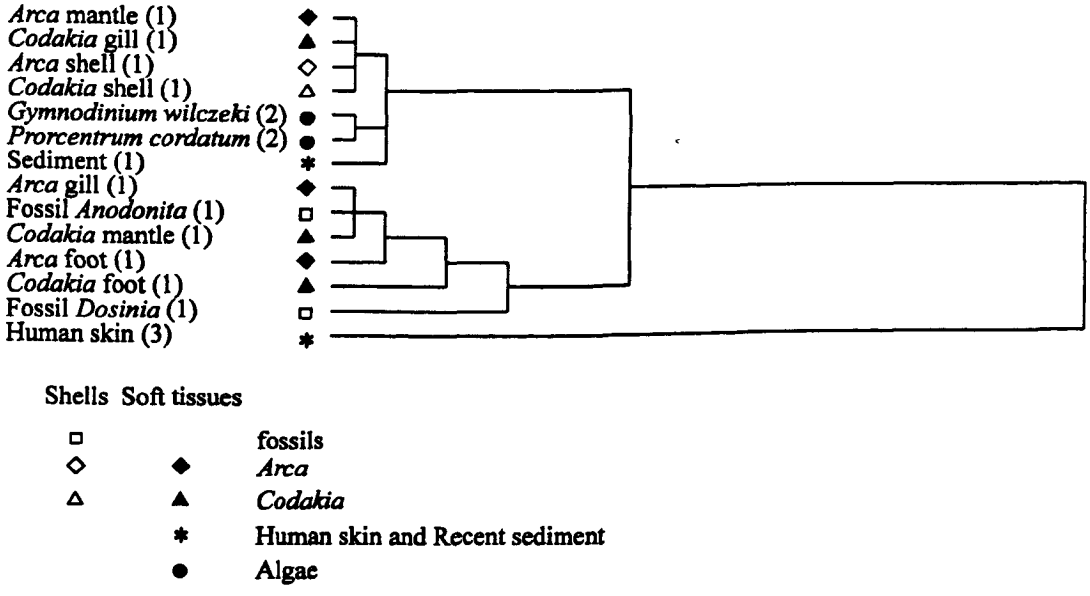


Figure 3. Dendrogram of reported fatty acids from molluscan soft tissues, live and fossil shells, algal and Human skin. References are identified in brackets; 1 = CoBabe & Pratt (1995); 2 = Nichols *et al.* (1984); 3 = Wertz & Downing (1991).

A dendrogram constructed from the FAMEs of the shell extracts, calcium carbonate blanks, the reported values of the saturated fatty acids of the soft tissues for the same species as analysed herein, the saturated fatty acids from two marine dinoflagellates and the free fatty acids from Human skin is shown in Fig. 4 (Data set is shown in Appendix 8). A cutoff value is shown which separates the samples into six groups. As previously observed (Fig. 3), no grouping of shell and soft tissues from the same species is observed. However, the majority of the *Littorina littorea* and *Nucella lapillus* shell extracts cluster together. This is due to the carbon number maximum of C_{18} of these extracts. The free fatty acids from Human skin are at a large distance from the shell extracts which indicates that although contamination is present as

revealed by the calcium carbonate blanks, the source may not be from skin contact. Although some of the blank extracts and the human skin free fatty acids form different groups to the other samples, this is not the case for all the blanks and the dinoflagellates are closely related to some shell extracts.

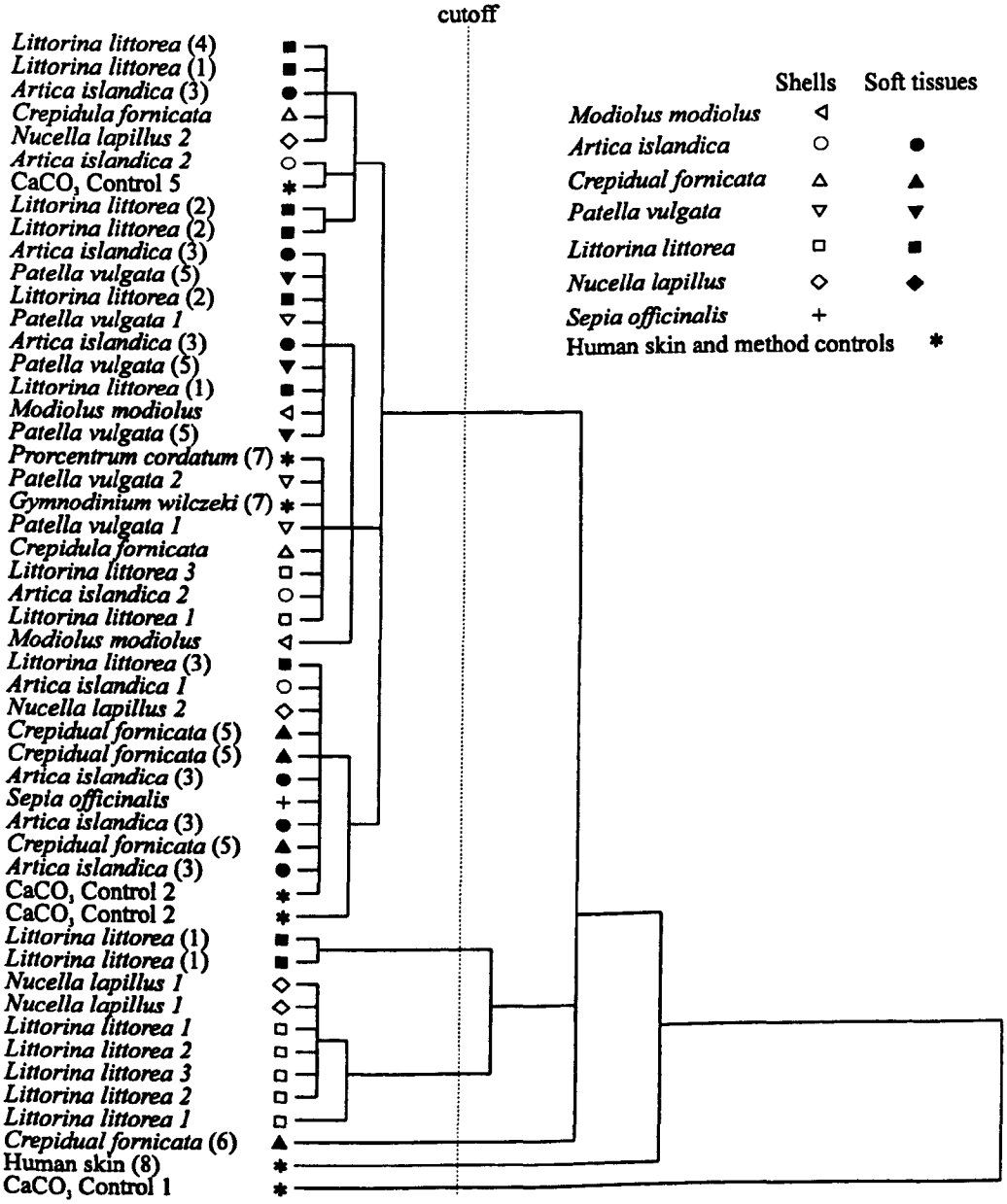


Figure 4. Dendrogram of shell and calcium carbonate blank FAME extracts and reported soft tissues, and outgroups. References are identified in brackets; 1 = Ackman *et al.* (1971); 2 = Brockerhoff *et al.* (1967); 3 = Ackman *et al.* (1974); 4 = Paradis & Ackman (1976); 5 = Gardner & Riley (1972); 6 = Herbreteau *et al.* (1994); 7 = Nichols *et al.* (1984); 8 = Wertz & Downing (1991).

Fig. 5 shows dendrograms using the same data as above (Fig. 4), except that the Class Gastropoda (Fig. 5a) and Bivalvia (Fig. 5b) are separated. No clustering below the Class level is observed. The grouping of some of the *Littorina littorea* and *Nucella lapillus* shell extracts is maintained, as observed in Fig. 4.

As no grouping of soft tissues with shells of the same species, or no grouping of shell extracts by Class, genus or species is observed, these analyses indicate that the bound saturated fatty acids from the shells are not suitable for detailed phylogenetic usage.

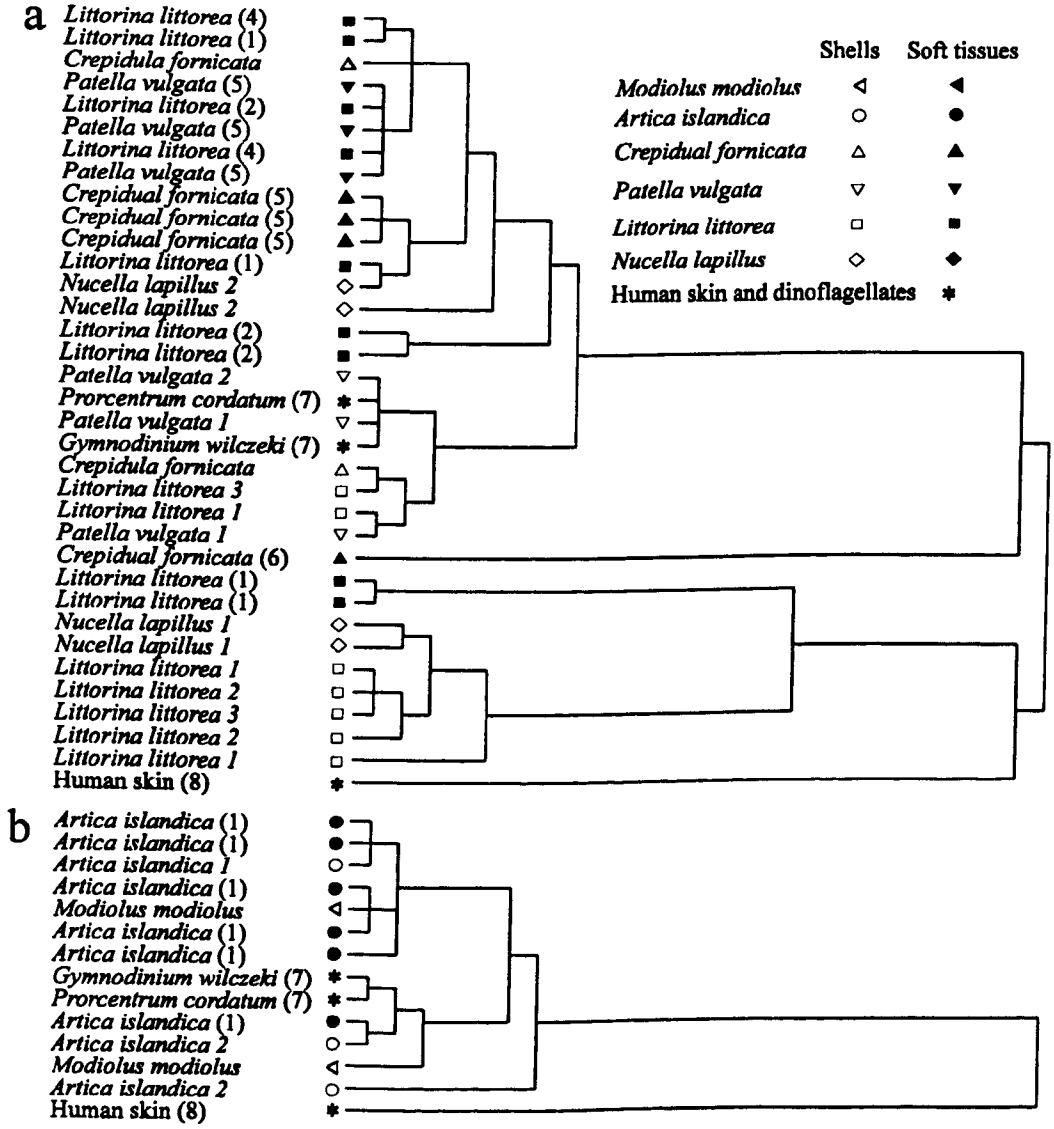


Figure 5. Dendrogram of shell and calcium carbonate blank FAME extracts and reported soft tissues, and outgroups. Split by Class, a) Gastropoda, b) Bivalvia. References are identified in brackets; 1 = Ackman *et al.* (1971); 2 = Brockerhoff *et al.* (1967); 3 = Ackman *et al.* (1974); 4 = Paradis & Ackman (1976); 5 = Gardner & Riley (1972); 6 = Herbreteau *et al.* (1994); 7 = Nichols *et al.* (1984); 8 = Wertz & Downing (1991).

β-Hydroxy fatty acids

The occurrence of these compounds only in the shells of *Littorina littorea* and *Nucella lapillus* (Chapter 2, page 70) is evidence for a possible phylogenetic rôle, via *de novo* biosynthesis. This phylogenetic distinction is expressed below the Class level as these shells are members of the Gastropoda and no other samples contained β-hydroxy fatty acids. Dietary influences can be excluded due to the exclusively herbivorous *Littorina littorea* and carnivorous *Nucella lapillus* as shown in Table 4.

Species	Habitat	Feeding mode	Diet
Crepidula fornicata	Sublittoral to 10 m	Cillary feeder	Phytoplankton, floating detritus
Nucella lapillus	Littoral	Carnivorous	Barnacles, juvenile mussels
Littorina littorea	Littoral to 60 m	Herbivorous	Sea weed, diatoms, lichen, algal detritus
Patella vulgata	Littoral	Herbivorous	Sea weed, diatoms, lichen, algal detritus
Sepia officinalis		Carnivorous	Shrimp, crab, fish
Artica islandica	Benthic	Filter feeder	Floating detritus
Modiolus modiolus	Benthic	Filter feeder	Floating detritus

Table 4. Habitat, feeding mode and diets of the species examined here. Data from Fretter & Graham (1962), Tebble (1966), Campbell (1976) and Graham (1988).

2) Steroids

The steroidal composition of the shell extracts is shown in Table 5. Data from all experimental protocols have been used in these analyses.

Extract	Species	25-Bisnorcholesta-5,22-dien-3b-ol	24-Methyl-27-norcholesta-5,22-dien-3b-ol	Cholesta-5,22-dien-3b-ol	Cholesterol	Cholesta-5,24-dien-3b-ol	24-Methylcholesta-5,22-dien-3b-ol	(R or S) 24-Methylcholest-5-en-3b-ol	Cholesta-3,5-dien-7-one	Cholest-5-en-3B-20-diol	Cholest-4-en-3-one	Unidentified steroidal ketone	24-Ethylcholest-5-en-3b-ol	24-Ethylcholesta-5,24(28)-dien-3b-ol	24-Methylcholesta-5,24(28)-dien-3b-ol	Cholest-5-en-3b-hydroxy-7-one	Sterane	Sum of remaining unidentified 3-OH D5 sterols
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	Q	X
Intracrystalline bound alkaline	*Artica islandica 1*	5.6		8.7	55.4		11.7						8.8					9.8
Intercrystalline bound alkaline	*Littorina littorea 1*				47.5	10.3				8.9			3.0				1.4	28.9
Intercrystalline bound alkaline	*Littorina littorea 2*				55.5	15.1			10.5	9.5					1.6			7.7
Intracrystalline bound alkaline	*Littorina littorea 2*				93.0	5.4												1.7
Intracrystalline bound acidic	*Littorina littorea 2*				72.4				4.1		10.4							13.1
Surficial	*Littorina littorea 3*				14.8				11.0		8.1	36.7				29.4		0.0
Intercrystalline	*Littorina littorea 3*			10.0	46.4	9.0								1.4				33.2
Intercrystalline bound alkaline	*Littorina littorea 3*				49.8	18.8			4.5	12.4			3.2	2.4				9.0
Intracrystalline free acidic	*Littorina littorea 3*				73.5	21.2												5.3
Intracrystalline bound alkaline	*Littorina littorea 3*				80.6													19.4
Intracrystalline bound acidic	*Littorina littorea 3*				88.8													11.2
Intercrystalline bound alkaline	*Nucella lapillus 1*				57.9			4.5	2.1	8.6								26.8
Intracrystalline bound alkaline	*Nucella lapillus 1*	2.1	4.5		73.2	1.1		6.3	1.6		4.9		1.5					4.4
Intracrystalline bound acidic	*Nucella lapillus 1*										100.0							0.0
Intercrystalline	*Nucella lapillus 2*				91.2			0.9										7.9
Intercrystalline bound alkaline	*Nucella lapillus 2*				94.9				1.8	0.0	2.7							0.6
Intracrystalline free acidic	*Patella vulgata 1*				96.1													3.9
Intracrystalline bound acidic	*Sepia officinalis*										100.0							0.0

Table 5. Steroids extracted from shells, as a percentage of the total steroids. Steroids are labelled and arranged A to P by inereasing retention times. Unidentified sterols have been combined and labelled X.

Multivariate statistical analysis of brachiopod steroids

A dendrogram produced from data reported by Clegg (1993) of the steroidal composition of brachiopod soft tissues and shells is shown in Fig. 6. A cutoff value is indicated which splits the samples into five major groups. In general, three groups are formed by the majority of the algae. The "control" which consists of cholesterol only, forms a separate group, and the brachiopod shells and soft tissues form another group. This group can be further divided into (i) the brachiopod soft tissues (■), (ii) two algae (●), and (iii) the brachiopod shells (□) and the *Artica islandica* (a bivalve mollusc) soft tissues (*). It appears that the brachiopod soft tissues are distinct from the molluscan soft tissues, although the brachiopod shell sterols are not similar to those from the brachiopod soft tissues.

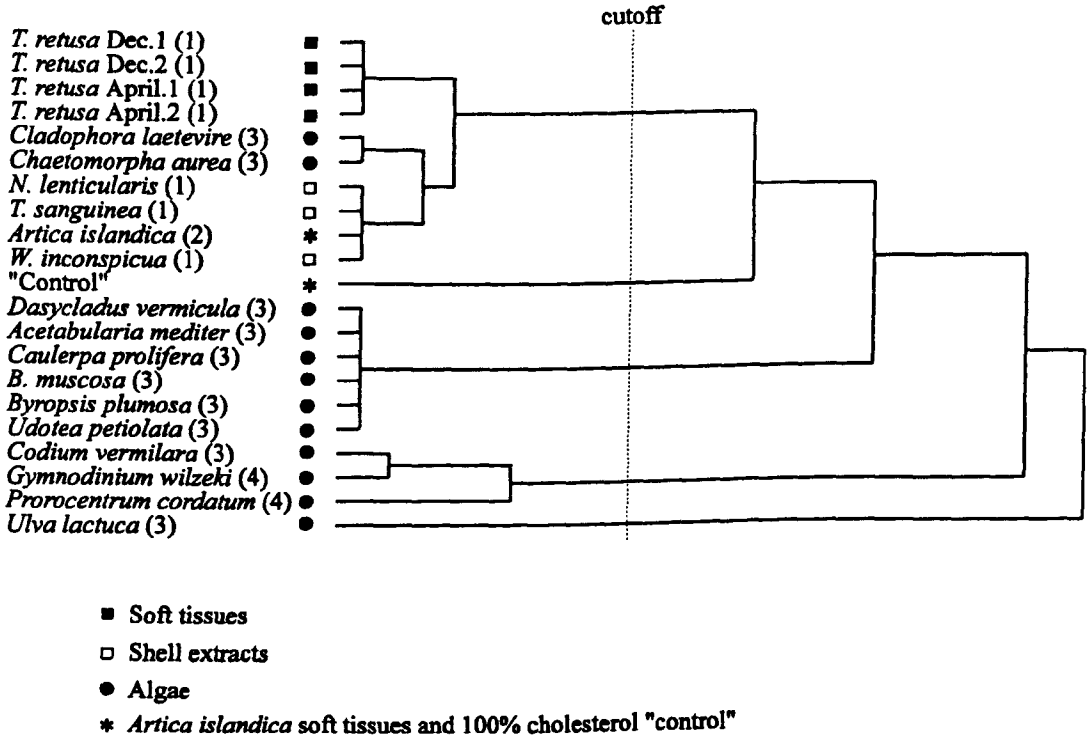


Figure 6. Dendrogram of reported sterol composition from shells and soft tissues of brachiopods, with outgroups. References are identified in brackets; 1 = Clegg (1993); 2 = Ackman *et al.* (1971); 3 = De Napoli *et al.* (1982); 4 = Nichols *et al.* (1984).

Multivariate statistical analysis of molluscan steroids

The first and second principal components for the shells examined here, reported molluscan and brachiopod (+) soft tissues, and marine algal and dinoflagellate (●) steroidal composition are shown in Fig. 7. (Data shown in Appendix 9, factors shown in Appendix 10). Where steroids are reported in the soft tissues but not found in the shells, they have been added to the unknown sterol group (X in Table 5) and each data set normalised to 100%. Combining the unidentified 3-OH Δ^5 sterols and the sterols which occur only in the soft tissues results in a potential loss of information. For the shell extracts, the only surficial extract from *Littorina littorea* 3 has been excluded as Chapter 2 has previously shown that this fraction is most likely to contain oxidised steroids and are therefore altered and are unlike the soft tissues, this excludes the components K (an unidentified steroidal ketone) and O (cholest-5-en-3β-hydroxy-7-one. The intracrystalline bound fraction from *Nucella lapillus* 1 was also excluded, as this sample is distinct from the others due to the presence of the steroid 24-methyl-27-norcholesta-5,22-dien-3β-ol (B).

The molluscan data have been labelled at the Class level. The *Artica islandica* (A) (Bivalvia) shell extract plots with the soft tissues from the bivalves, and separately from the other shell extracts. The gastropods *Littorina littorea* (L), *Nucella lapillus* (N) and *Patella vulgata* (P) shell samples, with the exception of one *Nucella lapillus* shell extract, plot over an area which includes the soft tissues of the gastropods. They remain distinct from the algal outgroups and other molluscan Classes. The cephalopod *Sepia officinalis* (S) does not plot with the cephalopod soft tissue sample, this may be due to the lack of cholesterol extracted from the endoskeleton shell.

As the soft tissues are likely to be the source of the shell lipids (Chapter 2), the use of the steroidal components present in the shells for phylogenetic analysis is limited by the composition of the soft tissues, this provides only limited phylogenetic distinctions. Only the molluscan Class Polyplacophora contains a phylogenetically distinct Δ^7 cholesterol (not extracted from the shells here) and there is a general Class level phylogenetic trend from complex sterol mixtures towards a sole cholesterol content with increasing evolutionary level (Morris & Culkin, 1977). Although distinct from algal and brachiopod outgroups, and having similar steroidal compositions to those reported for the soft tissues of the same molluscan Class, the shell steroids extracted in this study are of no further phylogenetic use. Techniques using other variables such as the shell morphology, used here to identify the shells, provide more phylogenetic information than the steroidal composition (Fig. 7a).

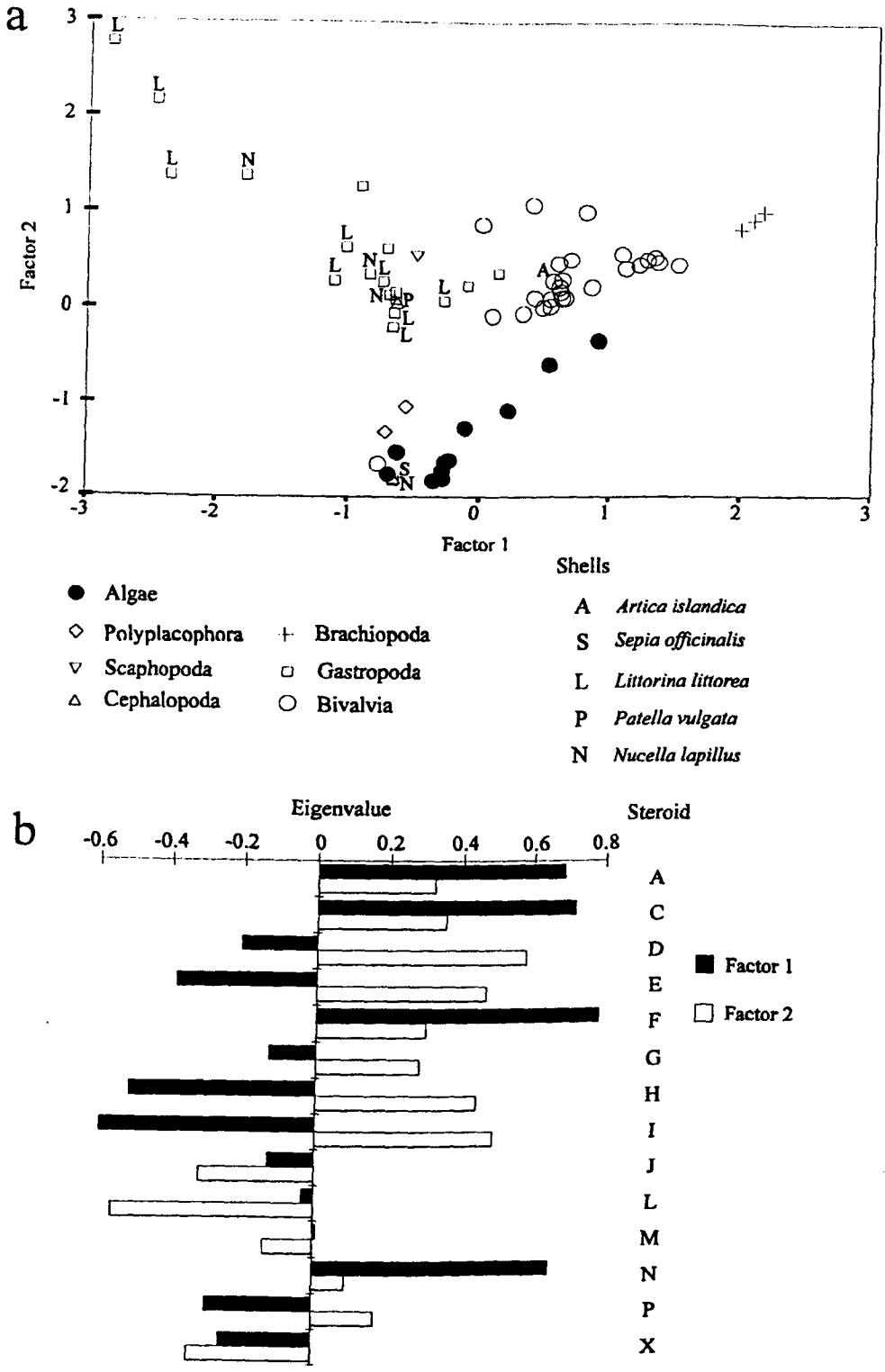


Figure 7. a) First and second principal components resulting from steroids from shells examined here and reported soft tissues. Shells are labelled with the species, soft tissues are plotted as brachiopods and by molluscan Class only. b) Eigenvalues for factors 1 and 2 for each steroid.

3) The use of sterols as dietary indicators

The use of sterols as biomarkers, where specific compounds are used to indicate a carbon pathway is used here to indicate dietary relationships. 24-Methylcholesta-5,22-dien-3β-ol (F) occurs solely in the *Artica islandica* shell extracts. This sterol is indicative of diatom, dinoflagellate or other algae (Volkman, 1986) and they may therefore be present in the diet of the *Artica islandica.* Volkman (1986) also states that diatoms are thought to be the primary source of cholesta-5,22-dien-3β-ol (C) which was extracted from *Littorina littorea* and *Artica islandica* shells. 24-Methylcholesta-5,24(28)-dien-3β-ol (N), only extracted from a *Littorina littorea* shell, is also indicative of diatoms or dinoflagellates (Volkman, 1986). 25-Bisnorcholesta-5,22-dien-3β-ol (A) is a typical phytoplankton sterol linked by diet to the soft tissues of mussels (Stoilov *et al.*, 1984), the extraction from the bivalve *Artica islandica* is therefore not surprising, if this sterol has no other sources then its extraction from the carnivorous *Nucella lapillus* implies a source via the mussel or barnacle prey. These data match the dietary habits of these molluscs shown in Table 4. However, as the steroidal composition of most organisms remains to be determined, these sterols may not be uniquely indicative of a specific dietary source.

24-Ethylcholesta-5,24(28)-dien-3β-ol (M) and 24-ethylcholest-5-en-3β-ol (L) are metabolites of phytosterol dealkylation to cholest-5,24-dien-3β-ol (E) which is a precursor to cholesterol (D) in *Patella vulgata* (Goad, 1978). With the exception of 24-ethylcholesta-5,24(28)-dien-3β-ol (M) these sterols are found in the *Patella vulgata* shell extracts. The other abundant sterols in the shells, such as 24-ethylcholest-5-en-3β-ol (L), 24-methylcholest-5-en-3β-ol (G) and cholesterol (D) have varied sources (Volkman, 1986), this precludes any identification of specific dietary sources.

The use of sterols from the shells for identifying dietary relationships has been shown to be worthy of further investigation and may be the only source of information relating to the diet in fossil shells. However *de novo* synthesis, the alteration of dietary sterols, symbiotic relationships and varied sources may add further complications.

CONCLUSIONS

For this particular sample set the bivalves have a saturated FAME carbon number maximum of C_{16} whilst the gastropods have either a C_{16} or C_{18} maximum. The gastropods are also observed to generally have a higher bound fatty acid content than the bivalves. These Class level differences are the only phylogenetic distinction observed by using the bound fatty acid distributions. The small sample size (3 bivalves and 8 gastropods) which was limited by the time consuming extraction procedure, may not make these results statistically significant.

The extraction of β-hydroxy fatty acids, only from *Littorina littorea* and *Nucella lapillus* shells, may indicate a possible phylogenetic distinction below the Class level. The steroids extracted from the shells show significant variation allowing phylogenetic analysis. The bivalve and gastropod shell extracts, whilst showing some variation, cluster near to the reported soft tissues of the same Class. The use of sterols as biomarkers confirms a diatom, dinoflagellate or other algal diet for *Artica islandica*. And similarly a diatom or dinoflagellate diet for *Littorina littorea*. The presence of a typical phytoplankton sterol in the carnivorous *Nucella lapillus* may indicate a dietary source via its mussel or barnacle prey.

CHAPTER 4. PRELIMINARY INVESTIGATIONS INTO HYDROCARBON POLLUTION IN MOLLUSC SHELLS.

ABSTRACT

The application of a methodology for the sequential extraction of lipids from molluscan shells has been used in a preliminary analysis of shell material for the presence of hydrocarbon pollutants. The shell *n*-alkanes require comparison of carbon number distributions and yields with an experimental calcium carbonate blank to ensure indigeneity. Different *n*-alkane distributions within two *Artica islandica* shell samples are attributed to the different sampling locations. Differences between *Patella vulgata* and *Littorina littorea* shells from the same environment have also been observed, indicating different *n*-alkane uptake by different species. Polyaromatic hydrocarbons and sterane biomarkers reported to be present in the soft tissues of *Patella vulgata* exposed to the *Braer* oil spill have been searched for in the shells of an exposed sample. These compounds have not been detected. No increase in the shell *n*-alkane yields or similarity in carbon number distribution with the spilt oil is observed. This suggests no hydrocarbon incorporation or depuration pathway into the shell.

INTRODUCTION

The soft tissues of bivalve molluscs have been routinely used to monitor contaminants from the local marine environment (Goldberg, 1980). A variety of accumulated organic pollutants, such as petroleum, polyaromatic and synthetic hydrocarbons have been revealed in the analyses of molluscan soft tissues (Goldberg, 1980; Mix, 1984). Hydrocarbons such as the *n*-alkanes (Mackie *et al.*, 1974), and polyaromatic hydrocarbons (Mix, 1984) are associated with anthropogenic sources but may also occur naturally (Brassell & Eglinton, 1980). The often unique "fingerprint" of fossil fuel biomarkers can be used for source correlation particularly for acute pollution events such as oil spills (e.g. Peters & Moldowan, 1993).

Bioconcentration of hydrocarbons, based on hydrophobic partitioning between water and the soft tissues, is further complicated by factors such as bioavailability, phylogeny, metabolisation and biotransformation (Barron, 1990). The food chain has also been shown to be an important factor in the uptake of pollutants such as polychlorinated biphenyls (Connolly, 1991). These effects are illustrated by different hydrocarbon pathways from the same oil spill having different effects on the soft tissues of different molluscan species (Boehm *et al.*, 1982). Blumer *et al.* (1970) found that the *n*-alkane distributions in the soft tissue of bivalves after an acute oil

spill could be distinguished from those occurring naturally by comparison with the same species in unaffected locations. However, Whittle *et al.* (1977) had difficulty identifying the origin of the *n*-alkanes from the soft tissues of a variety of marine organisms, including locations with a high *n*-alkane input. The hydrocarbon content of soft tissues from mussels from North Sea oil production platforms have been examined by Rowland and Volkman (1982) and Tibbetts *et al.* (1982). An unresolved complex mixture of hydrocarbons was observed, and although of similar yields to mussels from known polluted locations no increase was observed with the start of discharge of production waters. An *n*-alkane carbon number distribution from C_{16} to C_{31} was observed with an unusual even over odd carbon number predominance. This distribution did not match the production crude, but was more similar to the sediment. The sterane and triterpane distributions extracted from the soft tissues also differed from the production crude and most closely resembled a Middle Eastern oil. These results suggest that the use of *n*-alkanes alone cannot determine a specific source of chronic hydrocarbon pollution.

If the uptake of pollutant chemicals in the soft tissues is greater than the rate of their metabolisation and excretion then they will be accumulated over time. The hydrophobic nature of many organic pollutants may make them behave in a similar way to lipids within the soft tissues. If there is transfer of these pollutants to the biomineral during shell growth then accumulation will occur in the shell as shown for the soft tissue lipids (Chapter 2). It is therefore possible that a record of chronic pollution will be present in the shells from polluted environments, and will represent the accumulation history of these compounds over a longer time scale than the soft tissues. Incorporation into the shell could provide a mechanism for removal of the non-metabolisable or toxic compounds from the soft tissues.

A specific example of an acute oil spill has been investigated here to observe the hydrocarbon content of the shells of *Patella vulgata*. The incorporation of Gulfaks crude oil spilt from the *Braer* tanker (Wolff *et al.*, 1993; Ritchie & O'Sullivan, 1994) into the soft tissues of exposed *Patella vulgata* has been examined by Glegg and Rowland (1996). They reported the presence and the subsequent depuration from the soft tissues of the aromatic hydrocarbons; naphthalene, phenanthrene, alkylnaphthalenes and alkylphenanthrenes. Sterane biomarkers from the soft tissues (Glegg & Rowland, 1996) identified the hydrocarbon source as the *Braer* cargo by comparison to the production crude (Wolff *et al.*, 1993).

This Chapter reports a preliminary investigation into the potential of hydrocarbon pollutants entombed within the shells of Recent molluscs. Shells from both chronic and an example of an acute pollution event have been analysed. The compounds previously extracted from the shells and considered suitable for analysis are the *n*-alkanes (Chapter 2). The naphthalene, phenanthrene, alkylnaphthalenes, alkylphenanthrenes and sterane biomarkers detected in the soft tissues of *Patella vulgata* affected by the *Braer* oil spill (Glegg & Rowland, 1996) have also been investigated in an exposed shell sample.

METHOD

Samples

Live and Recent shells were collected as shown in Table 1. S. Rowland (Department of Environmental Sciences, University of Plymouth) provided *Patella vulgata* shells collected live from Duncan's Hole, Shetland on 16 May 1994 after the *Braer* tanker grounding on 5 January 1993 and the resulting oil spillage.

Shell extraction protocols

Fig. 1 illustrates the shell extraction protocols used. Two protocols (A and B) were employed. Protocol A was used for determining carbon number distributions and protocol B used for quantification of yields. The protocols differ in the method used for some solvent extractions, and concentrated extracts from protocol A were passed through a plug of alumina whilst those from protocol B were passed through a plug of cotton wool only (Chapter 1).

After initial cleaning treatments to remove surficial contamination, shells were sequentially extracted by the use of solvent extraction, saponification and full decalcification with further solvent extractions and saponification treatments. This results in the sequential release of increasingly protected free and bound lipids from within the shell. Shell samples were analysed in experimental batches, each with an inorganic calcium carbonate (calcite) blank. The purpose of this control is to determine the distribution, and where quantified, the yields of laboratory contamination introduced during extraction and analysis.

Species	Protocol	Age	Location
Batch 1			
Patella vulgata 1	A	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713.
Littorina littorea 1	A	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713.
$CaCO_3$ 1	A	-	Inorganic blank.
Batch 2			
Artica islandica 1	A	Recent	Ynys-las beach, Wales. Sheet 135, 909608.
Artica islandica 2	A	Recent	Unknown, Durham University.
$CaCO_3$ 2	A	-	Inorganic blank.
Batch 3			
$CaCO_3$ 3	A	-	Inorganic blank.
Batch 4			
Patella vulgata 2	B	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713.
Nucella lapillus 2	B	Recent	Bamburgh beach, England. Sheet 75, 181356.
Littorina littorea 3	B	Recent	Cullercoats Bay, Northumberland, England. Sheet 88, 364713.
$CaCO_3$ 4	B	-	Inorganic blank.
Batch 5			
Patella vulgata 3	B	Live	Duncan's Hole, Shetland.
$CaCO_3$ 5	B	-	Inorganic blank.

Table 1. Samples analysed this study. Species, extraction protocol used, sample age and sampling location. Shells are grouped by experimental batch with the calcium carbonate calcium carbonate blank. Map co-ordinates are from the Ordnance survey 1:50000 Landranger series. Live *Modiolus modiolus* were dredged at approximately 200m water depth from the Firth of Lorne and held in tanks with fresh sea water for five days until killed. *Artica islandica* 2 shells were dated by amino acid racemisation as Recent (G. Sykes personal communication).

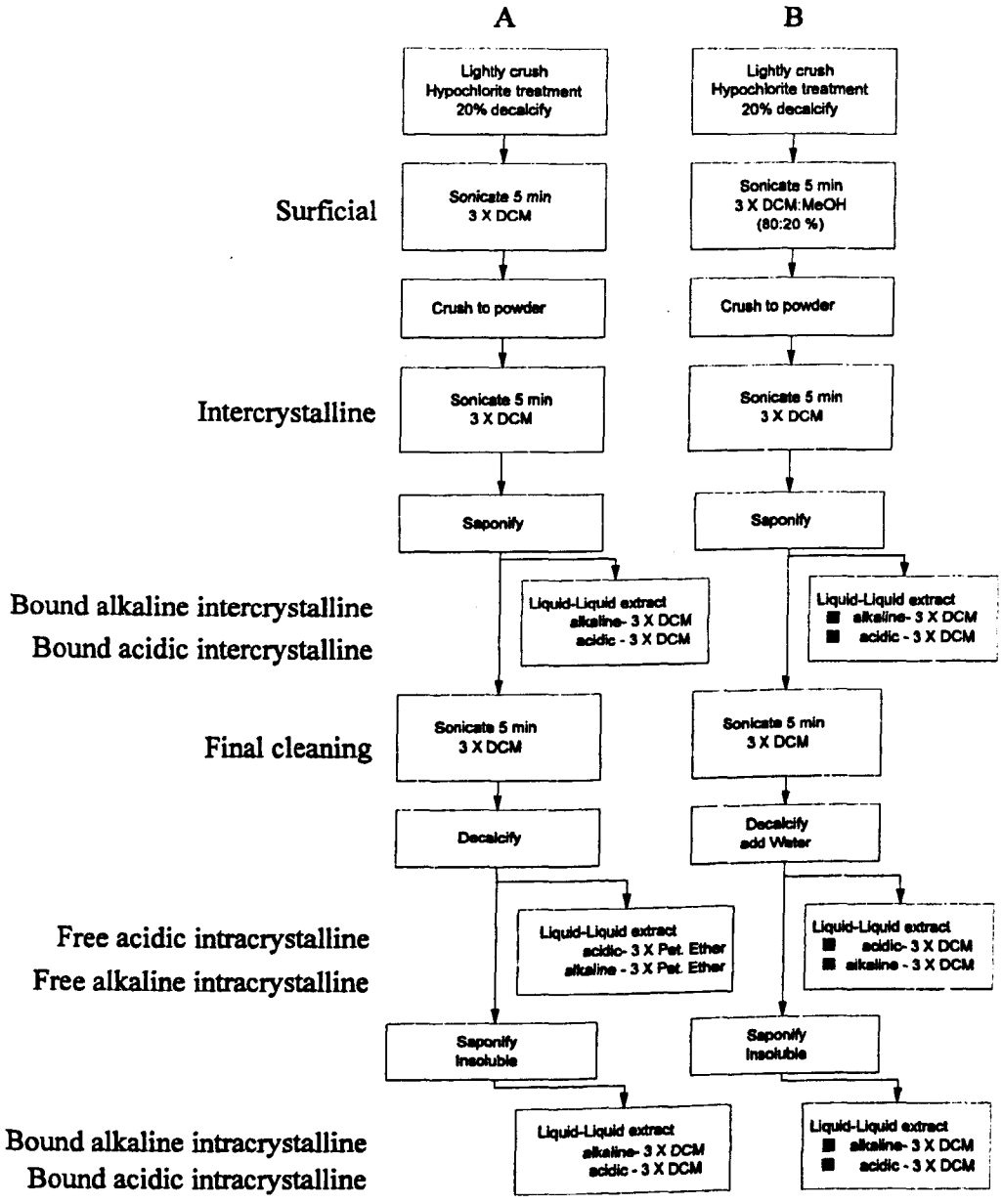


Figure 1. Flow diagram illustrating extraction protocols A and B. Extract names apply throughout the text as on this figure. The addition of surrogate standards before solvent extraction is indicated by; ■ = C_{36} *n*-alkane.

Gas chromatography (GC), gas chromatography - mass spectrometry (GCMS), the preparation of reagents and extracts and the use of surrogate standards for quantification were carried out as described in Chapters 1 and 2. With the exception of the surficial and intercrystalline extracts the *n*-alkanes were quantified with the C_{36} *n*-alkane surrogate standard, a relative response factor of 0.762 was previously calculated (Appendix 3). Additional GC-MS using selected ion monitoring (SIM) was employed for the analysis of the *Patella vulgata* 3 extracts, scanning for the diagnostic ions listed in Table 2. (Selected structures are shown in Appendix 11).

Diagnostic ions m/z	Compound
85	*n*-alkanes
92	toluene[3], methyl benzenes, ethylbenzene[3], xylene
114	benzo(a)anthracene[2]
126	benzo(a)pyrene[1 2], perylene[1]
128	ethylbenzene, napthalene[1 3]
138	Indeno(1,2,3-cd)pyrene-c[2]
142	alkylnapthalenes[1 3]
149	phthalates
178	alkylphenanthrenes[1 3], anthracene[2], phenanthrene[1 2 3]
191	polycylclic diterpanes, polycyclic triterpanes, hopanes
192	alkylphenanthrenes[1 3], alkylanthracenes
202	fluoranthene[1 2], pyrene[1 2]
217	steranes[3]
218	steranes[3]
219	alkylphenanthrene[1 3]
226	benzofluoranthene[1]
228	benzo(a)anthracene[2], chrysene[1 2]
252	benzo(a)pyrene[1 2], perylene, picene, benzofluoranthene[2]
253	monoaromatic steranes
256	benzanthracenes[1], dimethylchrysene
257	diasterenes
259	diasteranes, 13α and 17β steranes, dimethyl triaromatic steroids
278	picenes

Table 2. Diagnostic mass fragmentation ions of the polyaromatic and petroleum compounds investigated in *Patella vulgata* 3 shells. Those previously reported in molluscan soft tissues are identified as; 1 = Goldberg (1980), 2 = Hellou *et al.* (1993), 3 = Glegg and Rowland (1996).

RESULTS AND DISCUSSION

n-Alkanes

1) *n*-Alkane laboratory contamination

Hydrocarbons are ubiquitous and as discussed in Chapter 1 their contamination cannot be fully removed from any experimental workup procedure. For this reason, calcium carbonate controls were extracted and analysed with each experimental batch of shell samples. The calcium carbonate was previously Soxhlet extracted for 24 h. and then heated at 450°C for 24 h. The *n*-alkane content of the controls used with each experimental batch (1 to 5) are shown in Fig. 2. Each of the nine fractions (Fig. 1) extracted from each sample was normalised by dividing the GC peak area of the individual *n*-alkanes by the total *n*-alkane peak areas and then multiplying to a total of 100%. The stacked column graph (Fig. 2) shows the carbon number distributions for both the total and individual normalised extracts. The *n*-alkanes are not restricted to specific shell fractions and are extracted throughout the extraction protocol, although *n*-alkanes are not yielded from every extract as illustrated in Fig. 2 where surficial *n*-alkanes are extracted from controls 1 and 3 only. The prior Soxhlet extraction and heating of the calcium carbonate controls makes indigenous contamination unlikely, therefore laboratory contamination during extraction and analysis is the most likely source of these *n*-alkanes. Between each experimental batch the carbon number distributions are generally similar which indicates that the contamination is consistent and reliably indicated by each blank within each batch. The exact origin of this contamination is unknown and could be from more than one source. No odd over even carbon number distribution is observed which suggests a fossil fuel rather than a biological origin.

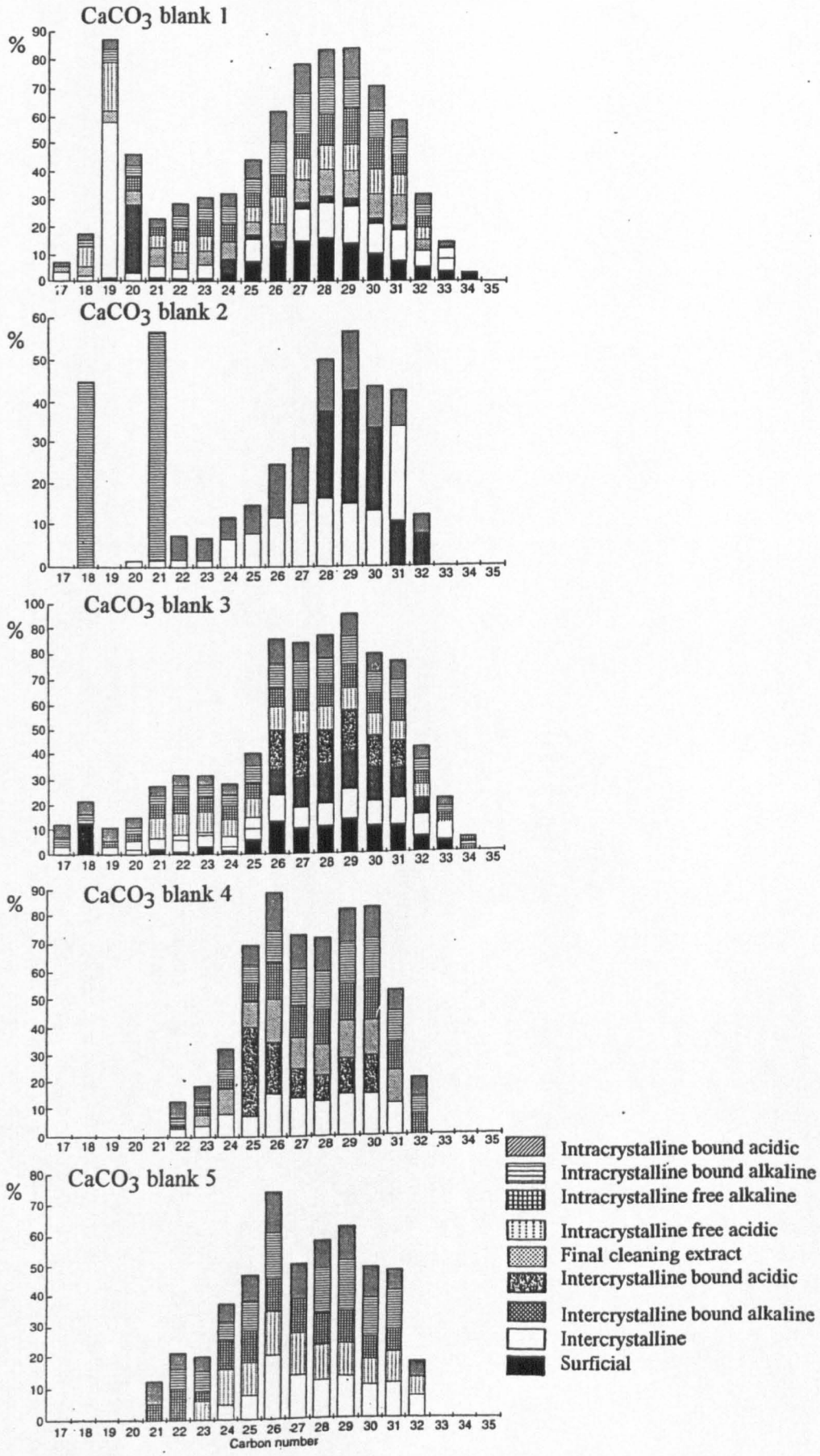


Figure 2. *n*-Alkane carbon number distributions of the five calcium carbonate controls extracted and analysed with each experimental batch. Each extract plotted with different column shading has been normalised to 100%.

2) *n*-Alkane carbon number distributions in shells

Extraction protocol A (Fig. 1) was used to extract shell *n*-alkanes without quantification. Therefore with the variation in background contamination and lacking information relating to yields, it is only possible to compare the normalised distributions of *n*-alkanes for shells within the same experimental batch where the laboratory contamination should be the same.

Patella vulgata and *Littorina littorea* shells sampled from Cullercoats Bay are shown in Fig. 3. The results from two experimental batches are shown. Samples a, b and c were extracted together using protocol A. Samples d, e and f were analysed using protocol B, the quantified yields are shown later, but for this figure the data have been normalised as for Fig. 2. The similar carbon number distributions in the calcium carbonate blanks and the shell extracts indicate that laboratory contamination may be the most significant input of *n*-alkanes to the shell samples. Combined with the variation of laboratory contamination between each experimental batch, as observed above (Fig. 2), this makes comparison of the carbon number distributions between experimental batches impossible.

Shells of different species (*Patella vulgata* and *Littorina littorea*) from the same environment are shown in Fig. 3. Those analysed in the same experimental batch are labelled b, c and e, f. Within the same experimental batch the laboratory contamination should be the same. These shells show different *n*-alkane carbon number distributions. The small differences could indicate discrimination between the uptake of *n*-alkanes into the shells of the different species.

Fig. 4 shows the *n*-alkane carbon number distributions for two different *Artica islandica* shell samples with the calcium carbonate blank. The shells were examined together in the same experimental batch and therefore only differ in their sampling location. The *n*-alkanes in the calcium carbonate blank differ from the shell extracts which indicates that the indigenous *n*-alkanes dominate the shell extracts. These two shell samples are the only examples which also show significant differences in carbon number distributions. This can be attributed to environmental differences. Although the *n*-alkanes do not provide enough information to assign a specific hydrocarbon source, the high carbon numbers and no odd over even carbon number distribution is indicative of a higher plant, coal or petroleum origin.

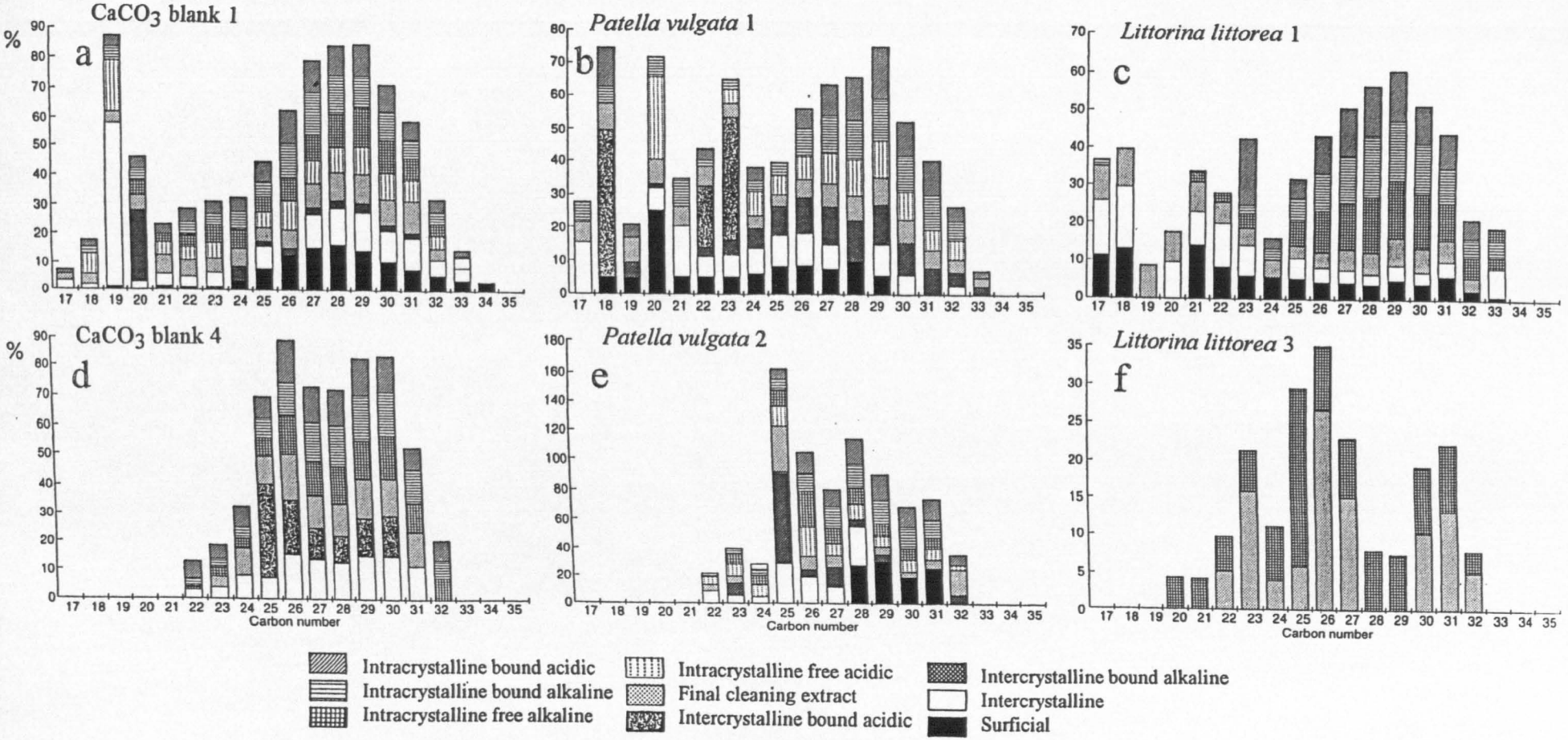


Figure 3. *n*-Alkane carbon number distributions for selected shell extracts. All shell samples are from Cullercoats Bay, the calcium carbonate blanks extracted and analysed with the shells are also shown. Samples a, b, c and samples d, e, f were analysed in the same experimental batch. Each extract plotted with different column shading has been normalised to 100%.

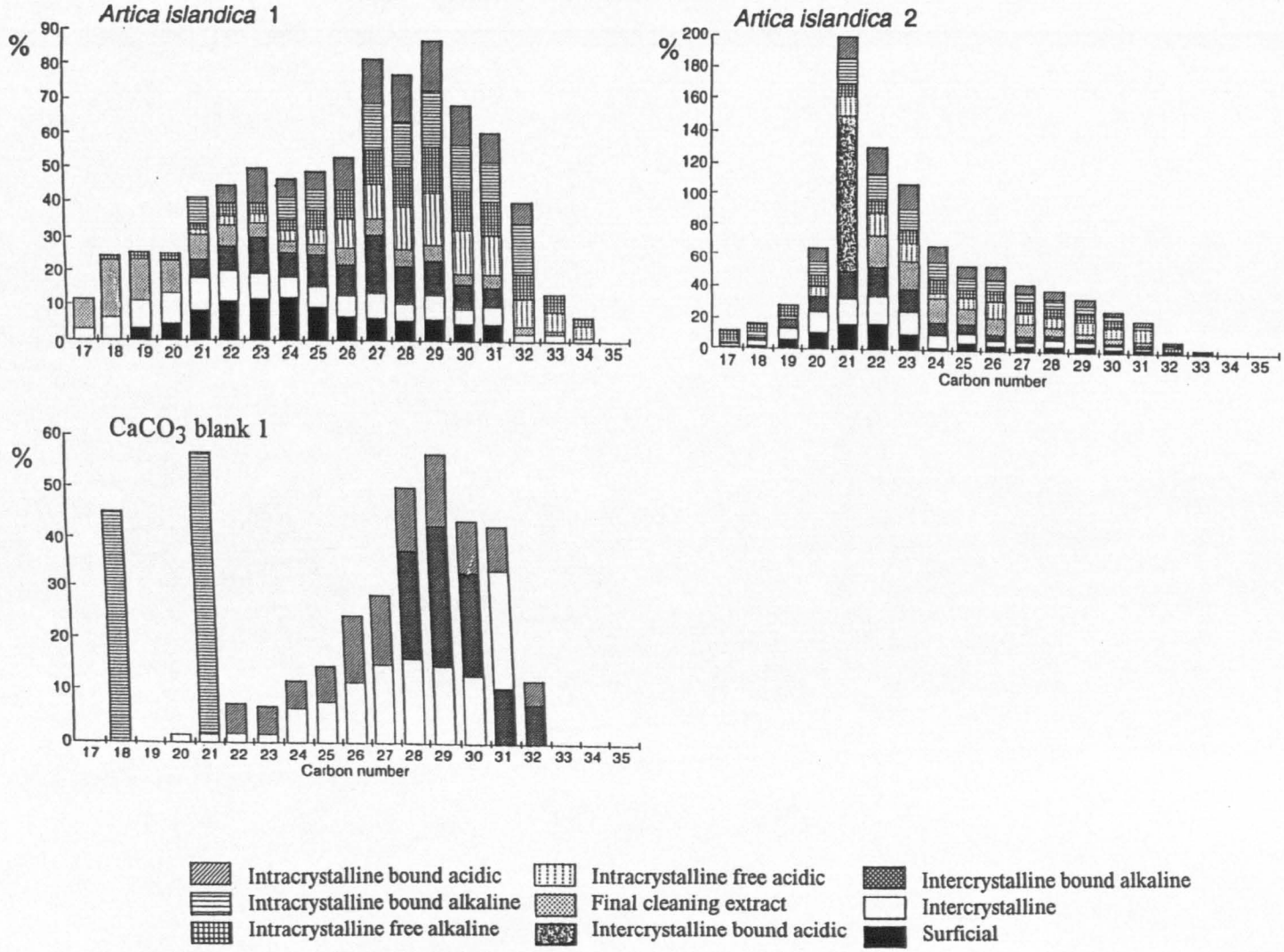


Figure 4. *n*-Alkane carbon number distributions of two *Artica islandica* shell samples from different locations. The shells were analysed in the same batch with the carbonate control also illustrated. Each extract plotted with different column shading has been normalised to 100%.

Fig. 5 shows partial GC traces for the intercrystalline fractions for *Artica islandica* 1. The *n*-alkanes are clearly distinguished in the intercrystalline fraction (a), however in the intercrystalline bound alkaline fraction (b) the yields of *n*-alkanes are lower and the C_{32} *n*-alkane is masked by coelution with cholesterol. The extraction of fatty acid methyl esters of higher yields than the *n*-alkanes (Chapter 2) in the intercrystalline bound acidic fraction (c) also makes it impossible to analyse the *n*-alkanes.

The GCMS m/z 85 partial mass chromatogram for the intracrystalline shell extracts are shown in Figs. 6 and 7. The mass chromatograms allow the homologous series of *n*-alkanes to be revealed in these total extracts. The intracrystalline fractions, released after full decalcification, are examined here as they are least likely to contain exogenous *n*-alkanes. The intracrystalline free extracts for *Artica islandica* 2 show few or no *n*-alkanes, this is probably due to the small amount of extracted material being fully used by previous GC analysis. Similarly, the mass chromatograms for the calcium carbonate blank show no *n*-alkanes. The background noise is increased in these analyses as the mass chromatograms have been normalised to the highest peak. The differences in carbon number distributions between the two shells is shown, and the similarities maintained within the same shell.

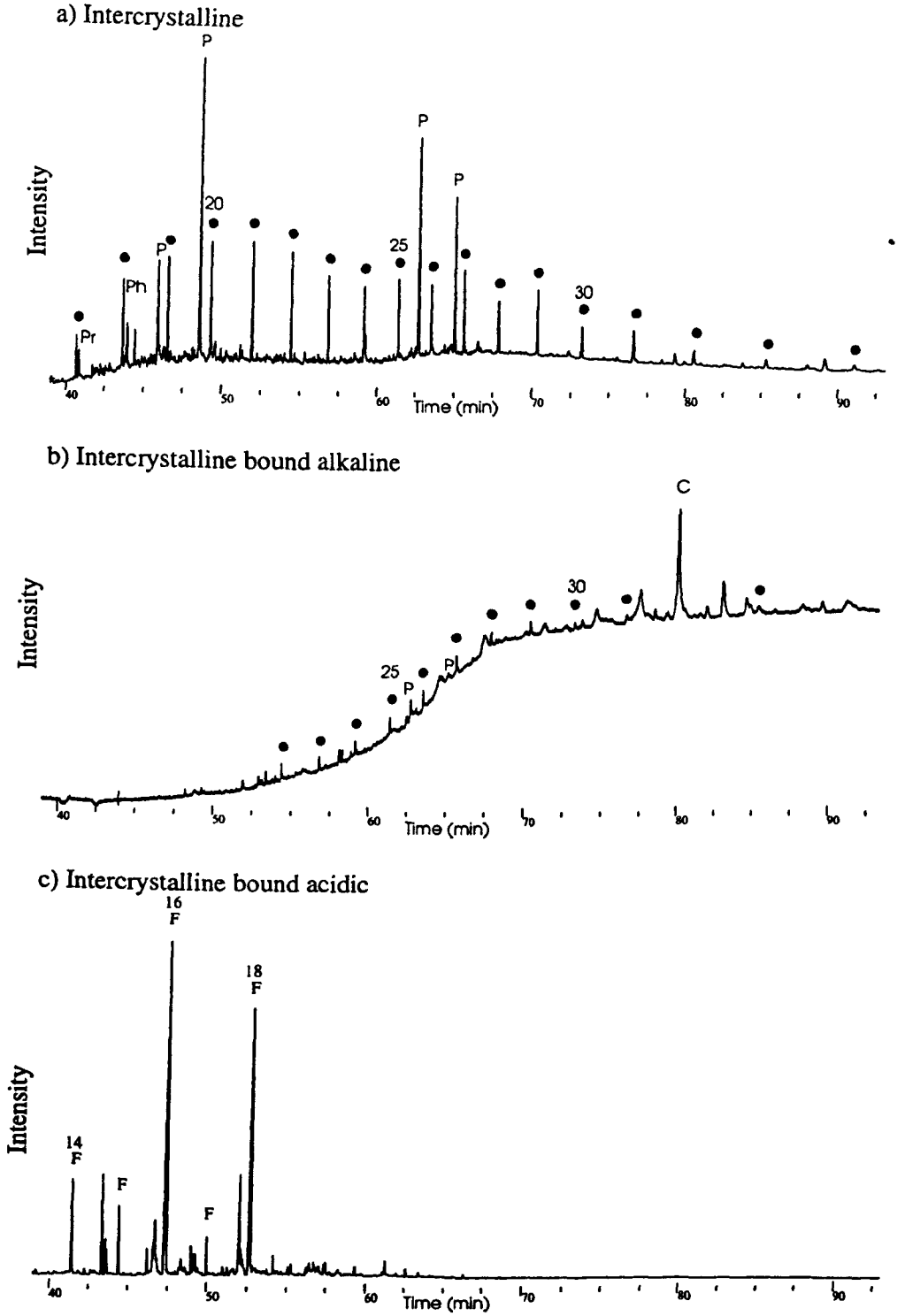


Figure 5. Partial gas chromatograms of intercrystalline fractions of *Artica islandica* 1. Peaks annotated by selected carbon numbers and, ● = *n*-alkanes, P = phthalate plasticisers, Pr = pristane, Ph = phytane, C = cholesterol and F = fatty acid methyl esters.

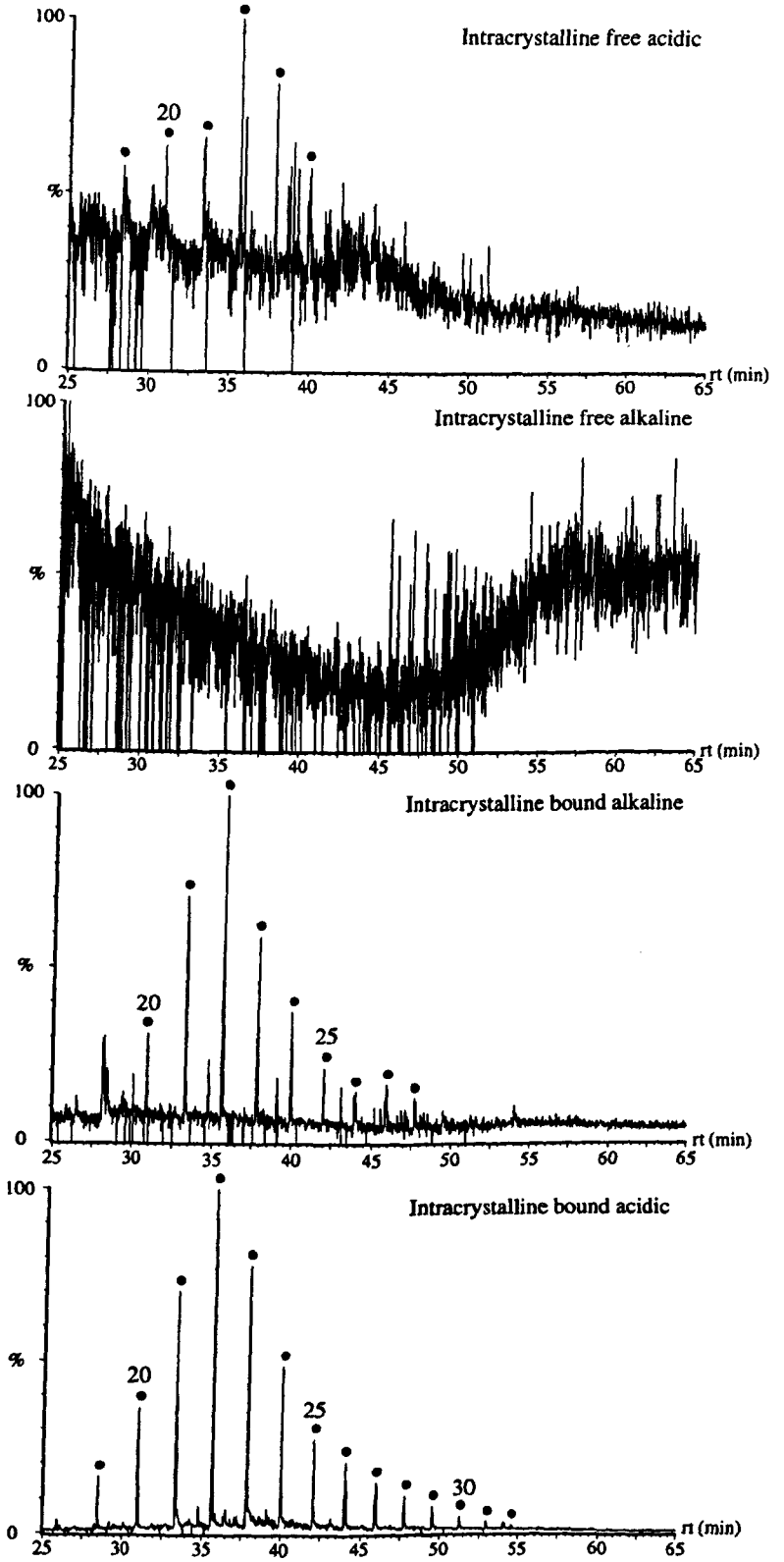


Figure 6. Partial mass chromatograms (m/z = 85) of intracrystalline *Artica islandica* 1 extracts showing the *n*-alkane distributions. *n*-Alkanes marked as ●, selected carbon numbers are also indicated.

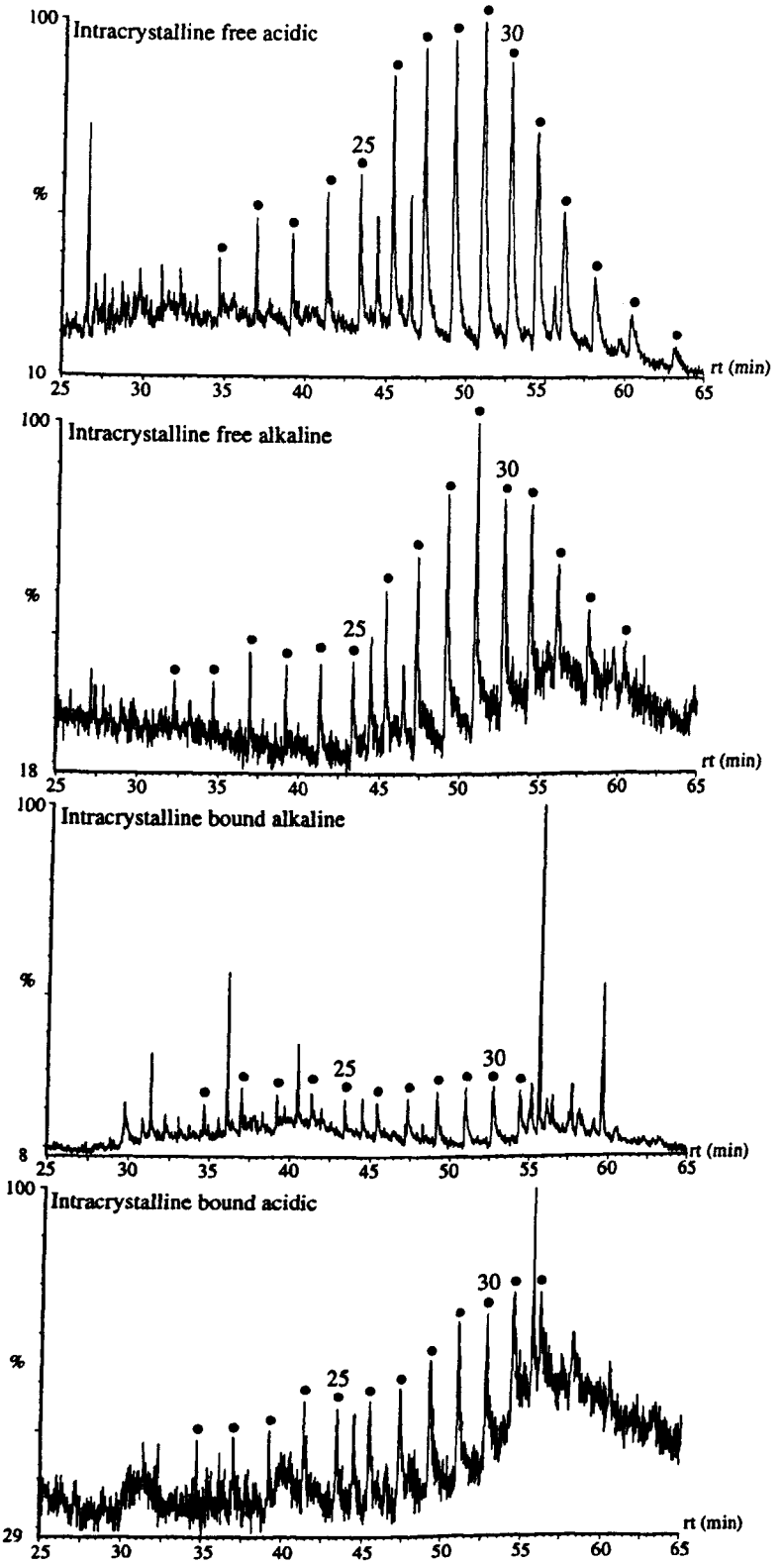


Figure 7. Partial mass chromatograms (m/z = 85) of intracrystalline *Artica islandica* 2 extracts showing the *n*-alkane distributions. *n*-Alkanes marked as ●, selected carbon numbers are indicated.

3) Quantification of *n*-alkanes

The majority of carbon number distributions are the same in the shells and the controls, and it is therefore difficult to interpret the data unless the carbon number distributions differ significantly (as shown for *Artica islandica* shells in Figs. 4, 6 and 7). Shell *n*-alkanes extracted using protocol B (Fig. 1) are quantified, this allows comparison with *n*-alkane yields from laboratory contamination (shown by the calcium carbonate controls). Comparison between different experimental batches is also possible. As shown in Fig. 5, coelution with other shell lipid components in these total GC analyses is a problem, and the use of mass chromatograms is limited by the small amounts of material extracted from the shells. Therefore the absence of *n*-alkanes reported in some quantified fractions may be due to coelution with other shell lipid components. Only those fractions where both shell and control *n*-alkanes are extracted are shown.

The yields (μg/g of extracted shell or calcium carbonate) of intracrystalline *n*-alkanes are shown in Fig. 8. When *n*-alkanes are extracted from the shell the *n*-alkanes from the same fraction of the control are also shown. These data show that the yields of *n*-alkanes are generally higher in the shell extracts than the calcium carbonate blank. This indicates that indigenous *n*-alkanes are present in the intracrystalline shell locations. However, for some fractions the laboratory contamination makes a significant contribution, and the intracrystalline bound acidic and intracrystalline bound alkaline yields for *Patella vulgata* 2 are similar to the control.

Comparison between shells from the same environment (*Patella vulgata* 2 and *Littorina littorea* 3, Cullercoats Bay), or between the same species from different environments (*Patella vulgata* 2 and 3) (Fig. 8) shows there are differences between the shell *n*-alkanes. No obvious pattern in extract fraction, yields or carbon number distribution is observed.

4) *n*-Alkanes in *Patella vulgata* shells exposed to the *Braer* oil spill

The methodology described above, using both a calcium carbonate blank and quantification to compare the *n*-alkanes from laboratory contamination to those extracted from the shell, has been applied to a *Patella vulgata* shell sample exposed to the *Braer* oil spill. The shells selected for analysis were collected live, 16 months after the *Braer* oil spill to allow for the possible deposition of these pollutants into the shells from the soft tissues. The chronic accumulation of *n*-alkanes into the soft tissues (Whittle *et al.*, 1977) and into the shells has been shown not to contain enough information to indicate the *n*-alkane source. However, Blumer *et al.* (1970) reported

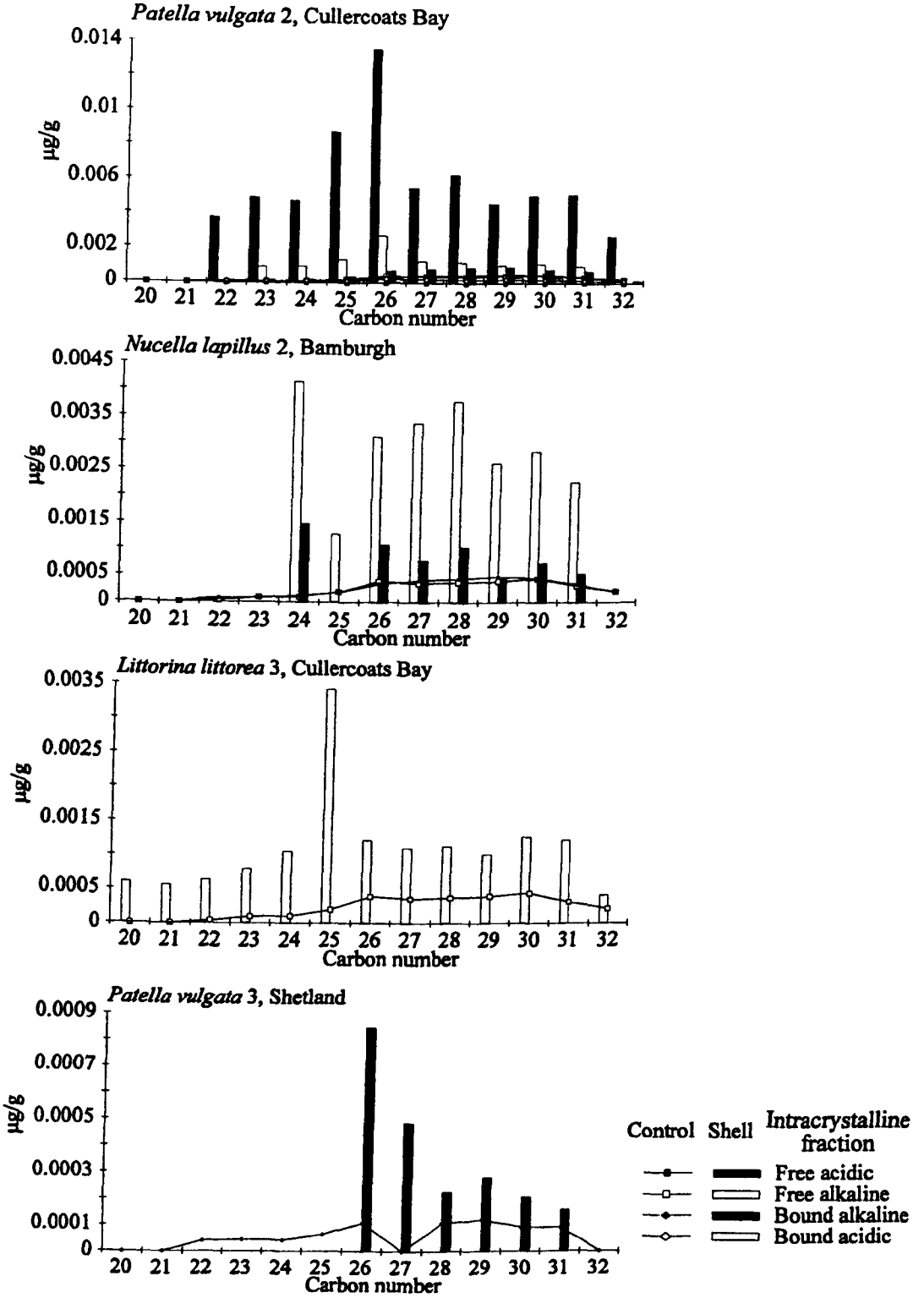


Figure 8. Intracrystalline *n*-alkanes plotted against carbon number. Quantified as µg/g. Yields from calcium carbonate blank, shown as joined symbols, are plotted on the same axis as the shell extracts shown as columns.

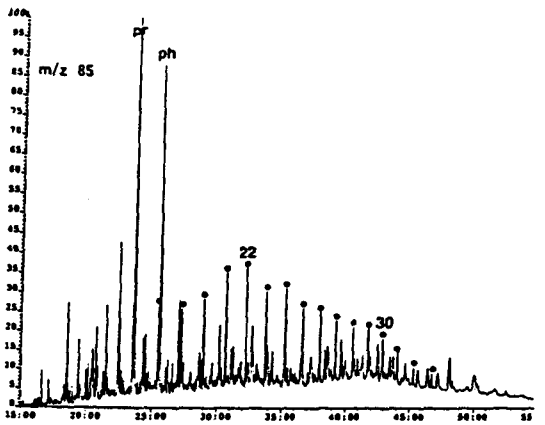
that by comparison with the same species in unaffected locations the *n*-alkanes from an acute oil spill could be identified in the soft tissues of bivalves. A comparison between the quantified shell *n*-alkanes of the gastropod *Patella vulgata* 3 exposed to the *Braer* oil spill and *Patella vulgata* 2 from Cullercoats Bay (Fig. 8) in fact shows a decrease in the *n*-alkane content for the oil exposed *Patella vulgata* 3. The distributions of *n*-alkanes are however different and are extracted from different shell fractions. Samples of beached oil and extracted water samples from Shetland are reported by Wolff *et al.* (1993) (reproduced in Fig. 9). These data show there is considerable variation between the *n*-alkane distributions present in the environment. With this uncertainty in the *n*-alkane distribution which may have been taken up by *Patella vulgata*, a comparison with the shell extracts was impossible.

Fossil fuel biomarkers and polyaromatic hydrocarbons

Glegg and Rowland (1996) found naphthalene, phenanthrene, alkylnaphthalenes and alkylphenanthrenes in the soft tissues of *Patella vulgata* exposed to the *Braer* oil spill. These and other selected aromatic pollutants (see Table 2) which have been previously encountered in molluscan soft tissues (Goldberg, 1980; Hellou *et al.*, 1993) and some fossil fuel biomarkers (e.g. steranes, diterpanes and triterpanes) were examined in the shell extracts. The low volatility and resistance to biodegradation of many of these compounds and their use with source correlation studies would make them particularly useful for evidence of oil spill contamination. None of these compounds were found in the shells.

Despite the incorporation of polyaromatic hydrocarbons into the soft tissues, the absence of specific *Braer* sourced hydrocarbons into the shells of *Patella vulgata* 3 could be due to three reasons: i) The animals had reached maturity and were no longer depositing shell material. This may be the case for these samples which were of adult-size. ii) A halt in mineralization could have occurred due to the toxic effect of the pollutants, not commencing until depuration from the soft tissues was complete. If so, this indicates that the biomineral cannot be used to deposit toxic compounds. iii) Biomineralization although shown to incorporate lipids from the soft tissues (Chapter 2), does not incorporate these pollutant compounds. Incorporation into the biomineral may be therefore be dependent on lipid functionality. However, the entrapment of *n*-alkanes indicates that hydrophobic attraction, possibly to the matrix proteins, may also be a mechanism for lipid incorporation.

a) Beached oil



b) Dissolved oil

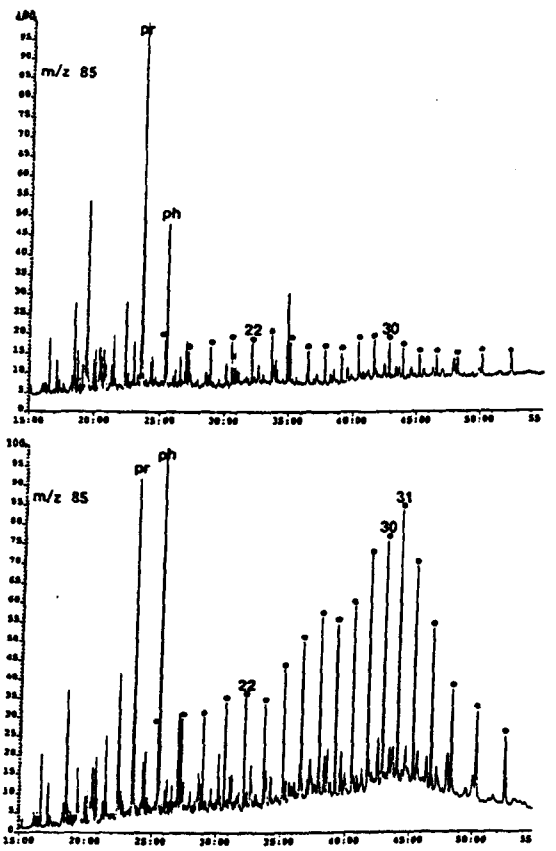


Figure 9. From Wolff *et al.* (1993) showing partial mass chromatograms (m/z = 85) for a) beached oil, and b) dissolved oil extracted from two water samples sampled near the wreck of the *Braer* tanker. *n*-Alkanes marked as ●, selected carbon numbers are also indicated, pr = pristane, ph = phytane.

CONCLUSIONS

n-Alkanes have been extracted from Recent mollusc shells. The use of this hydrocarbon class has two major problems; firstly the yields in the shells are low and secondly they are a common laboratory contaminant. The examination of carbon number distributions within an experimental batch where the contamination should be the same, the use of calcium carbonate blanks and quantification of yields enables some of this data to be used. Indigenous intracrystalline *n*-alkanes have been extracted.

Different gastropod species (*Littorina littorea* and *Patella vulgata*) from the same location show small differences in the shell *n*-alkane distributions. This may indicate a species-specific incorporation of *n*-alkanes into the shells. Two samples of the bivalve *Artica islandica* from different locations showed larger differences in the shell *n*-alkane distributions. This indicates that environmental differences can also be revealed by the shell *n*-alkanes. No source of the *n*-alkanes could be determined.

Although reported in the soft tissues, analysis of *Patella vulgata* shells exposed to the *Braer* oil spill found no naphthalene, phenanthrene or alkylnaphthalenes and alkylphenanthrenes in the shells. No other hydrocarbon pollutants were found. A decrease in the shell *n*-alkane yields was observed when compared to the same species from an unaffected location. The carbon number distributions of the spilt oil and the shell extracts were also dissimilar. There is therefore no evidence of transfer of *Braer* sourced hydrocarbons from the soft tissues to the shells.

CHAPTER 5. EXAMINATION OF THE UTILITY OF SHELL LIPIDS FROM THE FOSSIL RECORD.

ABSTRACT

Quaternary aged mollusc shells yield *n*-alkanes, *n*-alcohols, bound fatty acids and cholesterol. These have been extracted from both intercrystalline and intracrystalline locations within the shells. When compared with the extracts from Recent shells the yields of these lipids from fossil shells are significantly lower. The *n*-alkanes extracted from Quaternary shells are dominated by laboratory contamination, although some indigenous intracrystalline *n*-alkanes have been extracted. The bound fatty acids from intercrystalline sites within the fossils maintain their carbon number distribution but decrease in yields with increasing age; no diagenetic products were observed. The previously reported phylogenetic distinctions based on the bound fatty acids between the gastropods and bivalves are maintained for fossils. However, the information obtained from this analysis is limited by the small diversity of lipid distributions found in these fossil shells.

INTRODUCTION

Organic material in fossils has been reported by a variety of researchers (reviews by Curry, 1987; Logan *et al.*, 1991; Robbins *et al.*, 1993). The utility of fossil material for phylogenetic, environmental and diagenetic studies depends on the information content of the surviving organic material and how representative the fossil material is of the original biological molecules. Lipids contain less information than DNA, proteins or amino acids, but do have certain other advantages such as better geological preservation (Eglinton & Logan, 1991) and relative ease of analysis.

Decreasing amounts of organic material are generally found with increasing sample age (Hudson, 1967). This presents problems, as analysis nears the limits of detection and the levels of laboratory contamination become significant. Fossil samples have also been exposed for longer to potential contamination from the environment. Contamination has been shown to be a major problem in all biomolecular studies of fossil remains. Contaminating DNA (Pääbo *et al.*, 1989) and proteins (Walton & Curry, 1991) can be more readily distinguished than contaminating lipids (Chapters 2 and 4). This is due to the greater information content of proteins and DNA which allows their provenance to be distinguished. The use of calcium carbonate blank samples in this study allows the characterization of laboratory contamination, and with quantification, enables the identification of indigenous lipids.

The potential of fossil lipids to be retained and preserved from environmental contamination without the protection of intracrystalline entrapment is controversial, and although fossils may superficially appear exceptionally well preserved, they may contain little indigenous biomolecular material (Towe, 1980; Briggs *et al.*, 1995). Abell and Margolis (1982) reported no differences between the *n*-alkane content of Plio-Pleistocene vertebrate bones and the surrounding sediment, suggesting complete intermixing of *n*-alkanes between the fossils and the matrix. However, Evershed *et al.* (1995) reported the presence of indigenous acyl lipids, cholesterol and its degradation products in archaeological bones. Logan *et al.* (1995) found selective preservation of leaf wax lipids at the surface of exceptionally well preserved Miocene leaves, which were both characteristic of the species and distinct from the sediment.

Despite the reported extraction of lipids from fossil material without the protection of an inorganic structure, the intercrystalline and especially intracrystalline sites of biominerals offer the best preservation potential for indigenous lipidic material from fossil shells (Towe, 1980). The presence of primary aragonite in fossils and the original shell microstructure should be a good guide to excellent preservation, as the metastable aragonite is converted to calcite by inversion, dissolution or replacement (Land, 1967). However, the composition of trace elements in unrecrystallised fossil mollusc shells has been shown to vary due to diagenesis (Pilkey & Goodell, 1964; Ragland *et al.*, 1979), although this may be due to superficial adsorption at the crystal surfaces (Ragland *et al.*, 1979). Preservation of aragonite in shells from the Waipipi Shellbed, the oldest horizon sampled here, was reported by Mitchell *et al.* (1995). In addition, the presence of proteins and amino acids in shells from the same localities as those examined here suggests that the preservation of shell associated organic material is excellent (Curry *et al.*, 1991; Walton, 1992; Clegg, 1993; Mitchell *et al.*, 1995).

Quantification of the low molecular weight C_1 to C_5 hydrocarbons released after decalcification of a variety of Recent to Ordovician molluscan shells revealed that the yields of these compounds increased with sample age (Thompson & Creath, 1966). The alkane content of Mesozoic belemnites and one brachiopod were examined by Ivanov and Stoyanova (1972). After the removal of the surface and solvent washes, the lipids were solvent extracted from the decalcified hardparts. Normal, *iso*, *anteiso* and isoprenoid alkanes were found, with a range of uniformly distributed C_{14} to C_{35} *n*-alkanes. Stoyanova *et al.* (1980) used the same sample cleaning procedure as above followed by Soxhlet extraction and isolated alkanes from Palaeozoic trilobites. They achieved only a partial extraction before decalcification. It is therefore likely that the first extract was composed of the intercrystalline fraction. After decalcification they

obtained a further extraction of alkanes likely to have been derived from intracrystalline sites. Stoyanova (1984) examined Palaeozoic brachiopods and found a similar alkane distribution to the belemnites and brachiopod data reported above. These published results of *n*-alkane distributions have been found to be similar to the laboratory contamination encountered in this study (Chapter 4) and in the absence of published laboratory controls these data need to be treated with caution.

Fatty acids have been extracted from fossil algae of Lower Cambrian to Quaternary age using a method which included removal of surface material, decalcification, and solvent extraction (Das & Smith, 1968). They noted a reduction in the unsaturated relative to the saturated fatty acids in these algae, an observation supported in a comparison of the bound fatty acids extracted from Recent mollusc shells and the soft tissues (Chapter 2). Mesozoic belemnites have yielded saturated fatty acids; the C_{16} and C_{18} fatty acids were dominant over a C_{14} to C_{26} carbon number range, with an even over odd distribution and some branched components (Ivanov *et al.*, 1975). Palaeozoic brachiopods have yielded fatty acids with a carbon number range of C_{12} to C_{29} with a slight even over odd distribution (Stoyanova, 1984). CoBabe and Pratt (1995) found, by Soxhlet extraction without decalcification of a variety of Recent and Quaternary aged molluscan shells, cholesterol, fatty acids, phytadienes, ketones and *n*-alkanes. Lower yields were reported for the Quaternary shells.

As the methods used to extract the lipids in the previously published analyses used extraction with or without decalcification then only the intercrystalline, or a mixture of intercrystalline and intracrystalline lipids were being sampled. The intercrystalline fraction likely to be contaminated if accessible from the environment. The methodology used here allows the differentiation of the shell extracts into surficial, intercrystalline and intracrystalline fractions. This enables the removal and characterization of contaminated lipidic material from the surface of the shells, the environmentally accessible intercrystalline locations within the shell and the fully entombed intracrystalline shell locations.

Intracrystalline fatty acids have been extracted from a range of Quaternary brachiopod shells with the use of hypochlorite, Soxhlet extraction, decalcification and acidic hydrolysis to both remove post-depositional ingress and separate the different shell fractions (e.g. Curry *et al.*, 1991; Clegg, 1993). The intracrystalline lipids were found to be distinct from the intercrystalline free components. However, in these studies intercrystalline bound lipids were not fully removed as a saponification stage was not included before decalcification. These bound lipids could have been extracted with and

misinterpreted as the intracrystalline fraction. A decrease in the yields of lipids and loss of unsaturated fatty acids with increasing age was also noted in these brachiopod shells.

The studies of lipids associated with fossils has shown that these indigenous molecules can survive into the geological record. Changes in the lipid composition of fossil shells over time has been used to indicate biomolecular diagenesis. Thompson and Creath (1966) proposed that the C_1 to C_5 shell alkanes originated from amino acids, a process confirmed by the thermal decomposition of proteins by Philippi (1977). Conversely, the generation of longer chain *n*-alkanes from fatty acids within shells was suggested by Stoyanova *et al.* (1980) and Stoyanova (1984). Sykes *et al.* (1995) noted that the loss of proteins from mollusc shells stabilises to a constant level through time with losses of intercrystalline proteins by mobilisation and degradation leaving a constant intracrystalline component. This may similarly occur for the intercrystalline and intracrystalline lipids. The information gained from biomineral associated lipids has been used to differentiate between species (Thompson & Creath, 1966; Das & Smith, 1968; Logan *et al.*, 1995) and palaeodiets (Evershed *et al.*, 1995; CoBabe & Pratt, 1995).

This study presents the first systematic survey of lipids from fossil molluscs from a set of samples previously well characterized in terms of protein composition (Curry *et al.*, 1991; Walton, 1992; Clegg, 1993; Mitchell *et al.*, 1995). The aims of this Chapter are: i) to characterise lipids extracted from a variety of intercrystalline and intracrystalline locations of fossil shells, ii) to compare the lipids extracted from fossil shells with the phylogenetic, dietary and environmental conclusions derived from Recent samples, iii) to ascertain the changes in shell lipids with sample age and the best locations within the shell for preservation of lipids throughout the geological record, and iv) to assess the use of lipids associated with macrofossils in phylogenetic, palaeoecological and dietary studies.

METHODOLOGY

Sampling locations

Sampling was carried out in early May 1994 from Quaternary raised beach deposits near Wanganui, North Island of New Zealand (Fig. 1). This area has been described as "One of the most complete Quaternary stratigraphic records in the world" (Pillans, 1994 page 189). The locations have been intensively studied, with detailed information on ages (See Table 1) and the changing depositional environment (Fleming, 1953). The large amounts of shell material required for analysis are easily collected from a

variety of different molluscan and some brachiopod species. Shells were taken from exposed and often rapidly eroding outcrops. Species were identified by Dr Alan Beu (New Zealand Geological Survey, Lower Hutt, New Zealand). Table 1 shows a summary of the sampling locations and ages of the formations. There are some differences between the reported ages, particularly for the Kupe Formation. The dates used in this text are those of Walton (1992), as they cover the entire sample set Appendix 12 shows the stratigraphic order.

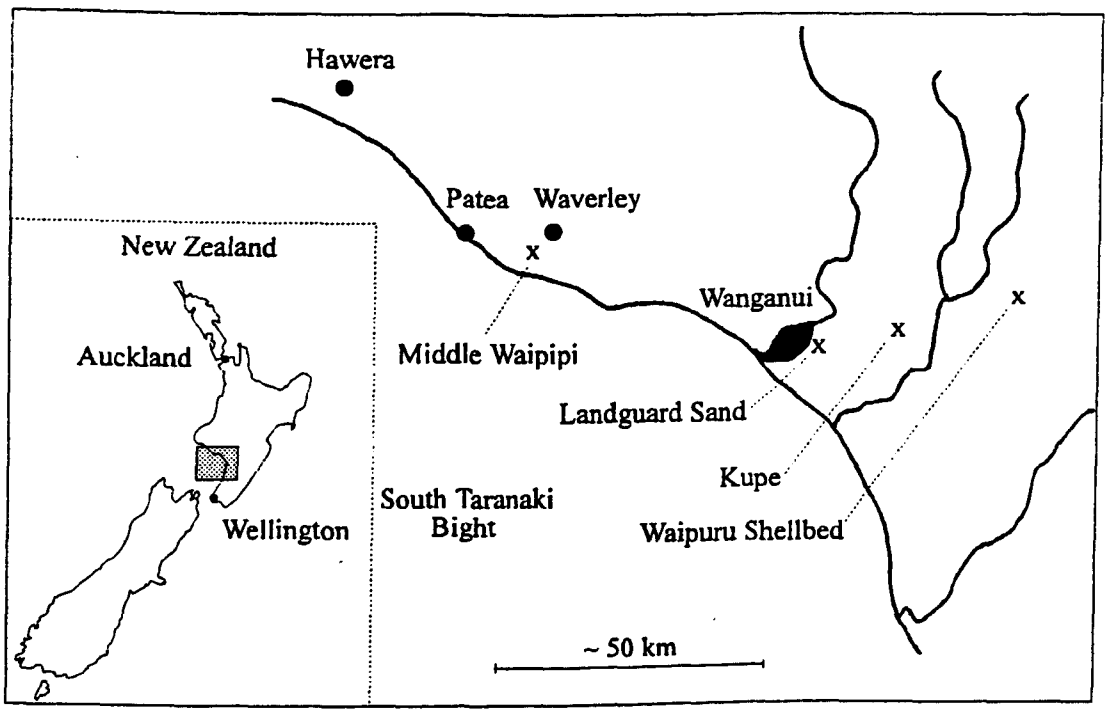


Figure 1. Location map showing sampling sites (x) of the fossil shells examined in this study. The inset shows an outline map of New Zealand, the shaded box indicating the area of the expanded map.

Table 2 shows the shells analysed in this study grouped by experimental batch, with the lithostratigraphy, age and extraction protocol. Extraction protocols A and B are outlined in Fig. 2. The calcium carbonate blanks are examined with each experimental batch. Where lipids are co-extracted in these blanks and the shell samples, the results from the calcium carbonate blank are also shown.

			New Zealand Topographical Map Edition 1, 1985	Beu & Edwards (1984)	Abbott & Carter (1994)	Pillans (1994)		Walton (1992)
Formation	**Member**	**Sampling location**	**Co-ordinates**	**O^{18} Stage**	**O^{18} Stage**	**O^{18} Stage**	**Age (Ma)**	**Age (Ma)**
Landguard	Landguard Sand	Landguard, Wanganui river	R22 / 831368	9?	-	9	0.3	0.34
Kupe	Pecten layer	Kauangaroa road, Whangaehu river valley	S22 / 028365	13	17	17	0.7	0.50
Waipuru Shellbed		Turakina valley road, Keilawarra farm land	S22 / 166446	Md	-	-	-	1.75
Waipipi	Middle Waipipi Shellbed	Mouth of Wairoa stream, Waipipi beach, Waverley	Q22 / 478521	-	-	-	-	2.60

Table 1. Formation, Member, sampling location and map co-ordinates of the shell samples analysed herein. Oxygen isotope stage ages and ages (Ma) for these samples are shown from a variety of sources.

Species	Phylum: Class	Formation or Member	Age (Ma)	Protocol
Batch 3				
Tiostrea chiliensis	Mollusca: Bivalvia	Kupe Formation, pecten layer	0.5	A
Pecten benedictus marwicki	Mollusca: Bivalvia	Kupe Formation, pecten layer	0.5	A
Tiostrea chiliensis	Mollusca: Bivalvia	Waipuru Shellbed	1.75	A
Crepidula "radiata"	Mollusca: Gastropoda	Waipuru Shellbed	1.75	A
Crepidula fornicata	Mollusca: Gastropoda	-	Recent	A
$CaCO_3$ 3 blank	-	-	-	A
Batch 5				
Tiostrea chiliensis	Mollusca: Bivalvia	Landguard Sand	0.34	B
Pecten tainui	Mollusca: Bivalvia	Landguard Sand	0.34	B
Tiostrea chiliensis	Mollusca: Bivalvia	Middle Waipipi	2.6	B
Neothyris lenticularis	Brachiopoda: Articulata	Middle Waipipi	2.6	B
$CaCO_3$ 5 blank	-	-	-	B

Table 2. Samples grouped by experimental batch. The Formation / Member and ages are listed for the fossil shells. Inorganic calcium carbonate blanks 3 and 5 were analysed with these experimental batches. Recent *Crepidula fornicata* shells from Walton on the Naze, Essex, England was also examined.

Shell extraction protocols

Fig. 2 illustrates the shell extraction protocols used. Two protocols (A and B) were employed. Protocol A was used for determining carbon number distributions and protocol B used for quantification of yields. The protocols differ in the method used for some solvent extractions, and concentrated extracts from protocol A were passed through a plug of alumina, those from protocol B were passed through a plug of cotton wool.

After initial cleaning treatments to remove surficial contamination, shells were sequentially extracted by the use of solvent extraction, saponification and full decalcification with further solvent extractions and saponification treatments. This results in the sequential release of increasingly protected free and bound lipids from within the shell. Shell samples were analysed in experimental batches, each with an inorganic calcium carbonate (calcite) blank. The purpose of the blank is to determine the distribution and yields of laboratory contamination introduced during extraction and analysis.

Gas chromatography (GC), gas chromatography - mass spectrometry (GCMS), the preparation of reagents and extracts, the use of internal standards for quantification, and multivariate statistical analysis were carried out according to Chapter 2.

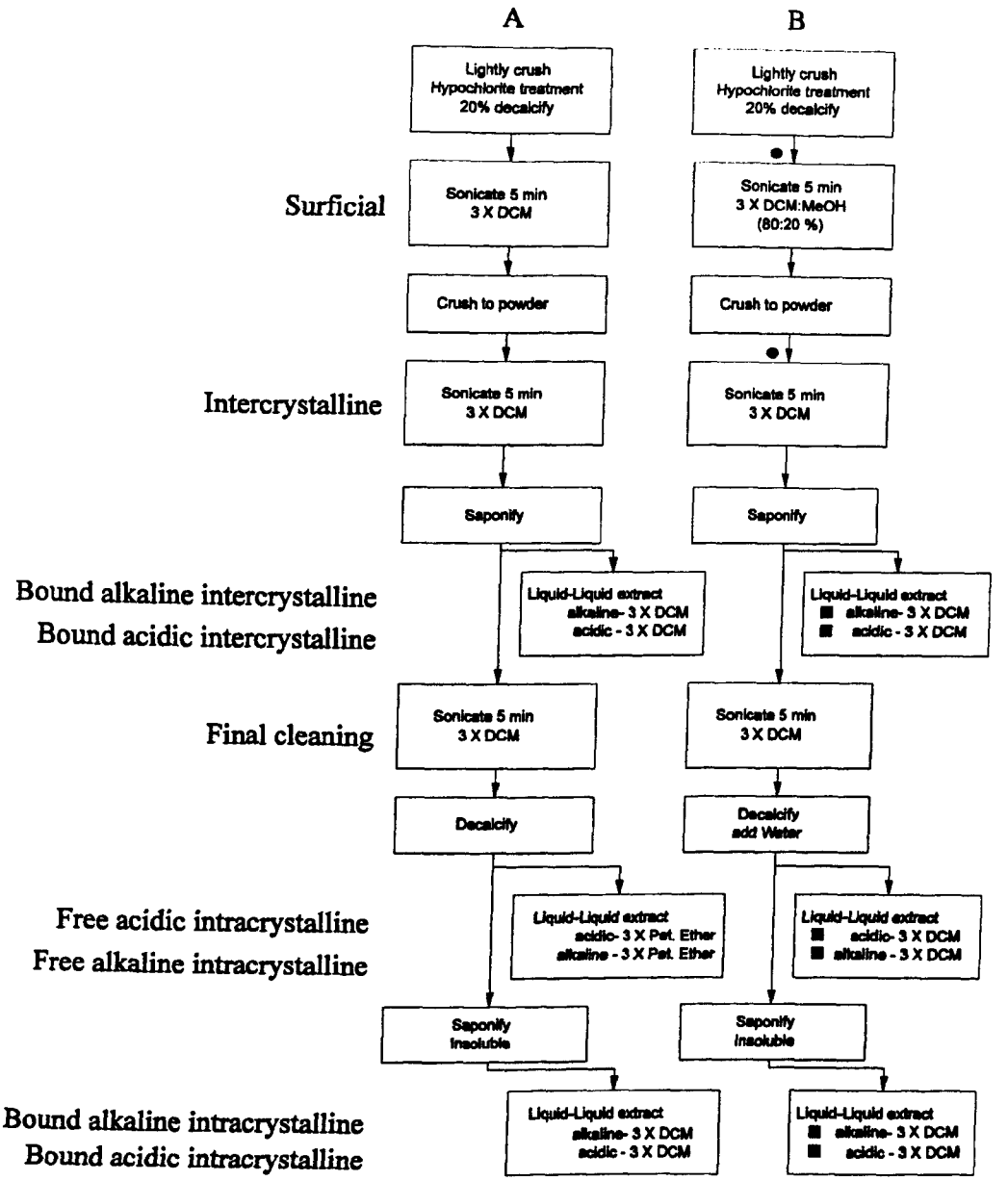


Figure 2. Flow diagram illustrating extraction protocols A and B. Extract names apply throughout the text as on this figure. The addition of surrogate standards is indicated by; lupeol = ●, C_{36} *n*-alkane = ■.

RESULTS AND DISCUSSION

n-Alkanes

1) Carbon number distribution

Examination of shell lipids from Recent *Crepidula fornicata* and fossil *Crepidula "radiata"* (1.75 Ma) allows a comparison of the effects of age on shells from the same genus. These shells were analysed in the same experimental batch and therefore the background contamination should be the same in each sample. As these shells were analysed using protocol A no quantification was possible. The relative abundance of the *n*-alkane carbon number distributions from each fraction of these shells with the calcium carbonate blank are shown in Fig. 3. The *n*-alkane homologues from each fraction were normalised by dividing the GC peak area of the individual *n*-alkanes by the total *n*-alkane peak areas and then multiplying to a total of 100%. The surficial extract has been excluded as this has previously been shown to include contamination from handling and initial cleaning treatments (Chapter 1). No *n*-alkanes with carbon numbers less than C_{17} were extracted.

The carbon number distributions between the shells and the calcium carbonate blank are generally similar. However there are minor differences between the shells, in particular the odd over even distribution from the intercrystalline fraction of the fossil *Crepidula "radiata"*. Without quantification it is difficult to ensure these are indigenous hydrocarbons.

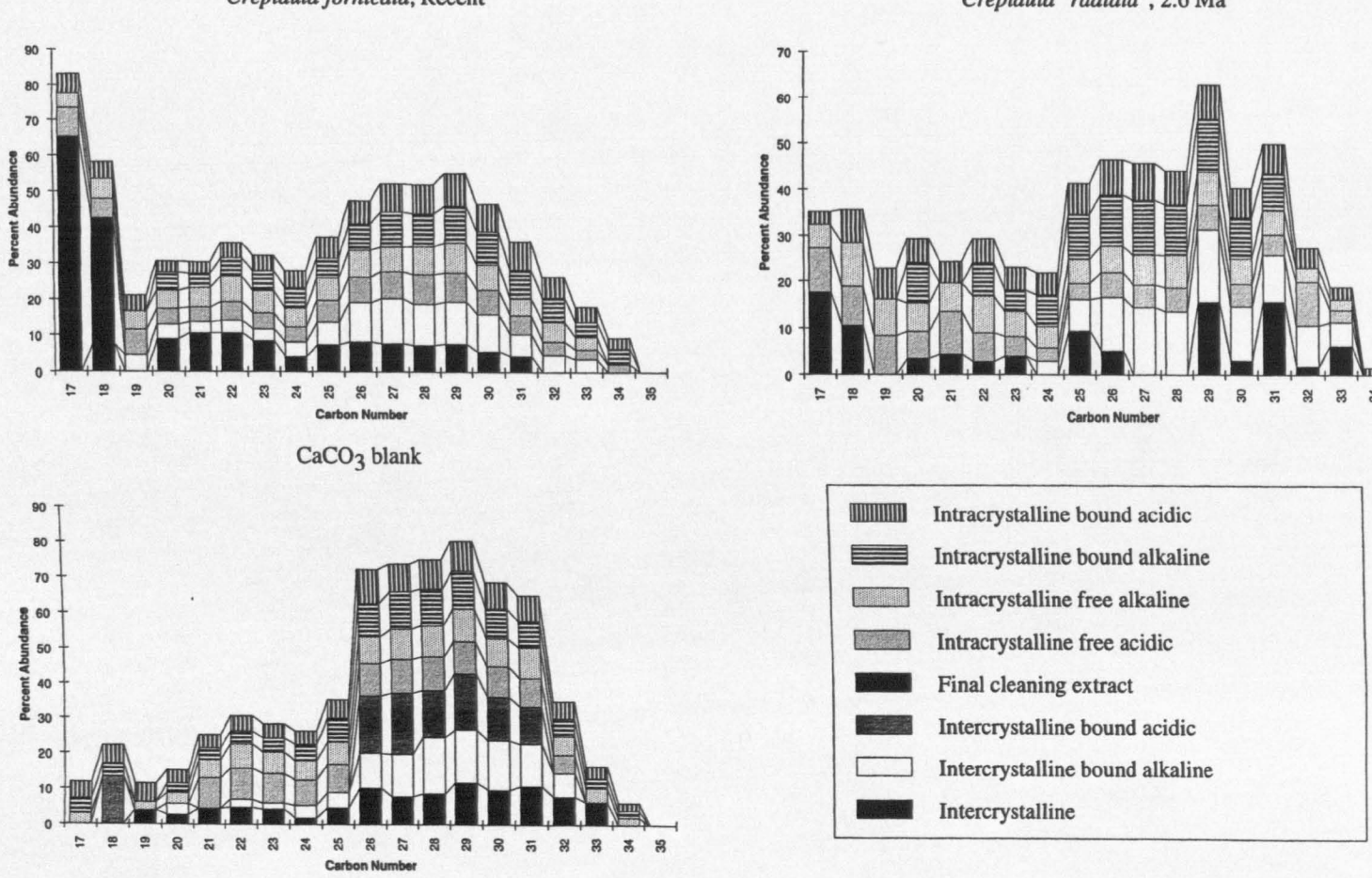


Figure 3. Percentage *n*-alkane carbon number distributions for Recent *Crepidula fornicata,* fossil *Crepidula "radiata"* shells and calcium carbonate blank.

2) Quantification

The use of extraction protocol B allows yields to be quantified. The carbon number distributions of the *n*-alkane yields (μg per g of sample extracted) for the fossil shells are shown in Fig. 4.

For the intercrystalline bound alkaline and intercrystalline bound acidic fractions, no *n*-alkanes were extracted from the calcium carbonate blank, this indicates indigenous *n*-alkanes were extracted from the shells. However, when *n*-alkanes are extracted from the calcium carbonate blank the yields from the blank are generally higher than the shell extracts, the exceptions are the intracrystalline bound extracts from *Tiostrea chiliensis* (0.34 Ma) and *Neothyris lenticularis* (2.6 Ma). The C_{21} *n*-alkane extracted from *Tiostrea chiliensis* (0.34 Ma) exhibits a higher yield than the blank and all the other fossil shell extracts. This carbon number may be indicative of an algal source, however other short chain *n*-alkanes are not present.

Although a comparison between different species, quantified *n*-alkanes extracted from fossils shells have lower yields than those from the Recent shells (Chapter 4, Fig. 8). Yields of *n*-alkanes in the fossil shells must be at the limits of detection as the yields from the shells are below the level of background contamination. These data exclude the use of the *n*-alkanes from fossil shells from any further analysis. The low yields in the fossil shells may indicate a loss of *n*-alkanes from the fossil material, or possibly that Recent hydrocarbon pollution may be incorporated in the Recent shells (Chapter 4), a process which is unavailable to fossil samples.

These findings imply that laboratory contamination may be the origin of *n*-alkanes in fossils from earlier reports (Ivanov & Stoyanova, 1972; Stoyanova *et al.*, 1980; Stoyanova, 1984) particularly as rigorous cleaning treatments or calcium carbonate blanks were not used or reported.

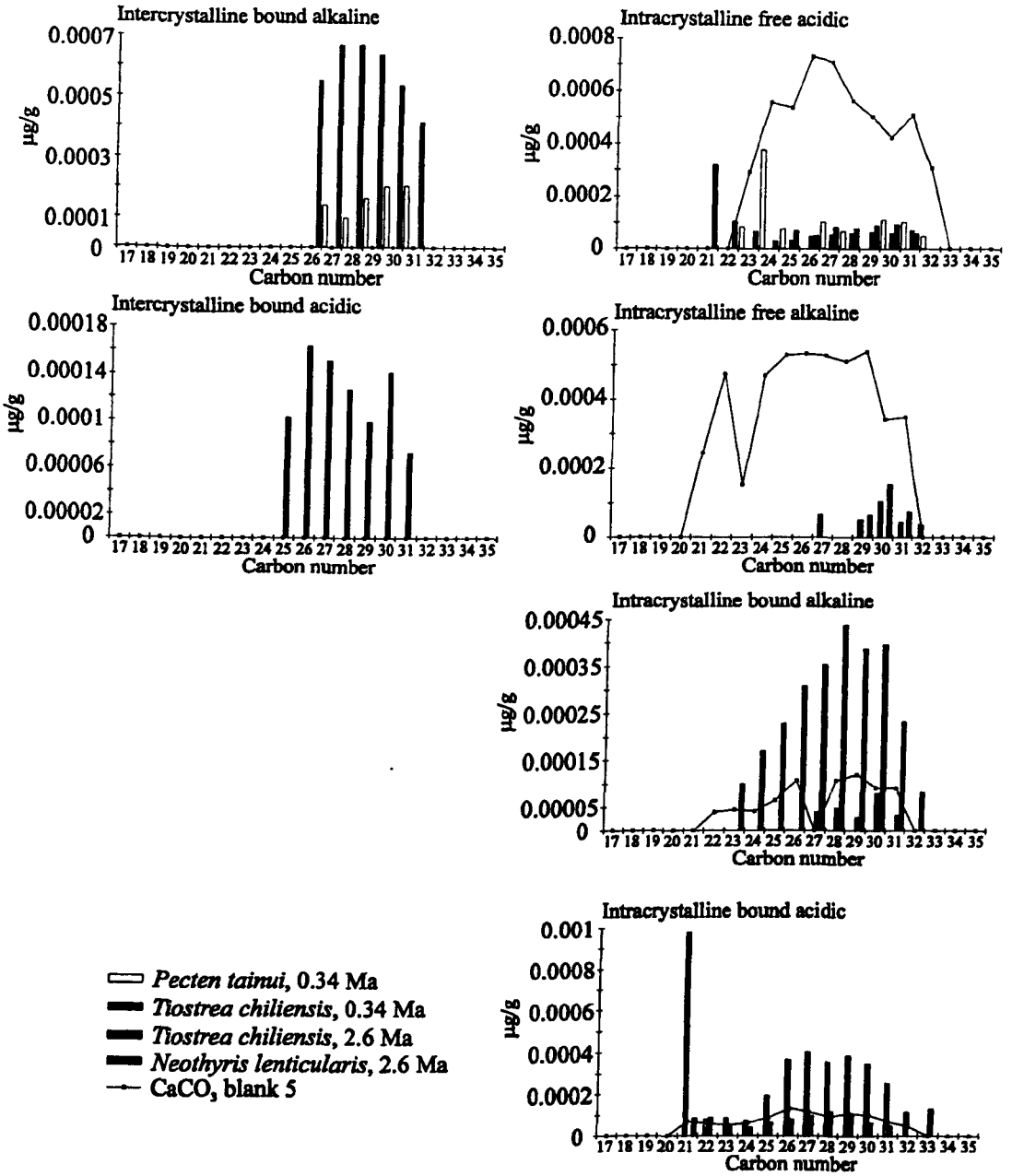


Figure 4. Yields (µg/g of material extracted) of *n*-alkane carbon number distributions, plotted for the fractions indicated. Shaded columns indicate the shell samples for each fraction. The calcium carbonate blank is shown by -■-.

n-Alcohols

Fig. 5 shows the relative abundance of the *n*-alcohol carbon number distributions extracted from Recent and fossil *Crepidula* shells, and fossil *Tiostrea chiliensis* shells. The *n*-alcohols have been extracted from the bound alkaline fractions of intercrystalline and intracrystalline shell locations. No *n*-alcohols were extracted from the blanks.

The intercrystalline fractions of all samples are similar. As these shells are from different locations, post-depositional contamination of the same *n*-alcohol composition can be excluded. An even over odd carbon number distribution is observed for all the shell extracts. The intracrystalline bound alkaline fraction of *Crepidula "radiata"* has a C_{16} carbon number maximum, all other intracrystalline bound alkaline fractions have a C_{18} carbon number maximum. These observations are similar to those for Recent shells (Chapter 2). With the exception of the presence of indigenous *n*-alcohols in fossil shells, these data do not provide any phylogenetic, palaeoecological or dietary information relating to the fossils.

Bound fatty acids

1) Carbon number distribution

Bound fatty acids are extracted from the shells as fatty acid methyl esters (FAMEs). The relative abundance of FAMEs from Recent and fossil shells of the genus *Crepidula* are shown in Fig. 6. No FAMEs were extracted from the calcium carbonate blank. The even over odd distributions and carbon number maximum of C_{16} in both intercrystalline and intracrystalline fractions are similar to those reported for soft tissues (Gardner & Riley, 1972; Herbreteau *et al.*, 1994; Appendix 13).

FAME distributions for both Recent and fossil samples are similar in both shell locations, although in the intercrystalline fraction a carbon number distribution from C_{12} to C_{18} is observed for the Recent sample whilst a C_{14} to C_{17} distribution is observed for the fossil sample, suggesting that the intercrystalline FAMEs of low yields are lost over geological time. This change does not occur for the inorganically entombed intracrystalline bound lipids.

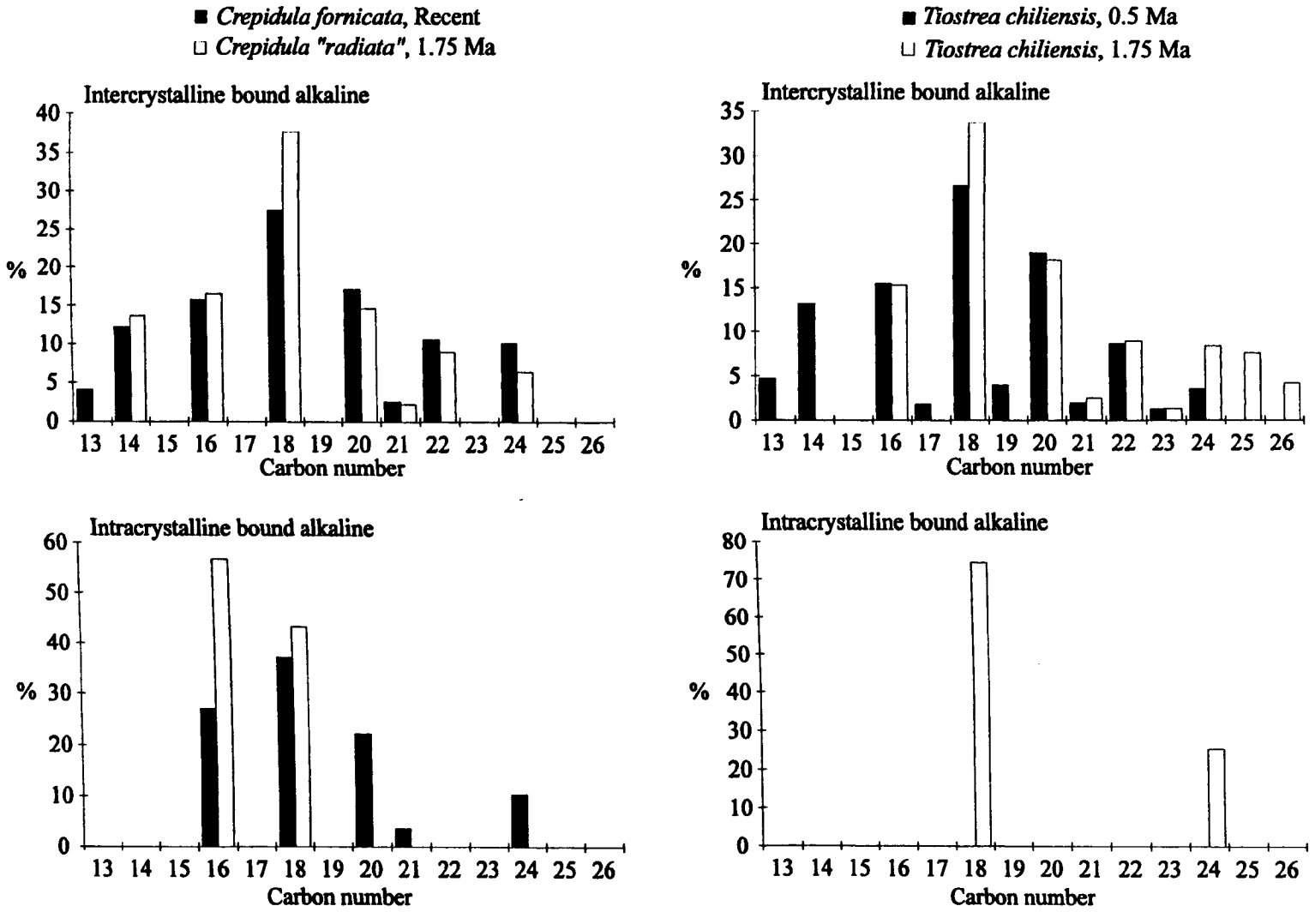


Figure 5. Percentage *n*-alcohol carbon number distributions. Extracts are from the bound alkaline fractions of intercrystalline and intracrystalline shell locations of Recent *Crepidula fornicata*, fossil *Crepidula "radiata"* and two fossil *Tiostrea chiliensis* samples.

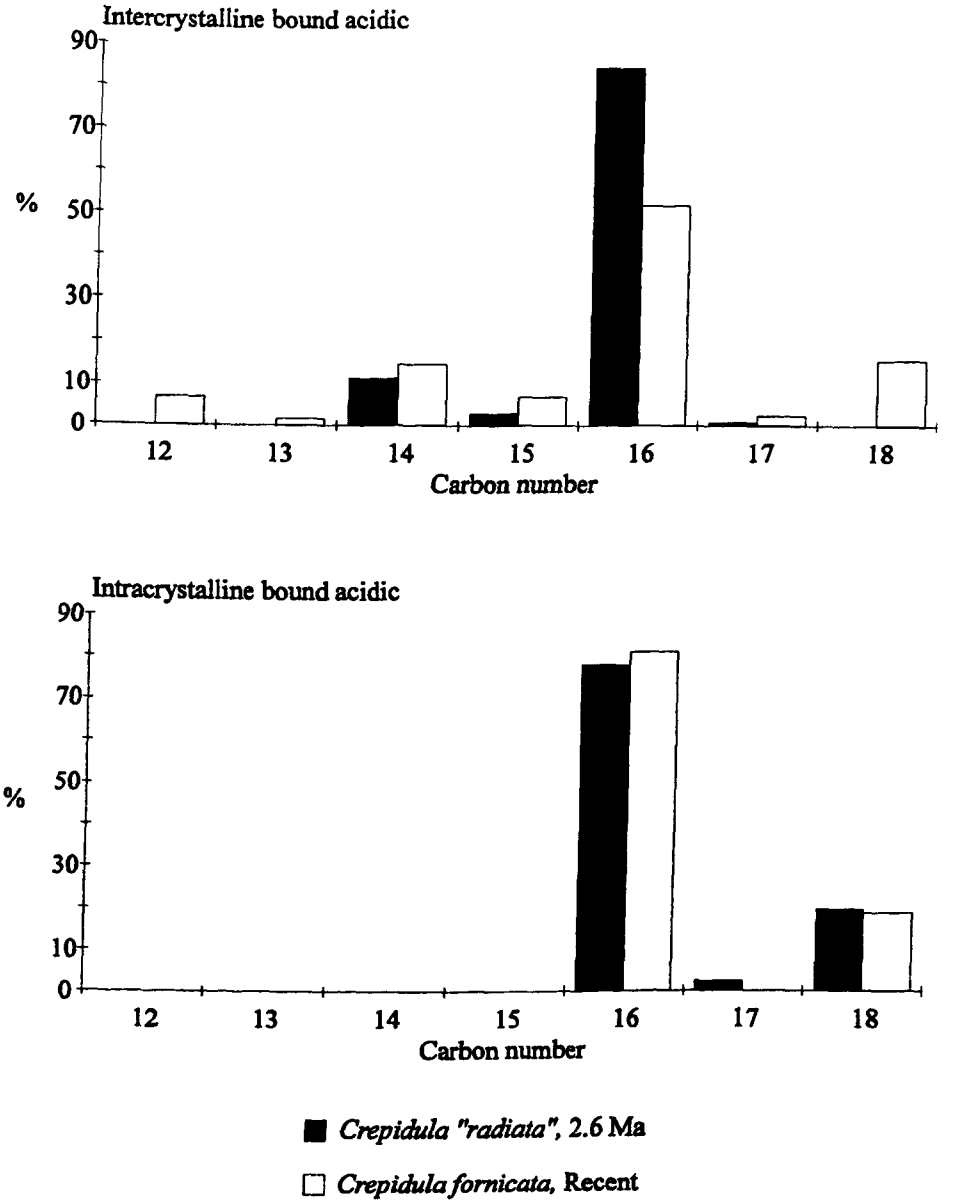


Figure 6. Percentage carbon number distributions of FAMEs from the bound acidic intercrystalline and bound acidic intracrystalline fractions of Recent *Crepidula fornicata* and fossil *Crepidula "radiata"* shells.

A comparison of the intercrystalline bound acidic FAMEs extracted from this study and the bound "intracrystalline" fractions from Clegg (1993) for shells of similar species are shown in Fig. 7a, and from the brachiopod *Neothyris lenticularis* of the same or similar ages in Fig. 7b. Clegg (1993) did not include a stage in the methodology for the release of bound intercrystalline lipids, so it appears likely that the intercrystalline bound lipids may have been misinterpreted as intracrystalline. Both the *Pecten tainui* and *Neothyris lenticularis* FAME carbon number maxima and

distributions are similar despite the use of different methodologies. The scallop (possibly *Pecten tainui*) and *Neothyris lenticularis* extracted by Clegg (1993) have a larger carbon number distribution of minor FAMEs than the shell samples examined herein.

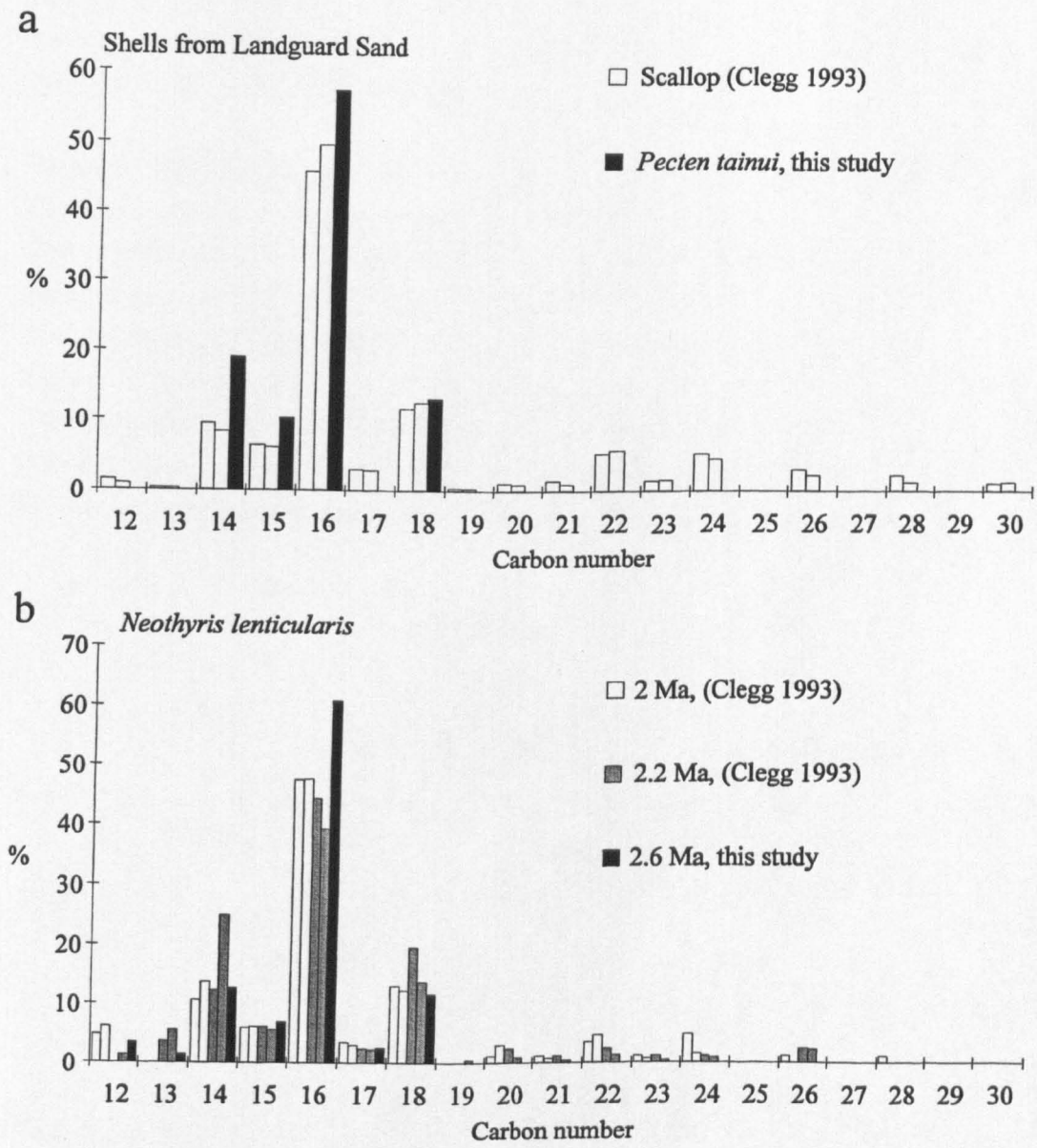


Figure 7. Comparison of percentage FAME carbon number distributions extracted from bound intercrystalline fractions in this study and bound "intracrystalline" fractions reported by Clegg (1993). a) Shells of similar species from the same location. b) *Neothyris lenticularis* (Brachiopoda) shells of similar or identical ages.

2) Quantification

Quantification was carried out for fossil samples using extraction protocol B. The total FAME yields (μg of total FAMEs per g of sample extracted) from shell fractions of Recent and fossil samples are shown in Fig. 8. As for the *n*-alkanes, the yields of FAMEs from the fossil shells are significantly lower than the Recent shells. However the shells are not of the same species, and the FAME yields are generally higher in gastropods than bivalves (Chapter 3).

The quantified yields (μg per g of sample extracted) of FAME carbon number distributions for the fossil shells with the calcium carbonate blank are shown in Fig. 9. The yields from the fossil shells are higher than the calcium carbonate blank. Extracts are made only from intercrystalline bound acidic fractions. The even over odd distribution is maintained for the fossil shells, with a carbon number maximum of C_{16} for both the bivalve species *Pecten tainui* and *Tiostrea chiliensis*. The distribution of FAMEs in *Neothyris lenticularis* (Brachiopoda, 2.6 Ma) is similar to that found in Recent bivalves. This similarity has also been observed for the fatty acid content of the brachiopod soft tissues (Clegg, 1993).

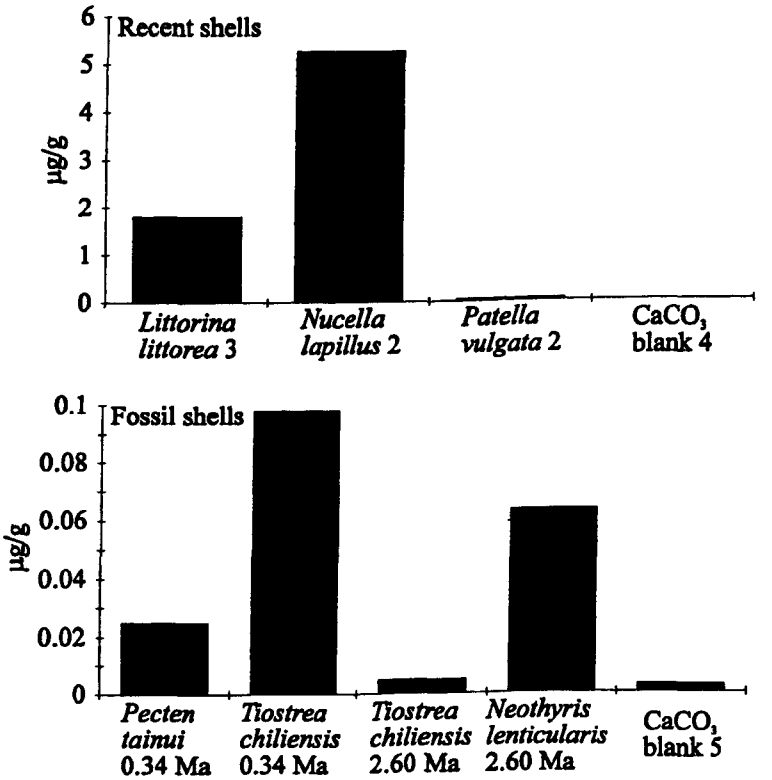


Figure 8. Total FAME yields (μg per g of sample extracted) from all shell fractions of Recent and fossil shells. Sampling locations of Recent shells are shown in Chapter 2.

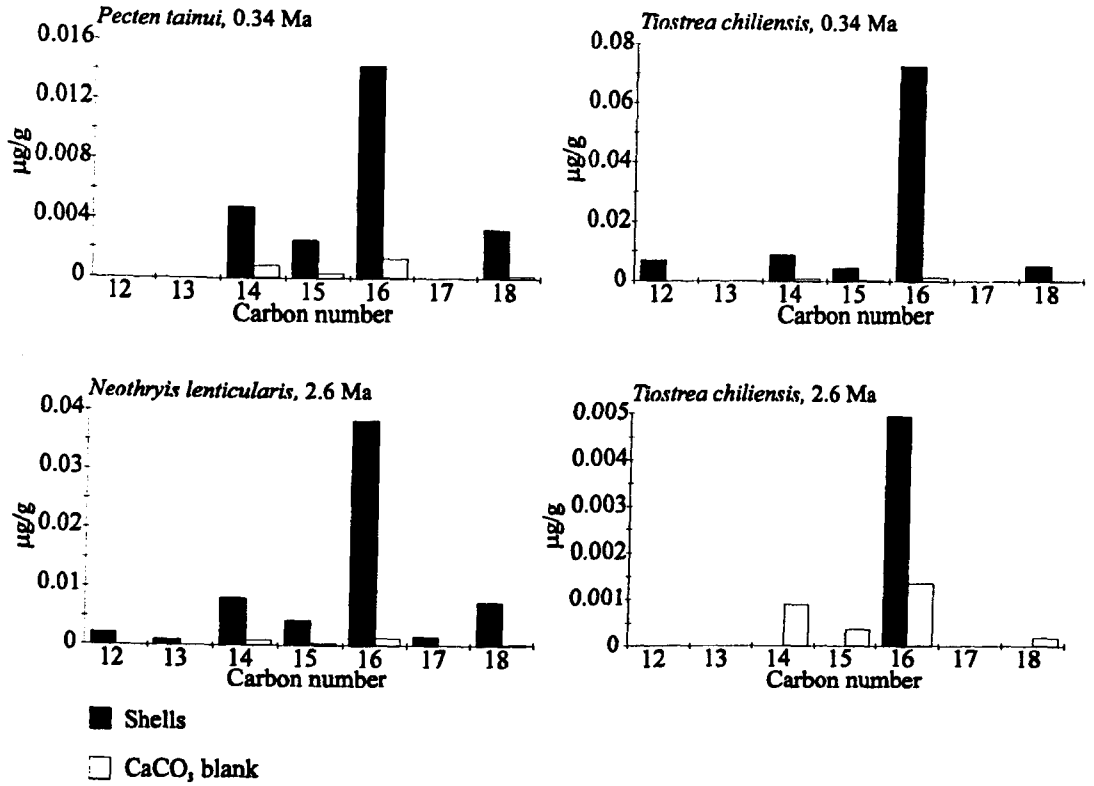


Figure 9. Yields (µg per g of sample extracted) of FAME carbon number distributions. All extracts are from intercrystalline bound acidic fractions.

3) Palaeoecological implications

Fleming (1953) provided a detailed analysis of the palaeoenvironments of deposition of the formations and members sampled here: The Landguard Sand (0.34 Ma) was deposited under offshore conditions some distance from the coast at 10 fathoms depth. The pecten layer of the Kupe Formation (0.5 Ma) was deposited on a sandy bottom at 6-15 fathoms. In the Waipuru Shellbed (1.75 Ma) mud-zone sediments formed a substratum for hard-bottom faunal assemblage with a series of communities. The Middle Waipipi Shellbed (2.6 Ma) was scoured from rapidly deposited soft-bottom sands and silts, in possible estuarine conditions.

The carbon number distributions of FAMEs from different fossil species from the same locality are shown in Fig. 10. These species include *Pecten tainui* and *Tiostrea chiliensis* from the Landguard Sand (0.34 Ma), *Neothyris lenticularis* (Brachiopoda) and *Tiostrea chiliensis* from the Middle Waipipi Shellbed (2.6 Ma). These species are presumed to be sharing the same, or a similar environment, although reworking of older material and the accumulation of shells from a variety of sources prior to incorporation into the sediment cannot be ruled out. There are large differences in the

FAME yields, and the carbon number distributions are not identical between shells of different species from the same locality. This indicates that post-depositional ingress from the surrounding matrix does not affect the intercrystalline FAMEs.

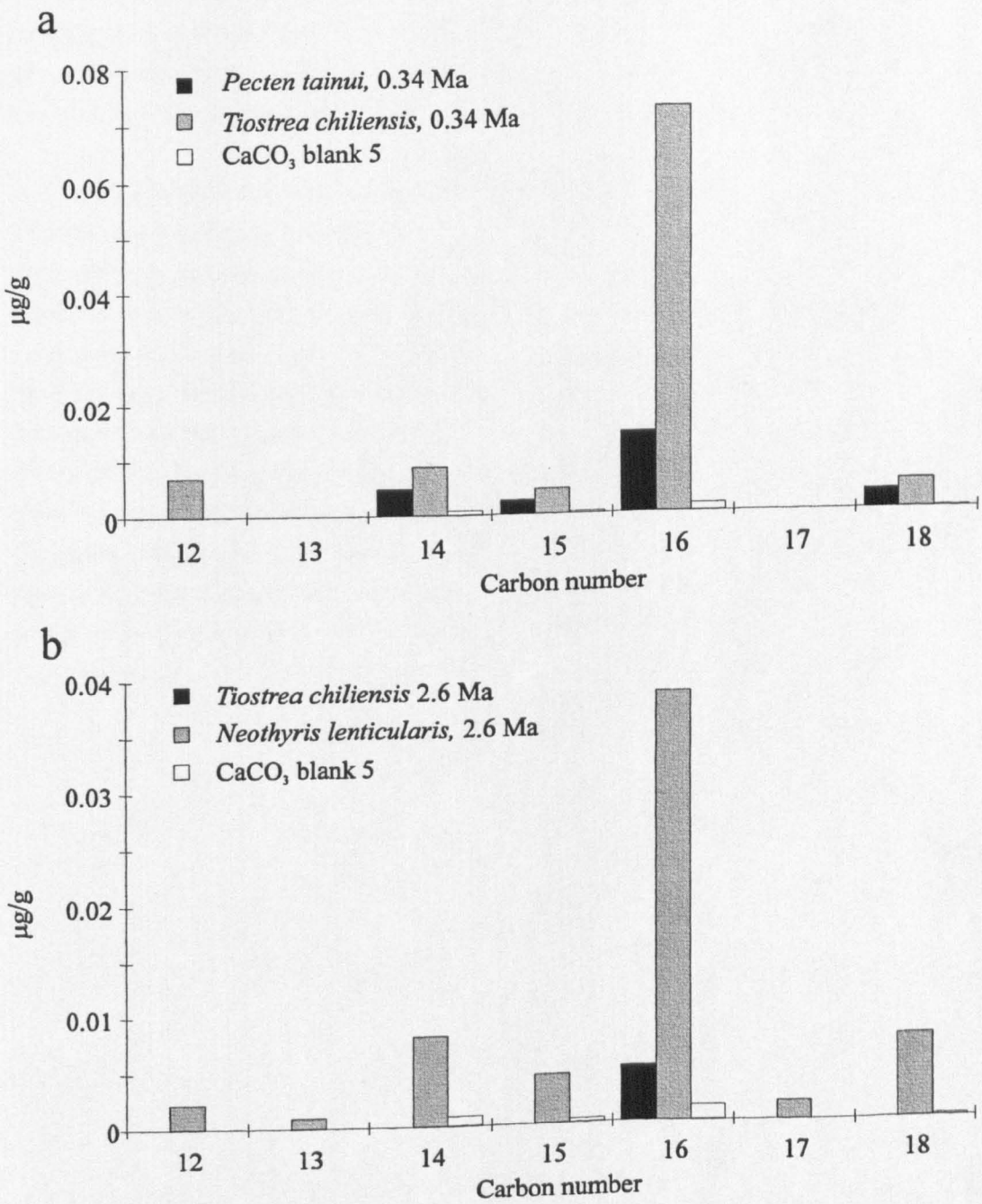


Figure 10. Carbon number distribution of FAME yields (μg per g of sample extracted) of shells of different species from the same location. a) Landguard Sand, 0.34 Ma. b) Middle Waipipi Shellbed, 2.6 Ma.

4) Phylogenetic implications

All the bivalve and the single brachiopod sample examined here exhibit a C_{16} bound fatty acid carbon number maximum (Figs. 6, 7, 9, 10, 11 and 12). No fossil gastropods were analysed. This result agrees with the findings of Chapter 3, where Recent bivalves were restricted to a FAME carbon maximum of C_{16}, whilst Recent gastropods exhibit either C_{16} or C_{18} maxima. This is indicative of phylogenetic distinctions using fossil FAMEs at the molluscan Class level, not at species or genera.

5) Changes in shell bound fatty acid content with age

To eliminate phylogenetic differences, the same species or genus must be compared. A comparison of samples of the same genus; *Pecten tainui* (0.34 Ma) and *Pecten benedictus marwicki* (0.5 Ma) is shown in Fig. 11. Although FAMEs were extracted from the quantified calcium carbonate blank the yields are less than from the shells. No FAMEs were extracted from the blank used with the unquantified data. A C_{16} intracrystalline bound fatty acid is found in the older *Pecten* (0.5 Ma) sample only. The loss of minor intercrystalline bound fatty acid components with increasing age is observed. This loss is similar to the changes observed between Recent and fossil *Crepidula* (Fig. 6). This is a specific loss of certain carbon numbers, and not a general loss taking the yields of minor components below the detection limit; the C_{18} FAME is present in the intercrystalline bound fraction of the 0.5 Ma shell and it is of similar yields to the C_{14} and C_{15} components which are present in the younger sample only.

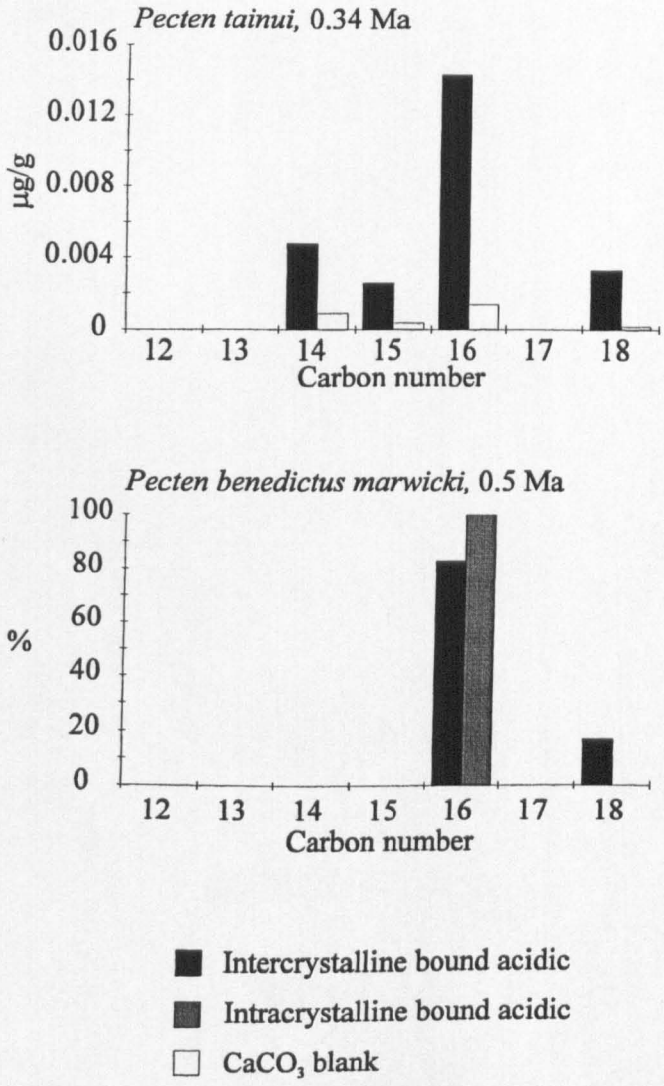


Figure 11. Carbon number distributions of quantified (µg/g of material extracted) and percentage FAMEs from *Pecten* shells from different locations. The column shading represents the extract fractions and the calcium carbonate blank.

Both the quantified and unquantified FAMEs in *Tiostrea chiliensis* shells of 0.34, 0.5, 1.75 and 2.6 Ma are shown in Fig. 12. Although similar, a decrease in the range of carbon number distributions in intercrystalline fractions with increasing age is observed for these samples. The quantified intercrystalline fractions (0.34 and 2.6 Ma) also show a reduction in yields with increasing age. Where intracrystalline FAMEs are extracted from the 0.5 and 1.75 Ma samples they are unquantified. The 0.5 Ma intracrystalline extract has a larger carbon number distribution, but the distributions and maxima of the other intracrystalline extracts are similar to the intercrystalline fractions.

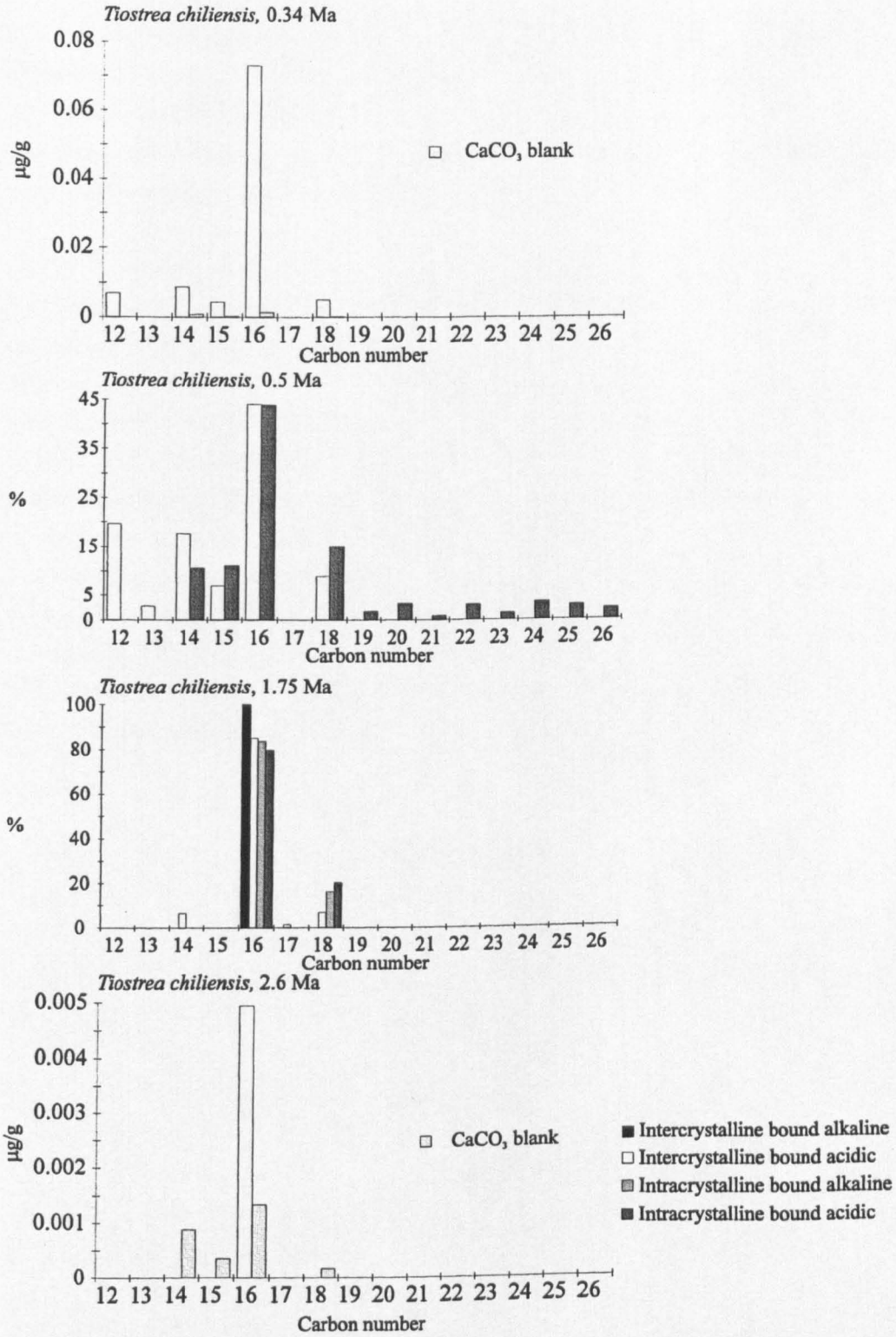


Figure 12. Carbon number distribution of FAME yields (µg per g of sample extracted) and percentage distributions from *Tiostrea chiliensis* shells of different ages. The column shading represents the extract fractions and the calcium carbonate blank.

If bound fatty acid degradation progressed by decarboxylation as proposed for sediments by Cooper and Bray (1963), then odd carbon numbered *n*-alkanes with one less carbon than the FAMEs would be expected. Although the FAMEs decrease in abundance and carbon number distributions with increasing age, there is no evidence for a corresponding increase in the yields of *n*-alkanes (Fig. 4).

Fig. 13 shows the percentage distributions of the saturated fatty acids extracted from fossil samples of algae (Das & Smith, 1968), belemnites (Ivanov *et al.*, 1975) and molluscs (CoBabe & Pratt, 1995). Although differences between molluscan Classes have been observed in this study, the carbon number distributions of all these fossils from different phyla are similar in their even over odd distributions and carbon number maximum of either C_{16} or C_{18}. This illustrates the limited information gained by using only the saturated fatty acids from fossils. These limited carbon number distributions may also make it difficult to distinguish between indigenous and contaminating fatty acids. In addition, the factors which are thought to determine the molluscan soft tissue fatty acid composition include the sex, sexual cycle, temperature, diet, food availability, the tissue type and phylogeny (Gardner & Riley, 1972; Joseph, 1982), these may be too interdependent to yield any useful information relating to the fossil being examined.

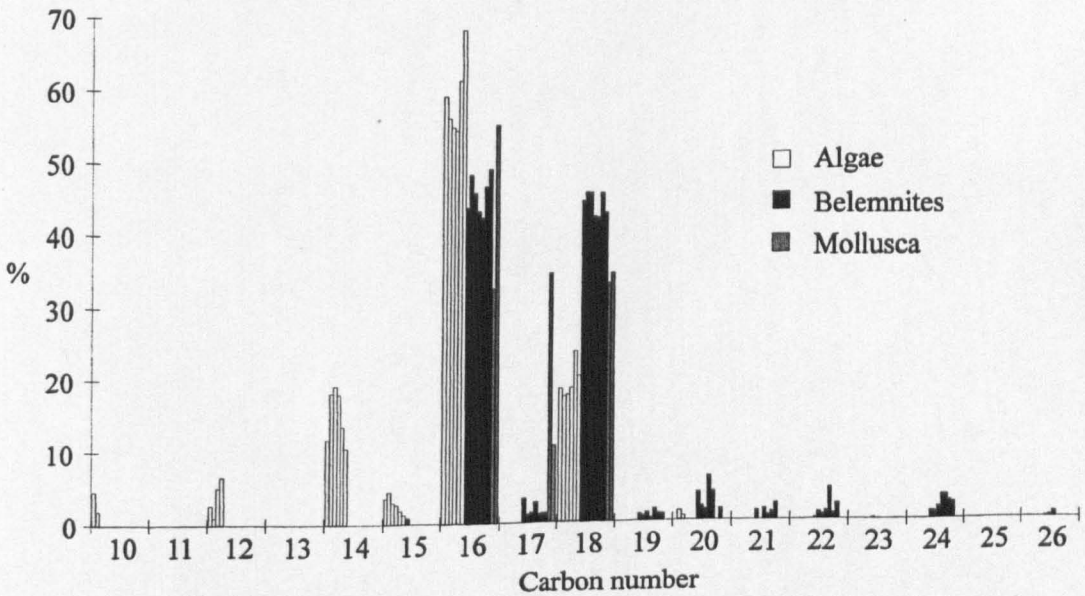


Figure 13. Percentage carbon number distributions of the saturated fatty acids reported for fossil algae (Das & Smith, 1968), belemnites (Ivanov *et al.*, 1975) and molluscs (CoBabe & Pratt, 1995).

Cholesterol

Quantified cholesterol yields (μg per g of sample extracted) from each fraction of the fossil shells are shown in Fig. 14. The calcium carbonate blank is shown with the shell extracts. The surficial (A) extract of the calcium carbonate blank yields the largest amount of cholesterol contamination, this is due to the initial cleaning treatments not removing absorbed lipids (Chapter 1). With the exception of *Neothyris lenticularis* the intercrystalline (B) and intercrystalline bound alkaline (C) shell fractions have the highest cholesterol yields. This is similar to the Recent shells examined in Chapter 2 where cholesterol dominates in the intercrystalline fraction. The yields of cholesterol in the fossils are lower than the Recent shells.

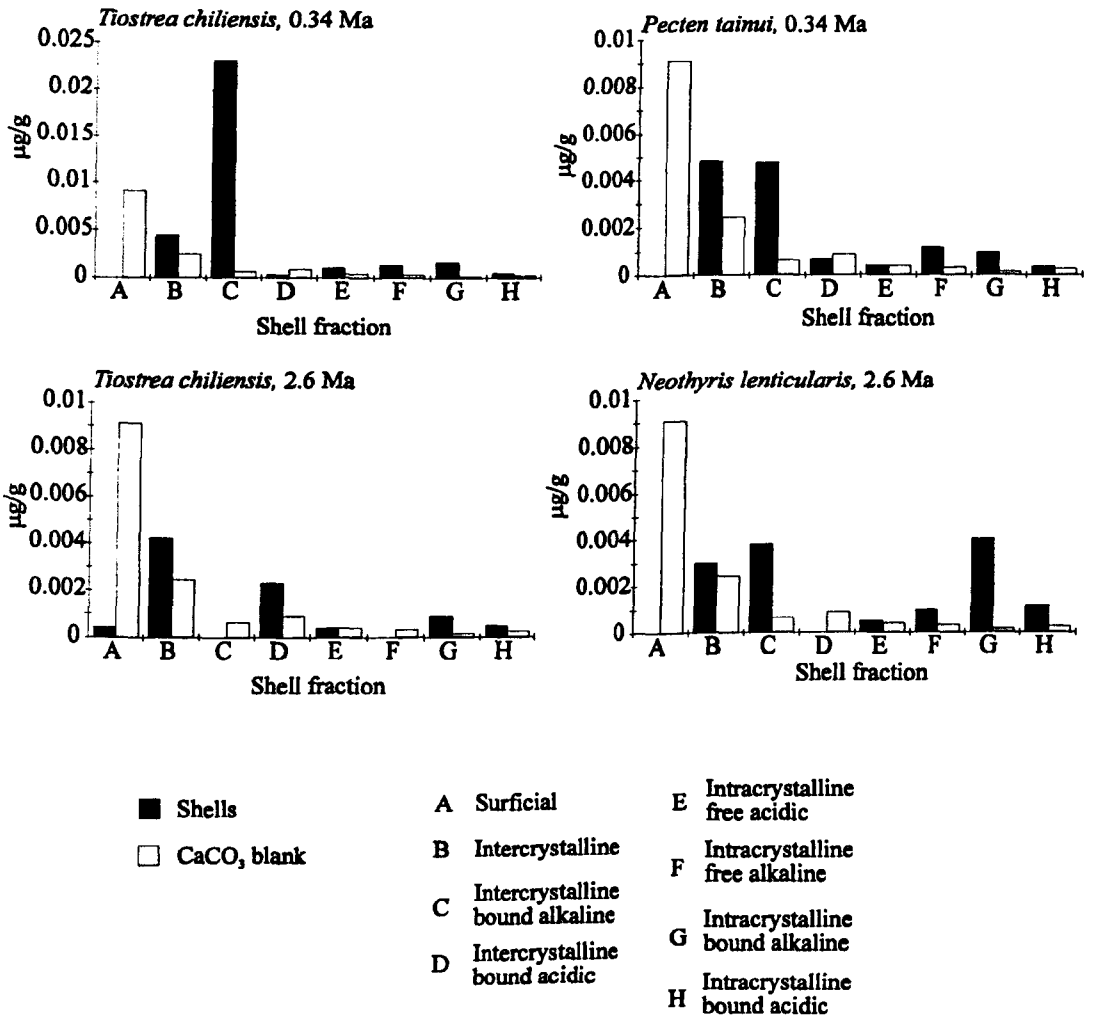


Figure 14. Cholesterol yields (μg per g of sample extracted) for fossil shells and the calcium carbonate blank. Plotted for the shell fractions A to H.

Cholesterol yields (μg per g of sample extracted) from *Tiostrea chiliensis* shells of different ages are shown in Fig. 15. The yields are shown for each fraction with the calcium carbonate blank. The yields from the surficial extract are higher in the control than the shells, and therefore this fraction is excluded. The intercrystalline bound alkaline fraction of the 0.34 Ma shell has the highest cholesterol yields. However, the cholesterol in the 2.6 Ma sample is coeluting with an unidentified component and is therefore not plotted. The remaining fractions show similar yields of cholesterol for both shells. Cholesterol therefore, unlike the FAMEs, remains at a constant level in the fossil shells. Cholesterol was the only steroidal component extracted from the fossil shells. This excludes the use of the steroidal biomarkers for the analysis of phylogeny and diet in fossils.

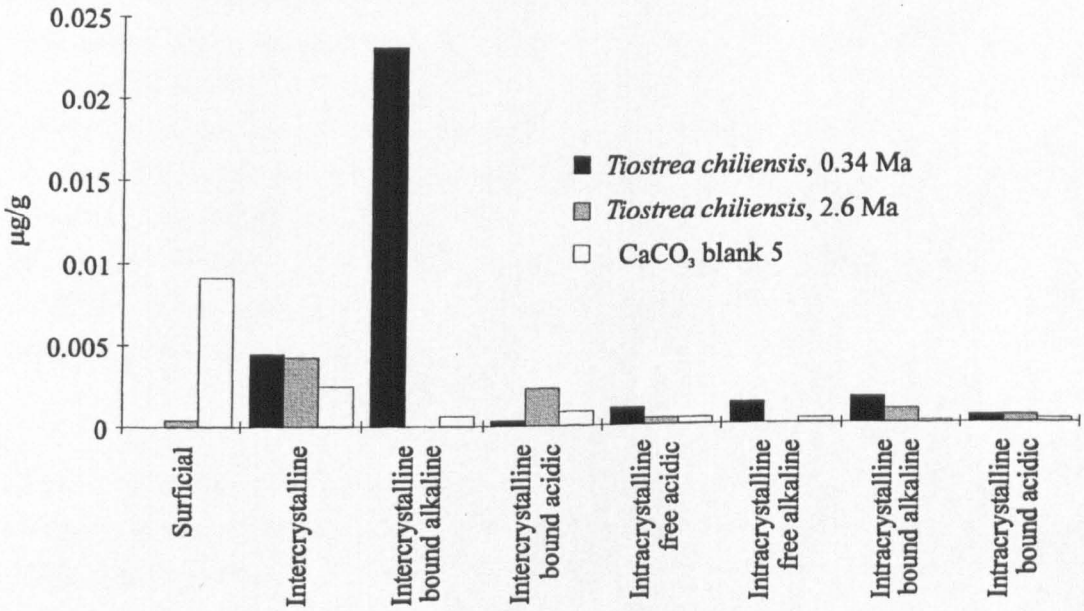


Figure 15. Cholesterol yields (μg per g of sample extracted) for *Tiostrea chiliensis* shells of different ages and the calcium carbonate blank.

The $\delta^{13}C$ values of fatty acids and cholesterol from Recent shells have been used by CoBabe and Pratt (1995) to distinguish between heterotrophic and chemosymbiotic bivalve shells and the sources of individual lipid components. The $\delta^{13}C$ values being dependent on the source of carbon to the mollusc. The question remains if this technique can distinguish between the variety of complicating factors previously mentioned, especially when extended to other molluscs which may not exhibit distinctive $\delta^{13}C$ values.

Mass-balance of lipids in intracrystalline shell locations

A reduction in yields of intercrystalline and intracrystalline *n*-alkanes, bound fatty acids and (except for cholesterol) the absence of steroids, has been observed in the fossil shells when compared with Recent samples. Lipid destruction may therefore be continuing throughout the fossil record and may not leave a constant intracrystalline component as proposed by Sykes *et al.* (1995). Although more data are required this could result in no lipids present in older material. This hypothesis questions the view that organic material in intracrystalline shell locations is protected from diagenetic processes. The reduction in lipid yields, the absence of some lipid components and diagenetic products could be due to migration of these components from the shells particularly from the environmentally accessible intercrystalline locations. For the fully entombed intracrystalline lipids losses should not occur by migration from the shell, although *in situ* diagenesis leading to complete destruction or condensation with other organic material may explain these losses. Further experimentation is needed to reveal the presence of condensed lipids in these fossils. Migration from intracrystalline locations is unlikely as the results of Thompson and Creath (1966) indicate that the short chain hydrocarbons produced by the diagenesis of shell matrix proteins are retained within the shell, and that if not truly intracrystalline then Sykes *et al.* (1995) and this study (Chapter 2) show the presence of extremely recalcitrant organic material within the shells.

CONCLUSIONS

The new extraction protocol previously used to examine the lipids from the shells of Recent molluscs has been applied to Quaternary fossil shells. A similar range of lipid components has been found in this older material.

Where shell yields are greater than the laboratory contamination the *n*-alkanes are considered indigenous in intracrystalline fractions. The yields of *n*-alkanes are lower in fossil shells compared to Recent shells. As yields in fossils are close to the yields of laboratory contamination then extreme care should by exercised in interpreting these results. Therefore the *n*-alkanes from fossils are an unreliable source of information relating to the fossils.

n-Alcohols extracted from fossil shells have an even over odd carbon number distribution with a C_{16} carbon number maximum. The carbon number distributions of the intercrystalline bound alkaline fraction were similar for all fossil samples, while the intracrystalline bound alkaline fractions were different. Although indigenous to the

shells and similar to the Recent shells examined here and elsewhere (Chapter 2), the fossil *n*-alcohols do not yield enough information to be of further use.

The bound fatty acids (extracted as FAMEs) with an even over odd distribution are extracted from the fossil shells, with extracts predominantly from intercrystalline shell locations. A comparison of the FAMEs from 0.34 and 2.6 Ma old *Tiostrea chiliensis* specimens shows that the yields are greatly reduced in the older shells with losses of minor FAME components. The FAME carbon number maximum is C_{16} for all the fossil shells, this supports the previous observation that the bivalves have a restricted C_{16} FAME carbon maximum only whilst gastropods can have C_{16} or C_{18} maxima (Chapter 3). This distinction at the Class level does not provide any information which was not previously known about the shells.

Cholesterol was the only steroid extracted from the fossil shells, which prohibited its use for the analysis of phylogenetic and dietary relationships. Cholesterol is extracted from all shell fractions, although in higher yields in intercrystalline fractions. The yields of cholesterol in the fossils are lower than the Recent shells, although remains at a constant level in 0.34 and 2.6 Ma old *Tiostrea chiliensis* fossil shells.

The intercrystalline bound and all the intracrystalline shell fractions are considered to be sufficiently protected to yield indigenous lipids in fossil shells. The intercrystalline locations within the fossil shells provide good sites for the preservation of bound lipids with carbon number distributions maintained and no evidence of post-depositional ingress from the surrounding matrix. However, the reduction in yields of the intercrystalline *n*-alkanes and FAMEs, the loss of minor FAME components and the complete loss of steroids (except cholesterol) over geological time indicates migration, condensation or destruction is not completely halted. There is no evidence for an increase in the *n*-alkanes with sample age from processes such as decarboxylation of fatty acids.

This study shows that despite the presence of indigenous lipids in fossil shells, the extracted lipids cannot be used to provide novel information relating to phylogenetic relationships, diets or the environment.

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GENERAL CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

1) Laboratory contamination is a major problem. This has to be both minimised and characterized in terms of yields, distributions and variability. Minimising contamination is possible using the methods described in Chapter 1. Further control may be achieved with the use of a clean room set up for the purpose. Extraction of a larger number of shells would increase the yields of indigenous shell lipids, however the increased amounts of reagents may also increase the levels of background contamination. Individual compound classes exhibit their own problems concerning contamination, such as the phthalate plasticisers which show large variability in their distributions and where they are extracted. The yields from background contamination and sample extracts need to be fully quantified, both surrogate and internal standards should be used. Data from the blanks and samples should be reported together.

2) Surficial, intercrystalline and intracrystalline fractions can be separately extracted. Compound specific gas chromatography isotope ratio mass spectrometry (GCIRMS) of the stable ^{12}C and ^{13}C isotopes of the lipids may indicate different lipid sources between the different shell fractions. This could also be a useful method for distinguishing between contamination and indigenous lipidic material. The use of GCIRMS may also aid the study of dietary and environmental lipid sources where the information is not sufficient from the carbon number distributions or yields.

3) To increase the numbers of samples it is possible to analyse, the time taken to complete the experimental protocol needs to be shortened by changes in the methodology. Retaining the cleaning stages of hypochlorite treatment, partial decalcification and surficial solvent extract are essential. Although there may be a risk of extracting some lipidic ingress in the intercrystalline sites, the shells could be fully decalcified, followed by saponification of the remaining insoluble material. This would result in the combined intercrystalline, intercrystalline bound, intracrystalline and intracrystalline bound fractions. Information would be lost concerning the location of the lipids within the shell, although only a single liquid-liquid extraction of the aqueous phase would now be required. However, liquid-liquid extraction was found to be the most time consuming and labour intensive stage. An alternative method using freeze drying is discussed in Appendix 14. Unfortunately in this laboratory the use of freeze drying was found to be a major source of contamination and produced irreproducible yields of extracted lipids.

4) Other compounds such as the direct analysis for triglycerides phospholipids, pigments, or once problems of contamination have been resolved, phthalate plasticisers may be worth investigating in the shells of molluscs. Bound lipids and insoluble residues could be examined using pyrolysis-GC.

5) A comparison of the soft tissue and shell lipids from the same sample would eliminate the different factors that affect the lipid composition when comparing reported values of the soft tissues and those of the shells examined in this work (Chapter 2). A good species for analysis would be *Mytilus edilus* (the edible mussel) of which the soft tissues are readily available and have been previously studied. Specific analysis of organs such as the mantle, and the triglyceride and phospholipid composition may provide information concerning the source of fatty acids in the shell.

6) If the accumulation of pollutants is species dependant, a study using *Mytilus edilus* which is used as a sentinel for a variety of pollutants bioconcentrated in the soft tissues (Goldberg 1980), may be a more reliable species for the accumulation of pollutants into the shell.

7) Scanning electron microscopy of the microstructure of the species examined here may answer the question as to whether it is the inorganic structure which gives higher yields and different lipid classes in the gastropods than the bivalves. The use of porosity measurements, or as examined here the deposition of a layer onto the crystals after hypochlorite treatment (Chapter 1), to assess the ease of penetration of the intercrystalline spaces would also be useful. These techniques may be used to select samples which can resist ingress.

8) The loss of sterols and β-hydroxy acids observed here when comparing Recent and fossil shells (Chapter 5), could be investigated over a shorter time period, perhaps with archaeological samples, this source of samples may also remove the uncertainty over the age of Recent shells as archaeological shells are most likely to be from a live food source and hence there would be a short time between use and deposition as waste material. Artificial diagenesis of living shells by the use of elevated temperatures and pressures may also be used to examine the loss of lipids from shells.

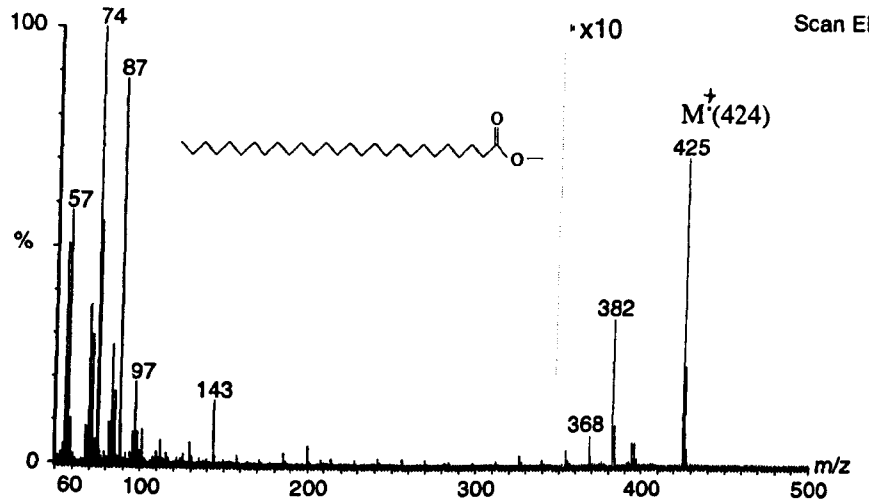
9) Reduced yields of *n*-alkanes, bound fatty acids and cholesterol were extracted from fossil shells. The expansion of the experimental protocol developed here to i) older samples, such as aragonitic ammonites which have probably not undergone reminrealisation shown by the presence of the aragonite, ii) to a variety of other mineralising systems such as bones and teeth, iii) to investigate mineralising systems which are under little biological control, such as stromatolites, or iv) to geological processes such as concretions.

10) The small fluid inclusions in molluscan shells were also investigated in this study. The inclusions were generally too small to observe any homogenisation of gas bubbles into the liquid phase with changes in temperature. This technique would be interesting to pursue as the temperature and salinity of fluid inclusion at formation could be ascertained, leading to a comparison with the pallial fluid salinity or environmental temperature at the time of shell deposition.

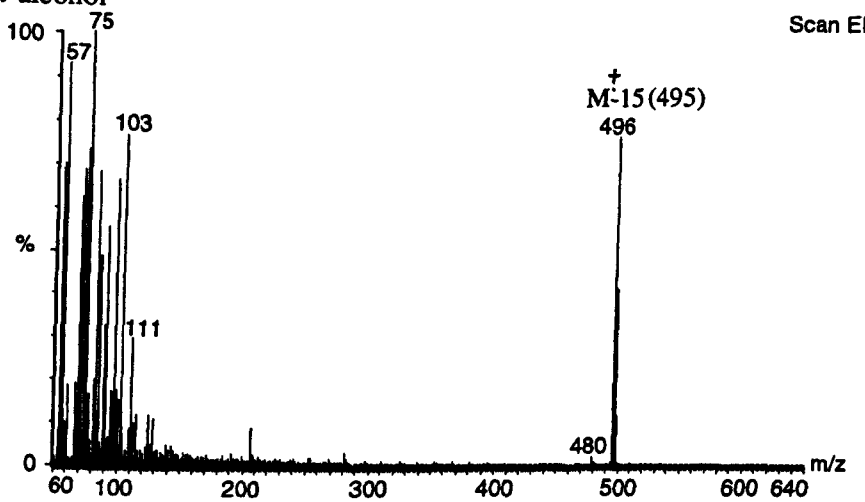
11) A preliminary attempt at analysis of the volatile composition of the fluid inclusions was tried by crushing a small quantity of the shell powder, cryogenic focusing in a cold trap followed by direct analysis by GC. Different FID responses were found with different shell samples, however these results were not reproducible and presented problems with contamination. GCMS analysis using the same technique produced large yields of water which masked the other components.

APPENDIX 1

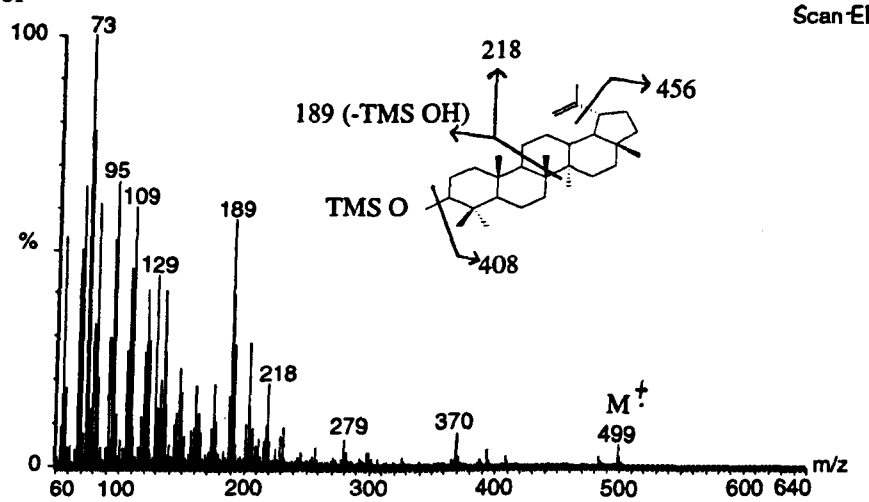
C_{27} FAME



C_{30} *n*-alcohol



Lupeol



APPENDIX 2

1) Recoveries of surrogate standards from shell and control extracts

The surrogate standards; C_{27} FAME (2.29 μg), C_{30} alcohol (3.32 μg), lupeol (2.63 μg) and C_{36} *n*-alkane (2.57 μg) were added to the samples before solvent extraction (Chapter 1, Fig. 1 Protocol B). If efficiently extracted the yields of these standards extracted from the samples would be expected to be the same as the original standard composition shown in Fig. 1. The extracted surrogate standards from experimental batch 4 are shown in Fig. 2. The ratio of the standards to the C_{36} *n*-alkane is shown, the C_{36} *n*-alkane is by definition one. The recoveries of the C_{27} FAME, C_{30} *n*-alcohol and lupeol vary relative to the C_{36} *n*-alkane and are not similar to the standard mixture added to the samples (Fig. 1). This suggests that inefficient extraction is occurring. Therefore, these surrogate standards may be carried over to the next sequential addition of surrogate standard. This would result in excess surrogate standard present in later extracts, resulting in the calculation of lower yields of indigenous shell lipids.

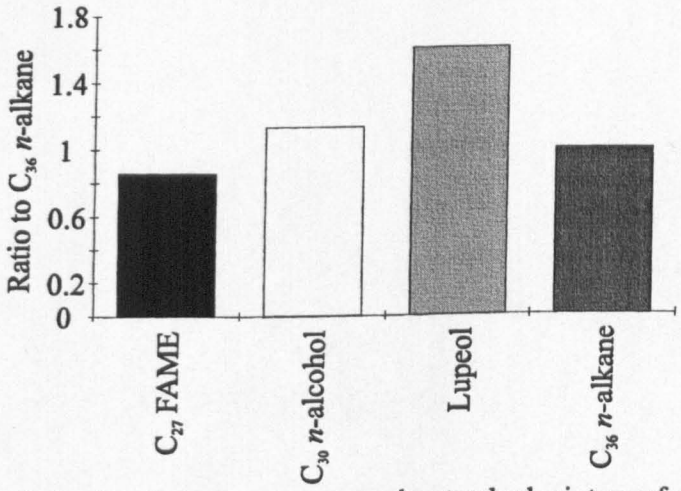


Figure 1. Flame ionisation detector response to the standard mixture of surrogate standards. GC peak areas are shown as the ratio to the C_{36} *n*-alkane.

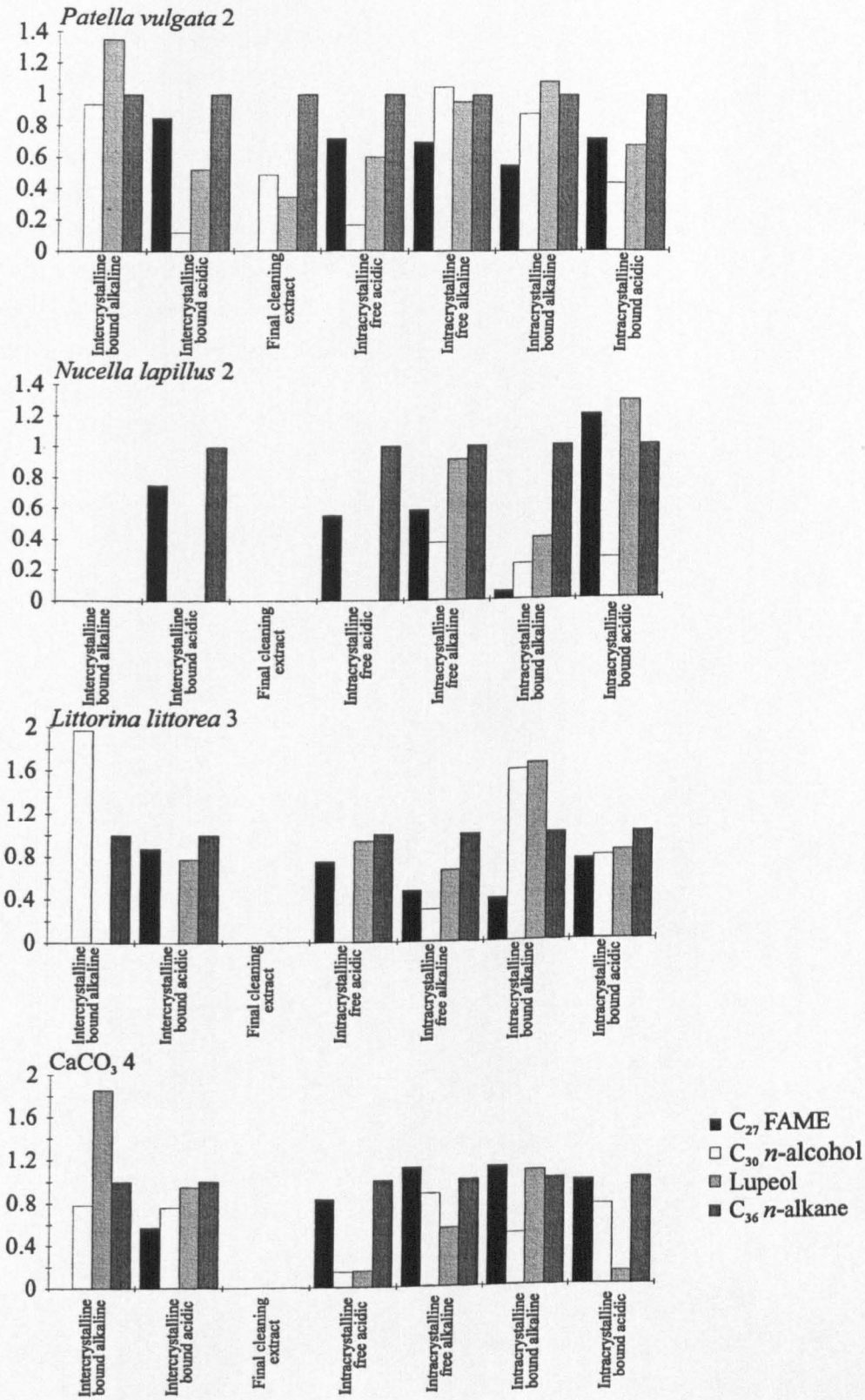


Figure 2. Ratio to the C_{36} *n*-alkane of surrogate standards for each sample analysed in experimental batch 4.

Table 1 shows the ratios of the surrogate standards extracted from experimental batch 4. The ratio has been repeated for each standard. The ratio of all the standards to the C_{36} *n*-alkane shows the least variation (shown by the coefficient of variation). This suggests that the C_{36} *n*-alkane exhibits the least variation in its efficiency of extraction. The smallest variation (30% coefficient of variation) is between the C_{27} FAME and C_{36} *n*-alkane, this is probably due to the similar hydrophobic nature of these compounds. The ratio of the C_{30} *n*-alcohol to the lupeol also shows a small variation, suggesting extraction dependent on the alcohol functionality. It is the partitioning behaviour between the aqueous and solvent phases during liquid-liquid extraction which is therefore responsible for these differences. These results exclude the C_{27} FAME, C_{30} *n*-alcohol and lupeol for use as surrogate standards.

	Mean	Standard deviation	Coefficient of variation (%)
C_{27} FAME	0.691	0.311	45
C_{30} *n*-alcohol	0.561	0.518	92
Lupeol	0.790	0.494	63
C_{36} *n*-alkane	1	0	-
C_{27} FAME	1.432	1.903	133
C_{30} *n*-alcohol	0.861	1.228	143
Lupeol	1	0	0
C_{36} *n*-alkane	1.733	1.891	109
C_{27} FAME	1	0	-
C_{30} *n*-alcohol	0.740	0.937	127
Lupeol	1.082	0.931	86
C_{36} *n*-alkane	1.422	0.433	30
C_{27} FAME	1.929	2.108	109
C_{30} *n*-alcohol	1	0	-
Lupeol	1.886	1.247	66
C_{36} *n*-alkane	2.524	2.301	91

Table 1. Ratios of surrogate standards extracted from intercrystalline bound and all intracrystalline fractions of samples analysed in experimental batch 4. Data are shown relative to the standard shown in bold typeface. Missing data have been excluded.

Fig. 3 shows the solubility in water of the C_{36} *n*-alkane, this shows a very low affinity for water due to its hydrophobicity and lack of functional groups. As the surrogate standards were added to the samples in 1 ml of DCM, the C_{36} *n*-alkane is unlikely to have partitioned into the aqueous phase from the solvent and would therefore have remained in the DCM solvent and been fully extracted.

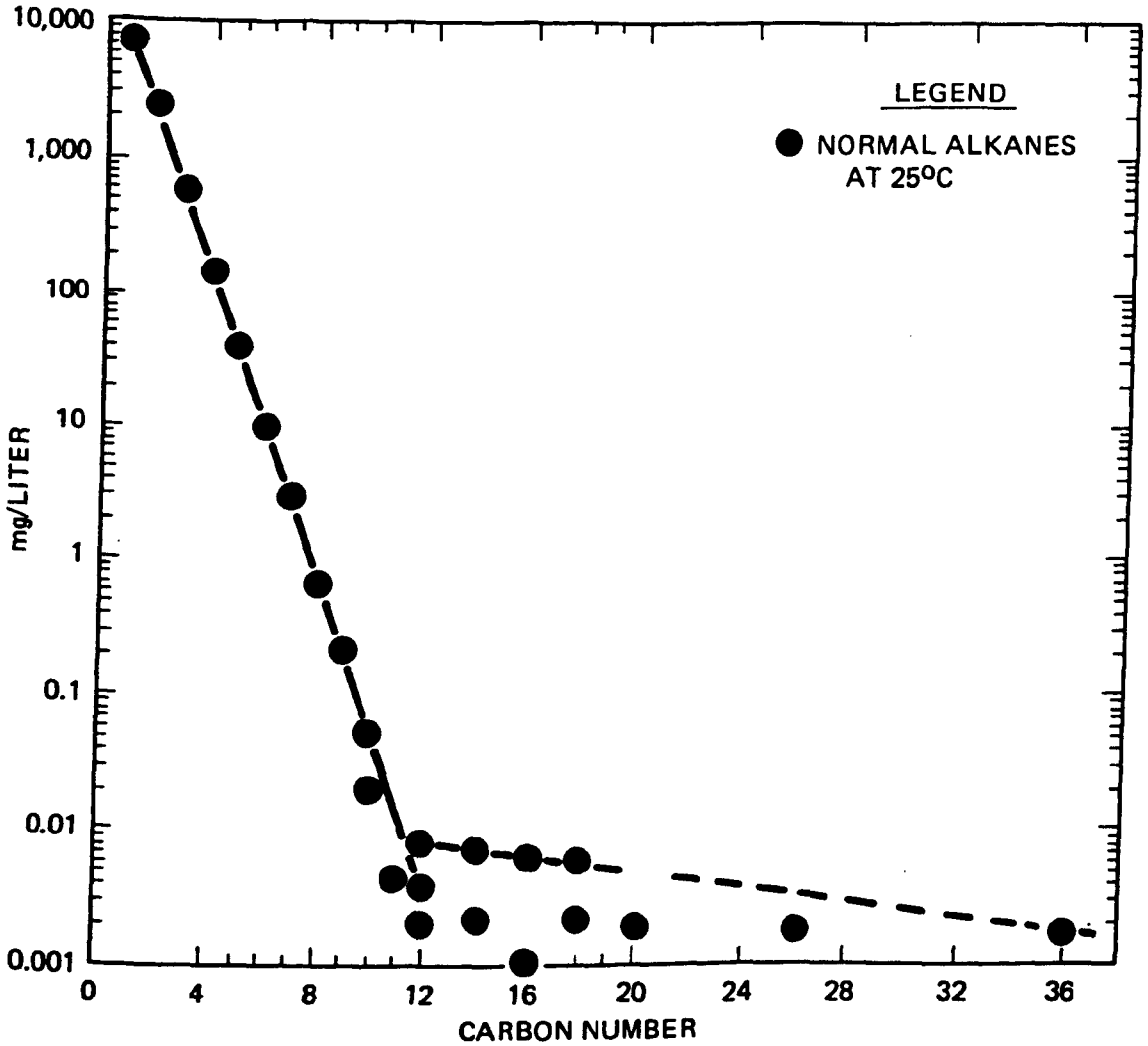


Figure 3. Adapted from McAuliffe (1978). Solubility of *n*-alkanes in water.

2) Prediction of surrogate standards remaining in the samples

A theoretical calculation to estimate the potential amounts of surrogate standards inefficiently extracted, remaining in the sample and therefore combined with the next addition of surrogate standard has been carried out. Table 2. illustrates the calculations used. For each fraction the amount of surrogate standard added to the sample is calculated by the amount of surrogate standard not extracted by the previous solvent extraction combined with the addition of new surrogate standard. This method assumes the C_{36} *n*-alkane standard is fully extracted as the other surrogate standards

are measured relative to this. Intercrystalline bound alkaline and intracrystalline free acidic fractions have only had the addition of the surrogate standard. The intercrystalline and intracrystalline fractions have been calculated separately, this assumes that there has been no carry over from intercrystalline to intracrystalline fractions. The final cleaning extract also shows some surrogate standards, carried over from addition of surrogate standards before liquid-liquid extraction of the aqueous phase, however as these amounts are small and the C_{36} *n*-alkane is not consistently present, this extract has not been included in the calculation. Negative amounts of carry over have been changed to zero. The extraction of no standards from the intercrystalline bound alkaline fraction of *Nucella lapillus* 2 has lead to the double addition the surrogate standard mixture to the intercrystalline bound acidic fraction for this sample.

Fraction	Surrogate standard added	Carry over to next fraction
Intercrystalline bound alkaline	Surrogate standard **= Added 1**	Added 1 - Extracted from Intercrystalline bound alkaline **= Carry over 1**
Intercrystalline bound acidic	Surrogate standard + Carry over 1 **= Added 2**	-
Final cleaning extract	-	-
Intracrystalline free acidic	Surrogate standard **= Added 3**	Added 3 - Extracted from Intracrystalline free acidic **= Carry over 2**
Intracrystalline free alkaline	Surrogate standard + Carry over 2 **= Added 4**	Added 4 -Extracted from Intracrystalline free alkaline **= Carry over 3**
Intracrystalline bound alkaline	Surrogate standard + Carry over 3 **= Added 5**	Added 5 - Extracted from Intracrystalline bound alkaline **= Carry over 4**
Intracrystalline bound acidic	Surrogate standard + Carry over 4	-

Table 2. Calculations used for estimating the amount of surrogate standard not extracted, and therefore carried over and added to the next sequential addition of surrogate standard.

Fig. 4 shows the amounts of surrogate standards calculated to have been added to each fraction (Table 2, carry over to next fraction). This calculation shows the increase of surrogate standard in the samples with each further addition of standard combining with the surrogate standard remaining from the previous addition of standard mixture. The increase of the C_{30} *n*-alcohol and lupeol shows that these compounds are inefficiently extracted from the samples. The C_{27} FAME also shows an increase with each fraction, but at a lower level than the C_{30} *n*-alcohol and lupeol.

3) Quantification of surrogate standards: Internal standard

To quantify the yields of extracted surrogate standards a C_{16} *n*-alkane (1.38 μg) internal standard was added prior to GC analysis to selected samples from experimental batch 4. The ratio of the C_{16} *n*-alkane internal standard to the surrogate standards is shown in Fig. 5. Only those samples with fractions identified on the x-axis have been analysed. These internal standard analyses were carried out after GC and GCMS analysis, the loss of material due to these previous analyses were calculated using the volumes used for dilution and on-column injection (Appendices 15-19). These corrected data are shown in Fig. 6. When adjusted for amounts of material previously removed more surrogate standard is calculated to be present in the extracts. Although the ratios of the surrogate standards is not constant, the expected increase of surrogate standard predicted by the carry over (Fig. 4) is not seen. The amounts of surrogate standards extracted in the final cleaning extract is very low (Figs. 5 & 6). Therefore the carry over of surrogate standards from one fraction to another is insignificant. The variation between fractions may be due to losses during workup.

Fig. 7 shows the yields (μg) of C_{36} *n*-alkane extracted from experimental batch 4. Quantification was carried out using the C_{16} *n*-alkane internal standard, a relative response factor (RRF) of 1.313 was used, calculated for C_{19} *n*-alkane to C_{36} *n*-alkane (Appendix 3). This RRF should be similar to that for the C_{16} *n*-alkane. Both the directly measured yields and yields adjusted for material used in previous analyses are shown.

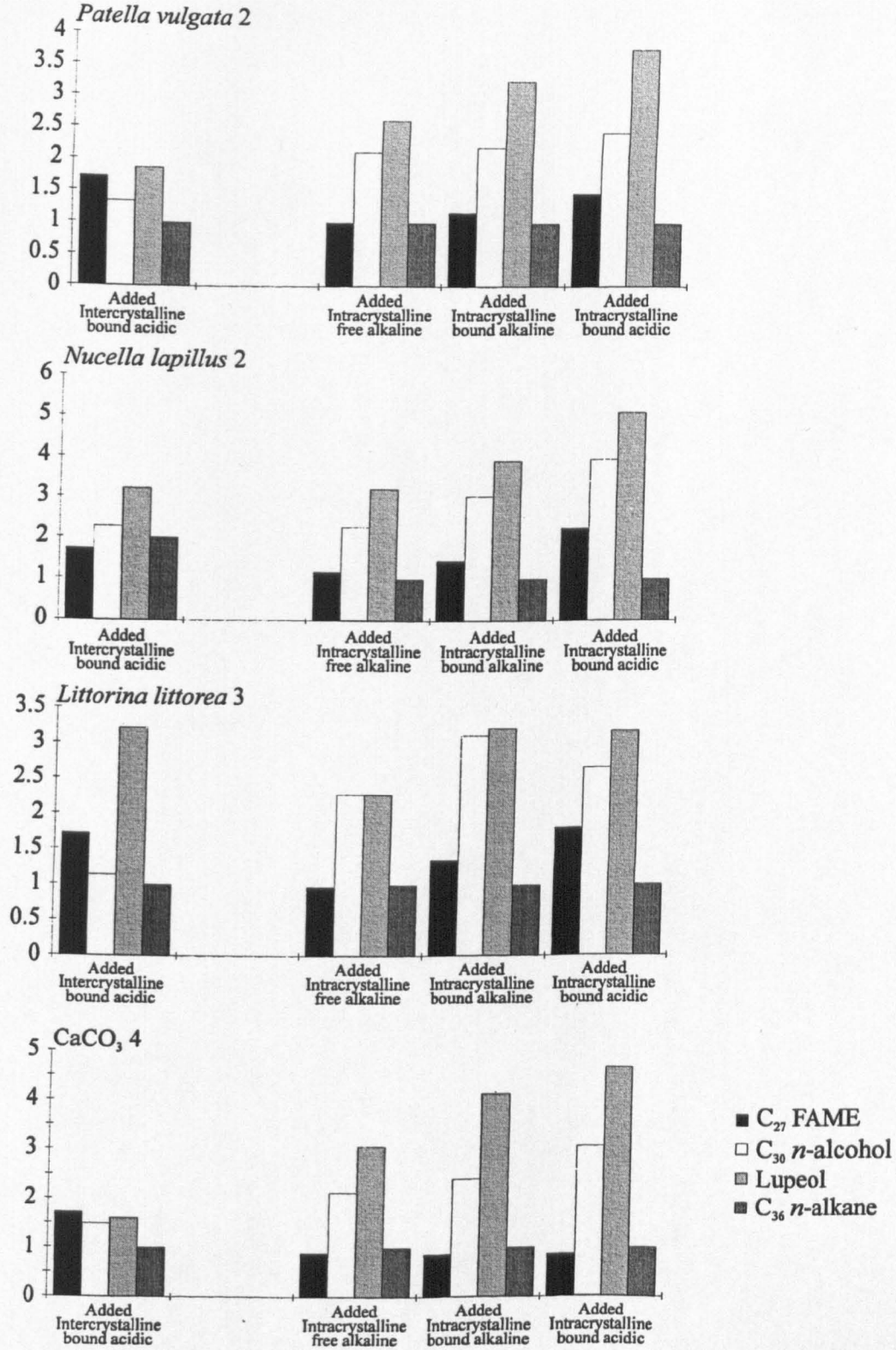


Figure 4. Ratios of surrogate standard to C36 *n*-alkane calculated to have been added to samples.

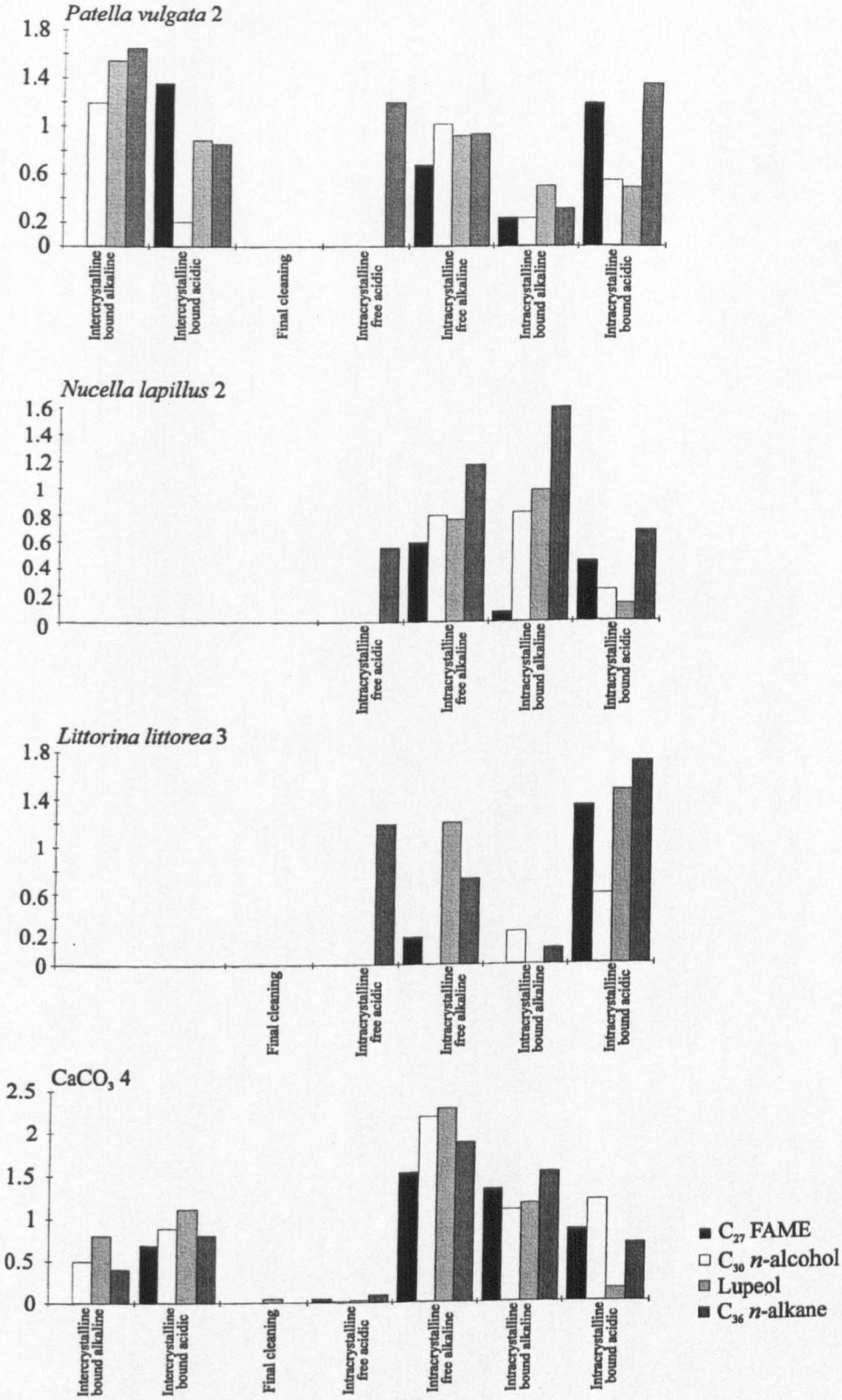


Figure 5. Ratio of C_{16} *n*-alkane internal standard to the surrogate standards.

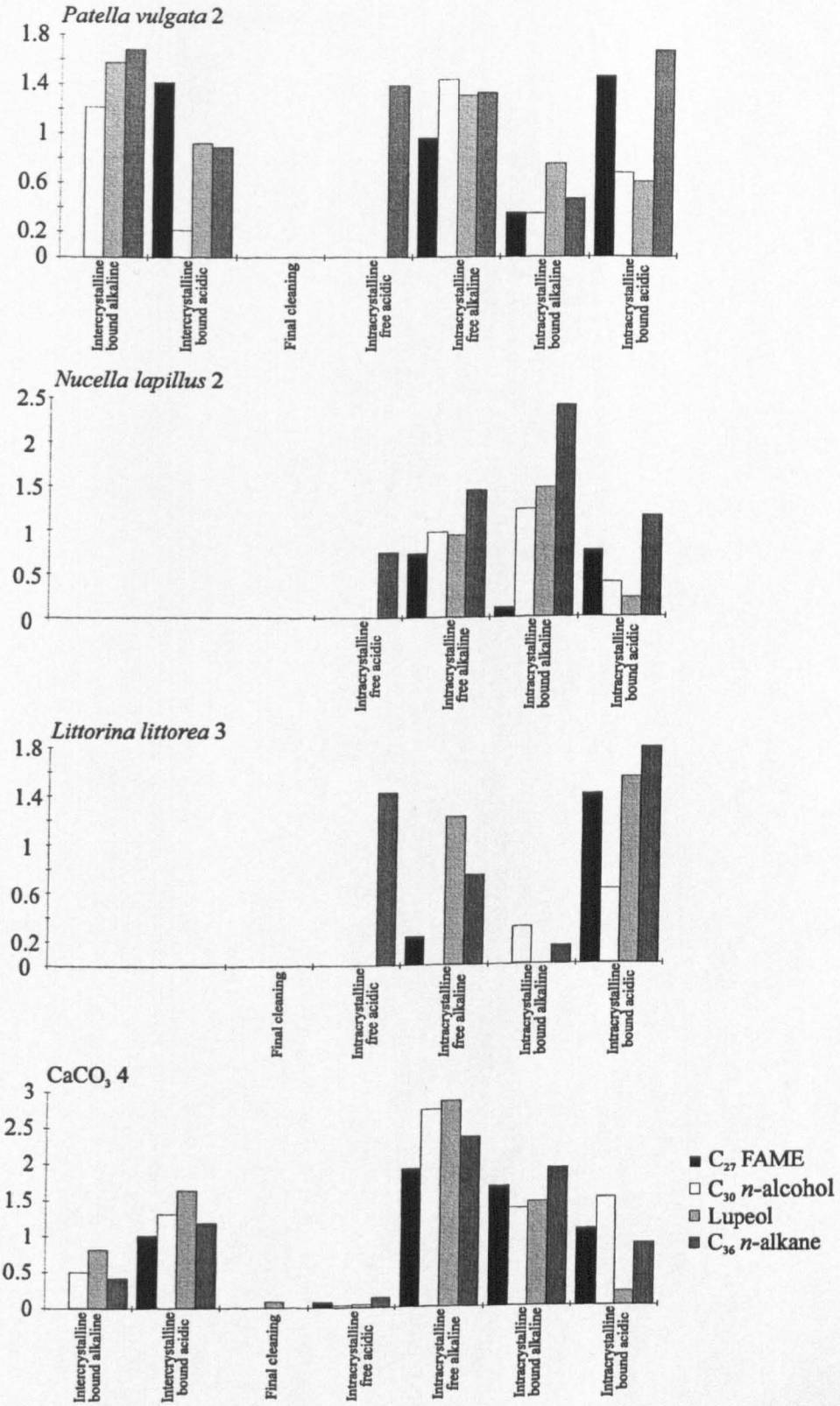


Figure 6. Ratio of C_{16} *n*-alkane internal standard to the surrogate standards adjusted for amounts previously removed for GC and GCMS analysis.

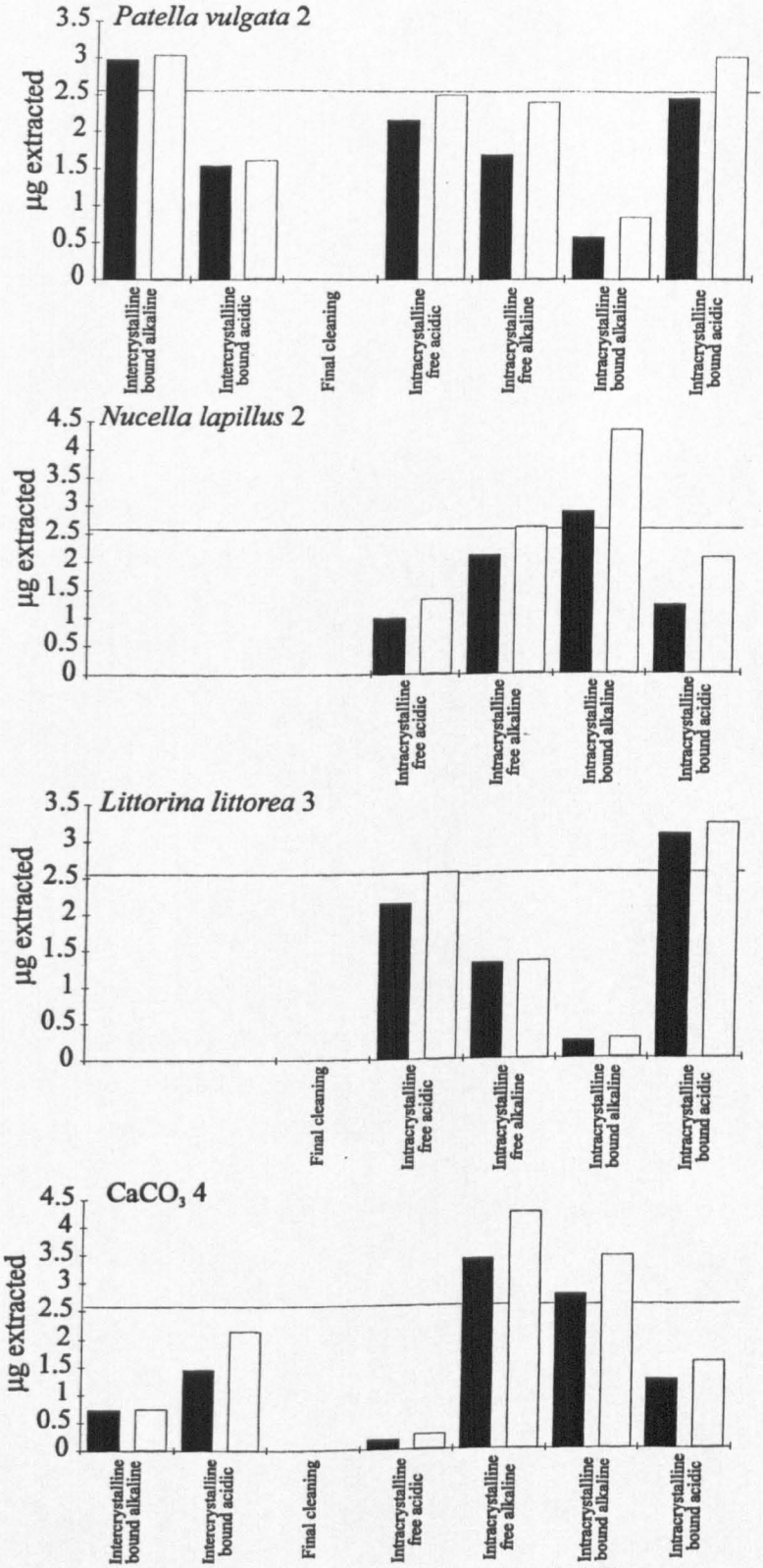


Figure 7. Yields (µg) of C_{36} *n*-alkane extracted from experimental batch 4. Black columns are yields directly measured, white columns show the data adjusted for removal of material for previous analysis by GC and GCMS. The line shows the amount (2.57 µg) of C_{36} *n*-alkane added to each sample (with the exception of the final cleaning extract).

The yields of the C_{36} *n*-alkane extracted from the intercrystalline bound alkaline fraction of *Patella vulgata* 2 are higher than the standard added (shown by the horizontal line, Fig. 7). This cannot be due to carry over as for this fraction this is the first addition of the internal standard. No similarities between the extracted fractions is observed. Experimental error may therefore account for extraction of excess standard, whilst low recoveries could also be explained by losses during experimental workup. The yields from the final cleaning extracts are zero, except for the *Patella vulgata* 2 sample where the C_{16} *n*-alkane coelutes which prohibits measurement of the yields. When adjusted for the loss of material used in previous analyses the yields are higher.

Using the above results the C_{36} *n*-alkane surrogate standard was chosen for use with quantification of the shell extracts. This surrogate standard was shown to be least effected by partitioning between the aqueous and solvent phases. It is also extracted from the majority of samples (missing only from the intercrystalline bound alkaline fraction of *Nucella lapillus* 2). The use of surrogate standards also accounts for material lost during workup and may be more reliable than the use of an internal standard which may introduce more errors, particularly with sample material already removed for analysis.

Implications for indigenous shell lipids

The surrogate standards; C_{27} FAME, C_{30} alcohol and lupeol have been shown to be inefficiently extracted by liquid-liquid extraction. This suggests that the indigenous FAMES, alcohols and sterols will behave in a similar way. They are however quantified in each extract by comparison with the C_{36} *n*-alkane surrogate standard. The indigenous *n*-alkanes have been shown to be unaffected.

The liquid-liquid extraction of fractions under both acidic and alkaline conditions was carried out to ensure the extraction of the polar lipid components, therefore the "double" extraction after the saponification, full decalcification and further saponification methods, should ensure that the indigenous lipids are fully extracted in either the acidic or alkaline fraction.

The intercrystalline and intracrystalline fractions have been shown to be distinct as the final cleaning extract contained few surrogate standards at low yields.

APPENDIX 3

Relative response factors

The flame ionisation detector (FID) response was measured as the gas chromatographic peak area (μV.s) for the surrogate standard (C_{36} *n*-alkane) and for the component to be quantified (Y).

Using the equation below, the amount of component Y in μg per gram of extracted shell was calculated. The calculation of relative response factors (RFF) is shown below.

$$Y\ (\mu g/g) = \frac{\{\text{Peak area of Y } (\mu V.s)\} \times \{\text{Amount of Standard added } (\mu g)\}}{\{\text{Peak area of Standard } (\mu V.s)\} \times \{\text{Amount of shell extracted } (g)\}} \times (RRF)$$

e.g. for the C_{22} *n*-alkane from the intracrystalline bound acidic extract of $CaCO_3$ 4 (see Appendix 18);

$$9.9 \times 10^{-5}\ (\mu g/g) = \frac{4054(\mu V.s) \times 2.57(\mu g)}{904690(\mu V.s) \times 88.5(g)} \times 0.762$$

Calculation of relative response factors

Relative response factors account for different FID responses to the different compound classes. RRF were calculated by measuring the peak areas of standard solutions of known weights using the equation below. The average of a number of analyses was used. The C_{19} *n*-alkane and C_{18} FAME standards were chosen to represent the similar homologues extracted from the shells.

$$RRF = \frac{\{\text{Peak area of Standard}\} \times \{\text{Weight of Y}\}}{\{\text{Peak area of Y}\} \times \{\text{Weight of Standard}\}}$$

$$\text{e.g. RRF } C_{36}\ n\text{-alkane to } C_{19}\ n\text{-alkane} = \frac{(289582 \times 23.487)}{(366085 \times 23.548)} = 0.789$$

	C_{19} *n*-alkane	C_{36} *n*-alkane	RRF C_{36}/C_{19}
Solution A mg/100ml	23.487	23.548	
Peak area (uV.s)	366085	289582	0.789
Peak area (uV.s)	289208	228722	0.789
Peak area (uV.s)	412393	356144	0.861
Solution B mg/100ml	20.103	21.53	
Peak area (uV.s)	154874	138841	0.837
Peak area (uV.s)	98618	74404	0.704
Peak area (uV.s)	100832	73413	0.680
Peak area (uV.s)	93795	67342	0.670
Average			**0.762**
Standard deviation			0.077
Coefficient of variation (%)			10.088

Table 1. Calculation of relative response factors for C_{36} *n*-alkane to C_{19} *n*-alkane. Two solutions (A and B) were analysed.

	C_{18} FAME	Cholesterol	C_{36} *n*-alkane	RRF C_{36}/Cholesterol	RRF C_{36}/C_{18}FAME
mg/50ml	10.201	9.509	9.416		
Peak area (uV.s)	415881	292636	363839	1.256	0.948
Peak area (uV.s)	139647	93589	112632	1.215	0.874
Peak area (uV.s)	290360	204869	254845	1.256	0.951
Average				**1.242**	**0.924**
Standard deviation				0.023	0.044
Coefficient of variation (%)				1.885	4.722

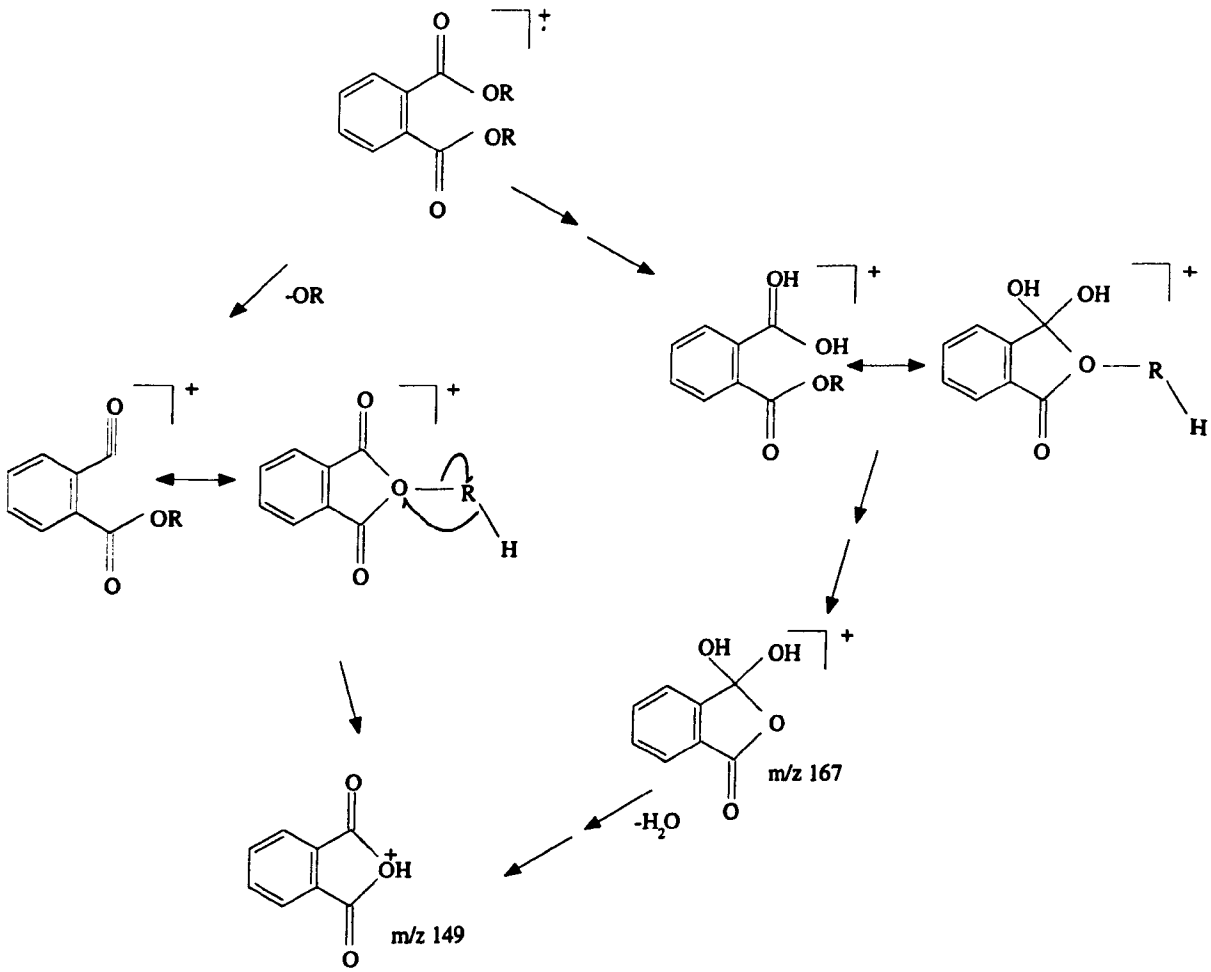
Table 2. Calculation of relative response factors for C_{36} *n*-alkane to cholesterol and C_{18} FAME.

	Lupeol	C_{19} *n*-alkane	Cholesterol	RRF Lupeol/C_{19}	RRF Lupeol/Cholesterol
mg/100ml	0.338	0.234	0.13		
Peak area (uV.s)	2053320	1234153	505612	1.152	1.562
Peak area (uV.s)	4690664	2910089	1099802	1.116	1.640
Peak area (uV.s)	1572252	964418	401601	1.129	1.506
Peak area (uV.s)	3894680	2599069	970665	1.037	1.543
Average				**1.108**	**1.563**
Standard deviation				0.050	0.057
Coefficient of variation (%)				4.478	3.631

Table 3. Calculation of relative response factors for lupeol to C_{19} *n*-alkane and cholesterol.

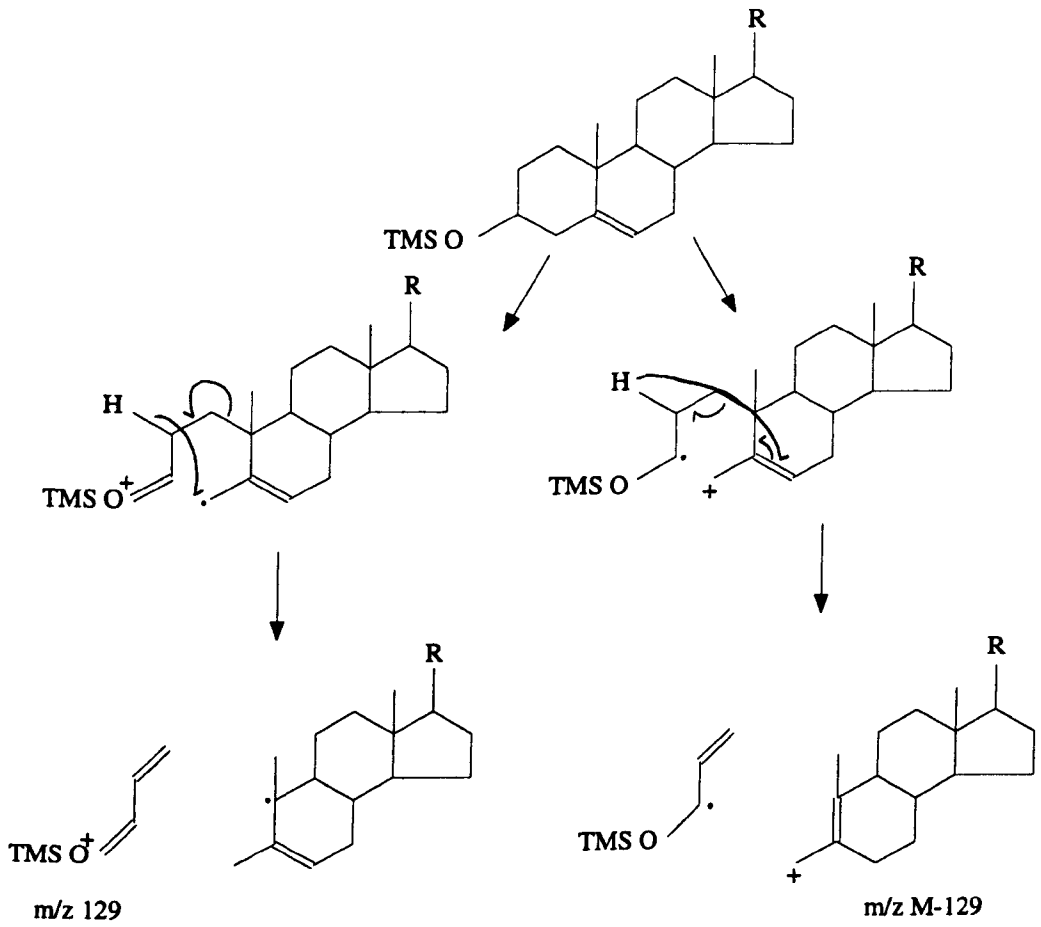
APPENDIX 4

Mechanism of fragmentation of dialkyl phthalate plasticisers (adapted from Safe and Hutzinger, 1973)



APPENDIX 5

Mechanism of fragmentation of sterols (from Brooks, 1979)



Species	Reference	Fraction	A	C	D	E	F	G	H	I	J	K	L	M	N	O
Artica islandica	Idler & Wiseman (1971)	Soft tissues	9.8	5.5	44.5	1.7	7.	4.2	0	0	0	0	3.3	2.4	21.6	0
Littorina littorea	Idler & Wiseman (1971)	Soft tissues	0.92	1.73	56.97	30.01	4.37	1.93	0	0	0	0	0	2.24	1.83	0
Littorina littorea	Voogt (1969)	Soft tissues	0.31	0	65.11	20.35	5.09	0	0	0	0	0	3.05	2.03	4.07	0
Littorina littorea 3		Surficial	0	0	14.81	0	0	0	11.04	0	8.09	36.67	0	0	0	29.38
Littorina littorea 3		Intercrystalline	0	14.93	69.65	13.53	0	0	0	0	0	0	0	2.05	0	0
Littorina littorea 3		Intercrystalline bound alkaline	0	0	54.51	20.54	0	0	4.92	13.61	0	0	3.51	2.63	0	0
Littorina littorea 3		Intracrystalline free acidic	0	0	77.61	22.38	0	0	0	0	0	0	0	0	0	0
Littorina littorea 3		Intracrystalline bound acidic	0	0	100	0	0	0	0	0	0	0	0	0	0	0
Littorina littorea 3		Intracrystalline bound alkaline	0	0	100	0	0	0	0	0	0	0	0	0	0	0
Ulva lactuca	De Napoli *et al.* (1982)		0	0	7.94	0	3.8	0	0	0	0	0	0	80.31	7.94	0
Cladophora laetevirens	De Napoli *et al.* (1982)		0	0	24.72	0	23.62	0	0	0	0	0	20.75	0	30.91	0
Chaetomorpha aurea	De Napoli *et al.* (1982)		0	0	30.03	0	10.23	0	0	0	0	0	27.01	0	32.72	0
Caulerpa prolifera	De Napoli *et al.* (1982)		0	0	9.32	0	0	0	0	0	0	0	85.51	0	5.18	0
Byropsis plumosa	De Napoli *et al.* (1982)		0	0	10.72	0	3.02	0	0	0	0	0	82.24	0	4.02	0
B. muscosa	De Napoli *et al.* (1982)		0	0	7.28	0	2.91	0	0	0	0	0	85.76	0	4.05	0
Udotea petiolata	De Napoli *et al.* (1982)		0	0	6.24	0	2.47	0	0	0	0	0	81.92	5.2	4.16	0
Codium vermilara	De Napoli *et al.* (1982)		0	0	0	0	0	0	0	0	0	0	100	0	0	0
Dasycladus vermicularis	De Napoli *et al.* (1982)		0	0	13.87	0	3.13	0	0	0	0	0	77.85	0	5.15	0
Acetabularia mediterranea	De Napoli *et al.* (1982)		0	0	14.13	0	2.17	0	0	0	0	0	76.09	0	7.61	0
Gymnodinium wilczeki	Nichols *et al.* (1984)		0	34.85	25.76	0	0	0	0	0	0	0	39.39	0	0	0
P. cordatum	Nichols *et al.* (1984)		0	0	9.98	0	5.94	0	0	0	0	0	0	0	84.09	0
"control"		100% cholesterol	0	0	100	0	0	0	0	0	0	0	0	0	0	0

Percentage distribution of steroidal components A to P, from shells extracted herein and from reported values in the literature

APPENDIX 7

Principal component eigenvalues and factors. Pairwise deletion of cases with missing values. SPSS for Windows release 5.0 (Jun. 20 1992) and release 6.0 (Jun. 17 1993). For figure 20. Chapter 2.

Factor	Eigenvalue	Pct of Var	Cum Pct
1	4.01679	28.7	28.7
2	2.34824	16.8	45.5
3	2.10660	15.0	60.5
4	1.42141	10.2	70.7
5	1.10018	7.9	78.5
6	1.06406	7.6	86.1
7	.87990	6.3	92.4
8	.58630	4.2	96.6
9	.44163	3.2	99.8
10	.03487	.2	100.0
11	.00000	.0	100.0
12	.00000	.0	100.0
13	.00000	.0	100.0
14	.00000	.0	100.0

Factor Matrix:

steroid	Factor1	Factor2	Factor 3	Factor4	Factor5	Factor6
A	-.13543	.46872	.73549	-.36353	.17878	.13125
C	-.08663	.10843	-.06694	-.52250	-.32686	-.06823
D	-.11957	.74543	-.32011	.06768	-.34058	-.27699
E	-.06405	.68936	-.31464	.18391	.19699	-.00567
H	-.22749	-.14220	.58852	.50768	.00821	-.16829
G	-.14719	.55169	.69230	-.33715	.18643	.12404
H	.95490	.12422	-.01636	.09216	.20333	.04918
I	.09331	.35534	-.35041	.29177	.64523	.19860
J	.98506	-.02465	.13842	-.03082	-.06865	-.03555
K	.98506	-.02465	.13842	-.03082	-.06865	-.03555
L	-.17937	-.75535	-.10188	-.41880	.43216	-.02172
M	-.05088	-.08403	.03797	.22319	-.33476	.90895
N	-.18117	-.20046	.58458	.53363	-.03361	-.22228
O	.98506	-.02465	.13842	-.03082	-.06865	-.03555

	Factor7	Factor8	Factor9	Factor10	Factor 11
A	-.06433	-.13276	.04031	.12764	.00002
C	.76273	.08477	.05294	-.00212	.00007
D	-.23749	-.21495	.16605	-.00958	.00028
E	.02195	.52720	-.26350	.03425	.00008
F	.10279	.32548	.42313	.00110	.00004
G	-.09165	.05465	-.04872	-.13087	.00001
H	.11831	-.05827	.05294	-.00401	.00004
I	.34499	-.24609	.14703	-.01235	.00002
J	-.02640	.04691	-.00855	.0011	.00004
K	-.02640	.04691	-.00855	.00118	.00004
L	-.11692	.10174	.01579	-.00099	.00032
M	.00259	.02812	.00702	-.00075	.00014
N	.26340	-.21616	-.36585	-.00224	.00016
O	-.02640	.04691	-.00855	.00118	.00004

APPENDIX 8

Data set of fatty acid compositions

Species	Reference	Tissue type / fraction	C10	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25	C26	C28
Gymnodinium wilczeki	Nichols et al. 1984	Outgroup	0	0	0	8	3	82	0	6	0	0	0	1	0	0	0	0	0
Human	Wertz & Downing 1991	Outgroup	0	0	0	0	0	9	1	11	1	7	2	7	8	33	6	10	3
Prorocentrum cordatum	Nichols et al. 1984	Outgroup	0	0	0	13	1	82	0	4	0	0	0	0	0	0	0	0	0
Arrica islandica	Ackman et al. 1974	Soft tissues	0	0	0	4	2	58	6	30	0	0	0	0	0	0	0	0	0
Arrica islandica	Ackman et al. 1974	Soft tissues	0	0	0	14	1	62	5	16	1	0	0	0	0	0	0	0	0
Arrica islandica	Ackman et al. 1974	Soft tissues	0	0	0	19	4	49	2	19	6	2	0	0	0	0	0	0	0
Arrica islandica	Ackman et al. 1974	Soft tissues	0	0	0	2	2	59	8	29	1	0	0	0	0	0	0	0	0
Arrica islandica	Ackman et al. 1974	Soft tissues	0	0	0	13	3	57	2	24	2	0	0	0	0	0	0	0	0
Arrica islandica	Ackman et al. 1974	Soft tissues	0	0	0	8	1	70	4	17	1	0	0	0	0	0	0	0	0
Crepidual fornicata	Gardner & Riley 1972	Soft tissues	0	0	0	7	0	57	5	30	0	0	0	0	0	0	0	0	0
Crepidual fornicata	Gardner & Riley 1972	Soft tissues	0	0	0	7	4	51	9	29	0	0	0	0	0	0	0	0	0
Crepidual fornicata	Gardner & Riley 1972	Soft tissues	0	0	0	10	3	52	6	28	0	0	0	0	0	0	0	0	0
Crepidual fornicata	Herbreteau et al. 1994	Soft tissues	38	0	0	26	0	26	0	9	0	0	0	0	0	0	0	0	0
Littorina littorea	Ackman et al. 1971	Soft tissues	0	0	0	7	1	19	1	27	1	34	0	10	0	0	0	0	0
Littorina littorea	Ackman et al. 1971	Soft tissues	0	0	0	10	1	45	3	41	0	0	0	0	0	0	0	0	0
Littorina littorea	Ackman et al. 1971	Soft tissues	0	0	0	3	0	16	1	26	0	41	0	11	0	0	0	0	0
Littorina littorea	Ackman et al. 1971	Soft tissues	0	1	0	17	2	62	2	15	1	0	0	0	0	0	0	0	0
Littorina littorea	Brockerhoff et al. 1967	Soft tissues	0	0	0	11	0	68	0	21	0	0	0	0	0	0	0	0	0
Littorina littorea	Brockerhoff et al. 1967	Soft tissues	0	0	0	33	0	63	0	4	0	0	0	0	0	0	0	0	0
Littorina littorea	Brockerhoff et al. 1967	Soft tissues	0	0	0	38	0	50	0	13	0	0	0	0	0	0	0	0	0
Littorina littorea	Paradis & Ackman 1976	Soft tissues	0	0	0	23	2	53	3	19	0	0	0	0	0	0	0	0	0
Patella vulgata	Gardner & Riley 1972	Soft tissues	0	0	0	12	2	68	2	17	0	0	0	0	0	0	0	0	0
Patella vulgata	Gardner & Riley 1972	Soft tissues	0	0	0	13	5	61	4	17	0	0	0	0	0	0	0	0	0
Modiolus modiolus	Gardner & Riley 1972	Soft tissues	0	0	0	11	6	66	5	11	0	0	0	0	0	0	0	0	0
Modiolus modiolus		Shell	0	0	2	14	8	57	3	15	0	0	0	0	0	0	0	0	0
Arrica islandica 1		Shell	0	0	0	0	0	71	0	0	0	0	0	3	0	8	13	5	0
Arrica islandica 2		Shell	0	0	0	8	6	48	3	35	0	0	0	0	0	0	0	0	0
Arrica islandica 2		Shell	0	0	0	25	18	48	0	5	0	4	0	0	0	0	0	0	0
Crepidula fornicata		Shell	0	0	0	0	0	74	11	15	0	0	0	0	0	0	0	0	0
Crepidula fornicata		Shell	0	7	2	15	7	52	2	16	0	0	0	0	0	0	0	0	0
Littorina littorea 1		Shell	0	0	0	0	0	81	0	19	0	0	0	0	0	0	0	0	0
Littorina littorea 1		Shell	0	0	0	0	0	74	6	21	0	0	0	0	0	0	0	0	0
Littorina littorea 1		Shell	0	1	0	1	0	17	4	74	1	0	0	3	0	0	0	0	0
Littorina littorea 1		Shell	0	1	0	3	0	27	4	60	4	0	0	0	0	0	0	0	0
Littorina littorea 2		Shell	0	8	1	9	2	24	6	49	0	2	0	0	0	0	0	0	0
Littorina littorea 2		Shell	0	1	0	3	2	26	6	62	0	0	0	0	0	0	0	0	0
Littorina littorea 3		Shell	0	2	1	5	2	31	5	53	0	0	0	0	0	0	0	0	0
Littorina littorea 3		Shell	0	0	0	0	0	87	0	13	0	0	0	0	0	0	0	0	0
Nucella lapillus 1		Shell	0	0	1	2	3	33	7	43	0	8	0	4	0	0	0	0	0
Nucella lapillus 1		Shell	0	0	0	1	2	25	6	50	0	15	0	0	0	0	0	0	0
Nucella lapillus 2		Shell	0	0	3	16	13	42	12	14	0	0	0	0	0	0	0	0	0
Nucella lapillus 2		Shell	0	1	1	2	5	45	9	38	0	0	0	0	0	0	0	0	0
Patella vulgata 1		Shell	0	0	1	7	4	77	0	10	0	0	0	0	0	0	0	0	0
Patella vulgata 1		Shell	0	0	0	6	0	74	3	16	0	0	0	1	0	0	0	0	0
Patella vulgata 2		Shell	0	0	0	11	4	85	0	0	0	0	0	0	0	0	0	0	0
Sepia officinalis		Shell	0	0	0	5	4	57	5	29	0	0	0	0	0	0	0	0	0
CaCO3 blank 1		Blank	0	0	0	0	0	0	41	0	59	0	0	0	0	0	0	0	0
CaCO3 blank 2		Blank	0	0	0	0	0	65	0	35	0	0	0	0	0	0	0	0	0
CaCO3 blank 2		Blank	0	0	0	0	23	42	0	35	0	0	0	0	0	0	0	0	0
CaCO3 blank 5		Blank	0	0	0	32	13	49	0	6	0	0	0	0	0	0	0	0	0

APPENDIX 9

Species	Reference	Tissue / fraction	Class	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	X
Ischnochiton sp.	Idler et al. 1977		Polyplacophora	0.	0.	0.	0.	26.67	0.	5.19	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	68.15
Pakcephorella velata	Idler et al. 1977		Polyplacophora	0.	0.	0.	0.	17.04	0.	1.12	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	81.84
Modiolus modiolus	Kahn & Goad 1983		Bivalvia	2.31	0.	0.	6.3	45.5	0.	24.94	0.	0.	0.	0.	0.	2.19	0.13	18.64	0.	0.	0.
Mytilus edulis	Jarzabski 1985		Bivalvia	5.63	0.	0.	0.	53.14	0.	15.24	0.	0.	0.	0.	0.	2.18	0.	17.93	0.	0.	5.89
Mya arenaria	Jarzabski 1985		Bivalvia	8.41	0.	0.	0.	49.53	0.	13.03	0.	0.	0.	0.	0.	3.44	0.	11.49	0.	0.	14.1
Cardium glaucum	Jarzabski 1985		Bivalvia	2.82	0.	0.	0.	53.7	0.	12.22	0.	0.	0.	0.	0.	4.	0.	17.27	0.	0.	9.99
Mytilus edulis	Kahn & Goad 1983		Bivalvia	4.43	0.	0.	10.27	48.19	18.09	10.62	0.	0.	0.	0.	0.	0.93	0.	7.47	0.	0.	0.
Macoma balthica	Jarzabski 1985		Bivalvia	3.23	0.	0.	0.	58.71	0.	11.88	0.	0.	0.	0.	0.	3.92	0.	7.04	0.	0.	15.22
Macoma balthica	Jarzabski et al. 1986	mantle & syphons	Bivalvia	6.21	0.	0.	6.21	46.8	0.	6.47	0.	0.	0.	0.	0.	3.99	0.	14.49	0.	0.	15.64
Pecten maximus	Kahn & Goad 1983		Bivalvia	4.1	0.	0.	9.	40.32	0.	26.34	0.	0.	0.	0.	0.	3.08	2.16	14.81	0.	0	0.
Macoma balthica	Jarzabski et al. 1986	residue	Bivalvia	6.48	0.	0.	5.92	47.71	0.	5.7	0.	0.	0.	0.	0.	3.14	0.	15.42	0.	0.	13.63
Cerastoderma edule	Kahn & Goad 1983		Bivalvia	2.2	0.	0.	4.73	33.96	11.65	14.29	0.	0.	0.	0.	0.	2.53	2.86	27.8	0	0.	0.
Mya arenaria	Kahn & Goad 1983		Bivalvia	2.2	0.	0.	3.41	47.25	7.36	16.7	0.	0.	0.	0.	0.	1.32	1.1	20.66	0.	0	0.
Chlamys opercularis	Kahn & Goad 1983		Bivalvia	4.6	0.	0.	9.85	39.83	0.	16.17	0.	0.	0.	0.	0.	1.93	0.75	26.87	0.	0.	0
Macoma balthica	Jarzabski et al. 1986	hepatopancreas	Bivalvia	6.37	0.	0.	5.2	45.65	0	5.84	0.	0.	0.	0.	0.	5.52	0.	14.12	0	0.	17.3
Gibulla divaricata	Stoilov et al. 1984		Gastropoda	0.	0.	0.	0.	100.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0	0.	0.	0.
Tritia reticulata	Stoilov et al. 1984		Gastropoda	0.	0.	0.	0.	100.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0	0.	0	0.
Macoma balthica	Jarzabski et al. 1986	muscle	Bivalvia	6.83	0.	0.	6.73	44.69	0.	6.1	0.	0.	0.	0.	0.	4.21	0	13.77	0.	0.	17.67
Nautilus sp.	Idler et al. 1977		Cephalopoda	0.	0.	0.	0.	94.12	0.	0.	0.	0.	0.	0.	0.	4.2	0	0.	0.	0	1.68
Macoma balthica	Jarzabski et al. 1986	whole	Bivalvia	5.64	0.	0.	5.54	47.65	0.	5.43	0.	0.	0.	0.	0.	4.91	0.	13.06	0.	0.	17.76
Cardium edule	Stoilov et al. 1984		Bivalvia	1.15	0.	0.	5.01	58.39	0.	18.77	0.	0.	0.	0.	0.	4.17	0.	12.51	0.	0.	0.
Macoma balthica	Jarzabski et al. 1986	gills	Bivalvia	5.15	0.	0.	7.63	51.75	0.	6.91	0.	0.	0.	0.	0	4.85	0.	4.85	0.	0.	18.87
Donax julianae	Stoilov et al. 1984		Bivalvia	1.13	0.	0.	9.26	42.18	0.	25.31	0.	0.	0.	0.	0.	3.6	0.	18.52	0.	0.	0.
Rapana thomasiana	Stoilov et al. 1984		Gastropoda	3.06	0.	0.	0.	82.65	0.	7.14	0.	0.	0.	0.	0.	0.	2.04	5.1	0.	0.	0.
Terebratulina retusa	Clegg 1993	December I	Brachiopoda	15.24	0.	0.	13.72	25.91	0.	19.82	0.	0.	0.	0.	0.	6.	2.64	12.8	0.	0.	3.86
Mytilus galloprovincialis	Stoilov et al. 1984		Bivalvia	2.85	0.	0.	13.21	51.83	0.	14.23	0.	0.	0.	0.	0.	3.66	0.	14.23	0.	0.	0.
Terebratulina retusa	Clegg 1993	April I	Brachiopoda	18.5	0.	0.	15.45	25.61	0.	21.04	0.	0.	0.	0.	0.	6.61	2.44	6.5	0.	0.	3.86
Terebratulina retusa	Clegg 1993	April II	Brachiopoda	17.77	0.	0.	15.23	24.77	0.	19.8	0.	0.	0.	0.	0.	7.61	2.23	7.31	0.	0.	5.28
Ensis siliqua	Kahn & Goad 1983		Bivalvia	3.34	0.	0.	8.92	33.23	0.	22.39	0.	0.	0.	0.	0.	0.91	1.01	30.19	0.	0.	0.
Archidoris montereyensis	Idler et al. 1977		Gastropoda	1.41	0.	0.	3.63	60.89	6.65	3.02	0.3	0.	0.	0.	0.	2.72	0.	21.37	0.	0.	0.
mussel	Pang et al. 1992	From hydrocarbon seep	Bivalvia	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	100.
Dentalium entale	Idler et al. 1977		Scaphopoda	0.5	0.	0.	2.21	89.77	0.	1.91	2.61	0.	0.	0.	0.	1.6	0.	1.4	0.	0.	0.
Tapes rugatus	Stoilov et al. 1984		Bivalvia	3.	0.	0.	8.	43.	0.	24.	0.	0.	0.	0.	0.	1.	0.	21.	0.	0.	0.
Venus gallina	Stoilov et al. 1984		Bivalvia	2.	0.	0.	0.	57.	0.	22.	0.	0.	0.	0.	0.	2.	0.	17.	0.	0.	0.
Mya arenaria	Stoilov et al. 1984		Bivalvia	4.	0.	0.	0.	49.	0.	20.	0.	0.	0.	0.	0.	2.	0.	25.	0.	0.	0.
mussel	Pang et al. 1992	Not seep samples	Bivalvia	0.	0.	0.	0.	64.57	0.	25.78	4.75	0.	0.	0.	0.	0.	0.	0.	0.	0.	4.91
Artica islandica	Idler & Wiseman 1971		Bivalvia	9.8	0.	0.	5.5	44.5	1.7	7.	4.2	0.	0.	0.	0.	3.3	2.4	21.6	0.	0.	0.
Ulva lactuca	De Napoli et al. 1982		Algae	0.	0.	0.	0.	7.94	0.	3.8	0.	0.	0.	0.	0.	0.	80.31	7.94	0.	0.	0.
Cladophora laetevirens	De Napoli et al. 1982		Algae	0.	0.	0.	0.	24.72	0.	23.62	0.	0.	0.	0.	0.	20.75	0.	30.91	0.	0.	0.
Chaetomorpha aurea	De Napoli et al. 1982		Algae	0.	0.	0.	0.	30.03	0.	10.23	0.	0.	0.	0.	0.	27.01	0.	32.72	0.	0.	0.
Caulerpa prolifera	De Napoli et al. 1982		Algae	0.	0.	0.	0.	9.32	0.	0.	0.	0.	0.	0.	0.	85.51	0.	5.18	0.	0.	0.
Byropsis plumosa	De Napoli et al. 1982		Algae	0.	0.	0.	0.	10.72	0.	3.02	0.	0.	0.	0.	0.	82.24	0.	4.02	0.	0.	0.
B. muscosa	De Napoli et al. 1982		Algae	0.	0.	0.	0.	7.28	0.	2.91	0.	0.	0.	0.	0.	85.76	0.	4.05	0.	0.	0.
Udotea petiolata	De Napoli et al. 1982		Algae	0.	0.	0.	0.	6.24	0.	2.47	0.	0.	0.	0.	0.	81.92	5.2	4.16	0.	0.	0.
Codium vermilara	De Napoli et al. 1982		Algae	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	24.23	0.	0.	0.	0.	75.77
Dasycladus vermicularis	De Napoli et al. 1982		Algae	0.	0.	0.	0.	13.87	0.	3.13	0.	0.	0.	0.	0.	77.85	0.	5.15	0.	0.	0.
Acetabularia mediterranea	De Napoli et al. 1982		Algae	0.	0.	0.	0.	14.13	0.	2.17	0.	0.	0.	0.	0.	76.09	0.	7.61	0.	0.	0.
Gymnodinium wilczeki	Nichols et al. 1984		Algae	0.	0.	0.	2.3	1.7	0.	0.	0.	0.	0.	0.	0.	2.6	0.	0.	0.	0.	93.4
Prorocentrum cordatum	Nichols et al. 1984		Algae	0.	0.	0.	0.	4.2	0.	2.5	0.	0.	0.	0.	0.	0.	0.	35.4	0.	0.	57.9
Littorina littorea	Idler & Wiseman 1971		Gastropoda	0.92	0.	0.	1.73	56.97	30.01	4.37	1.93	0.	0.	0.	0.	0.	2.24	1.83	0.	0.	0.
Littorina littorea	Vougt 1969		Gastropoda	0.31	0.	0.	0.	65.11	20.35	5.09	0.	0.	0.	0.	0.	3.05	2.03	4.07	0	0.	0.
Artica islandica 1		Intracrystalline bound alkaline	Bivalvia	5.56	0.	0.	8.69	55.43	0.	11.68	0.	0.	0.	0.	0.	8.81	0.	0.	0	0	9.82
Littorina littorea 1		Intercrystalline bound alkaline	Gastropoda	0.	0.	0.	0.	47.5	10.34	0.	0.	0.	8.92	0.	0.	2.99	0.	0.	0.	1.38	28.86
Littorina littorea 2		Intercrystalline bound alkaline	Gastropoda	0.	0.	0.	0.	55.51	15.14	0.	0.	10.55	9.52	0.	0.	0.	0.	1.57	0.	0.	7.7
Littorina littorea 2		Intracrystalline bound alkaline	Gastropoda	0.	0.	0.	0.	92.96	5.36	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	1.68
Littorina littorea 2		Intracrystalline bound acidic	Gastropoda	0.	0.	0.	0.	72.43	0.	0.	0.	4.09	0.	10.39	0.	0.	0.	0.	0.	0.	13.09
Littorina littorea 3		Surficial	Gastropoda	0.	0.	0.	0.	14.81	0.	0.	0.	11.04	0.	8.09	36.67	0.	0.	0.	29.38	0.	0.
Littorina littorea 3		Intercrystalline	Gastropoda	0.	0.	0.	9.95	46.44	9.02	0.	0.	0.	0.	0.	0.	0.	1.37	0.	0.	0.	33.22
Littorina littorea 3		Intracrystalline bound alkaline	Gastropoda	0.	0.	0.	0.	49.77	18.75	0.	0.	4.49	12.43	0.	0.	3.2	2.4	0.	0.	0.	8.95
Littorina littorea 3		Intracrystalline free acidic	Gastropoda	0.	0.	0.	0.	73.53	21.21	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	5.26
Littorina littorea 3		Intracrystalline bound alkaline	Gastropoda	0.	0.	0.	0.	80.57	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	19.43
Littorina littorea 3		Intracrystalline bound acidic	Gastropoda	0.	0.	0.	0.	88.81	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	11.19
Nucella lapillus 1		Intercrystalline bound alkaline	Gastropoda	0.	0.	0.	0.	57.91	0.	0.	4.53	2.15	8.56	0.	0.	0.	0.	0.	0.	0.	26.84
Nucella lapillus 1		Intracrystalline bound alkaline	Gastropoda	2.13	0.38	4.5	0.	73.24	1.07	0.	6.29	1.61	0.	4.89	0.	1.49	0.	0.	0.	0.	4.4
Nucella lapillus 1		Intracrystalline bound acidic	Gastropoda	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	100.	0.	0.	0.	0.	0.	0.	0.
Nucella lapillus 2		Intercrystalline	Gastropoda	0.	0.	0.	0.	91.18	0.	0.	0.94	0.	0.	0.	0.	0.	0.	0.	0.	0.	7.89
Nucella lapillus 2		Intercrystalline bound alkaline	Gastropoda	0.	0.	0.	0.	94.89	0.	0.	0.	1.82	0.01	2.67	0.	0.	0.	0.	0.	0.	0.61
Patella vulgata 1		Intracrystalline free acidic	Gastropoda	0.	0.	0.	0.	96.14	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	3.86
Sepia officinalis		Intracrystalline bound acidic	Cephalopoda	0.	0.	0.	0.	0.	0.	0.	0.	0.	0.	100.	0.	0.	0.	0.	0.	0.	0.

APPENDIX 10

Principal component eigenvalues and factors. Pairwise deletion of cases with missing values. SPSS for Windows release 5.0 (Jun. 20 1992) and release 6.0 (Jun. 17 1993). For figure 7. Chapter 3.

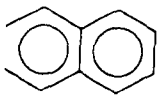
Factor	Eigenvalue	Pct of Var	Cum Pct
1	3.02055	21.6	21.6
2	1.98084	14.1	35.7
3	1.41048	10.1	45.8
4	1.21428	8.7	54.5
5	1.09570	7.8	62.3
6	1.02345	7.3	69.6
7	.99052	7.1	76.7
8	.95703	6.8	83.5
9	.86458	6.2	89.7
10	.72809	5.2	94.9
11	.32112	2.3	97.2
12	.23860	1.7	98.9
13	.15475	1.1	100.0
14	.00000	.0	100.0

Factor Matrix:

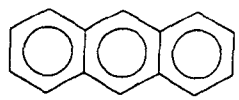
steroid	Factor1	Factor2	Factor 3	Factor4	Factor5	Factor6
A	.68439	.31992	.24227	-.19922	.14316	-.07405
C	.71357	.35195	.26680	-.17921	.14062	-.07488
D	-.20548	.57962	-.69872	-.08014	-.13089	-.02515
E	-.38639	.46783	.13364	.18243	.04997	.02263
F	.78395	.29918	.08698	.13185	-.05005	.00357
G	-.12645	.28294	-.28364	-.05307	-.04243	.10395
H	-.51422	.44233	.28235	.24693	.18959	-.11619
I	-.59824	.49240	.49420	.10716	-.00909	-.03308
J	-.12595	-.31797	.02215	-.00025	.84176	-.22520
L	-.03105	-.56325	.18737	.54747	-.34860	-.29800
M	.00660	-.13508	.10147	.26468	.18725	.91024
N	.65587	.08952	.16073	.26648	-.16720	.05642
P	-.29381	.17077	.44166	-.17861	-.26509	.04666
X	-.25539	-.34400	.30948	-.73309	-.17914	.11127

	Factor7	Factor8	Factor9	Factor 10	Factor 11	Factor 12	Factor 13
A	.09934	.00764	.39212	-.09040	.25216	-.26340	-.00865
C	.02185	-.09498	.30884	.05906	-.09496	.34711	.01993
D	-.19881	-.12931	.05194	-.22071	.03080	.02299	-.01664
E	-.19121	-.20893	.06777	.69809	.00228	-.07228	.01778
F	.01091	.14201	-.25473	-.01449	-.39099	-.17351	-.00911
G	.45667	.73817	.06428	.2075	.03974	.04381	.06287
H	.40104	-.24710	-.11472	-.25117	-.02031	-.01148	.22604
I	.10550	.15364	-.05646	-.16640	.00257	.03679	-.27530
J	-.20462	.25143	-.13413	.00785	.00970	.00487	-.00190
L	.10171	.09119	.34273	.00750	-.01679	.00856	.00965
M	-.00596	-.02925	.16736	-.09323	-.00969	.00626	.00467
N	-.02303	.02384	-.57361	.06150	.30164	.09371	.01288
P	-.62659	.38376	.03851	-.14614	.00330	-.00136	.14954
X	.27939	-.13277	-.16971	.11132	-.03498	-.02360	.01057

APPENDIX 11



Naphthalene



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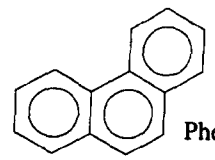
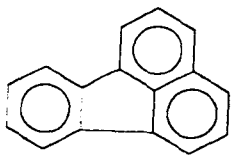
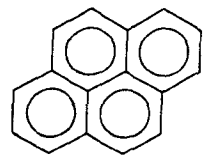


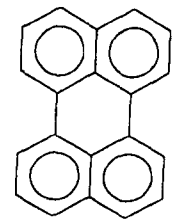
Phenanthrene



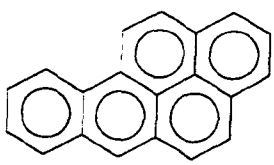
Fluoranthene



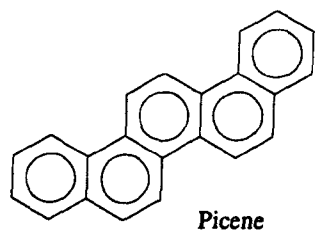
Pyrene



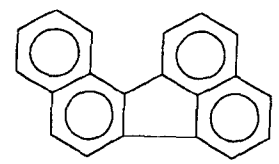
Perylene



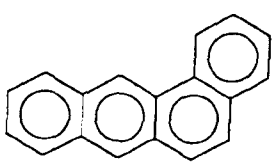
Benzo(a)pyrene



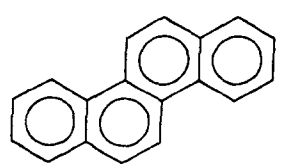
Picene



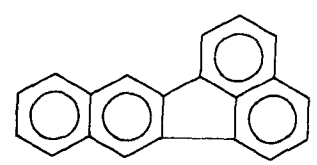
Benzo(j)fluoranthene



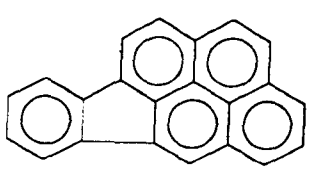
Benzo(a)anthracene



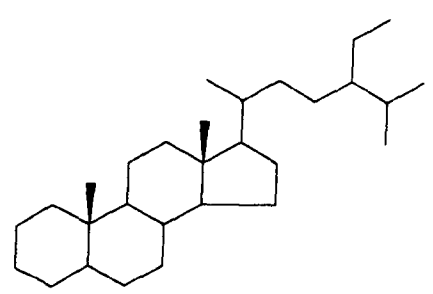
Chrysene



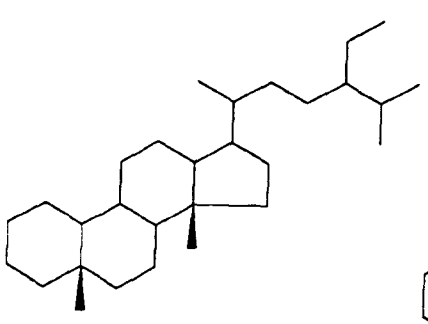
Benzo(k)fluoranthene



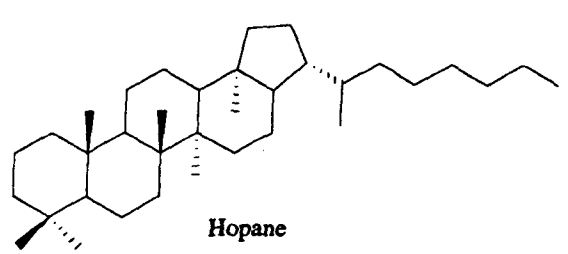
Indeno(1,2,3-cd)pyrene-c



Sterane



Diasterane



Hopane

APPENDIX 12

Stratigraphic order of Quaternary raised beach deposits near Wanganui, North Island of New Zealand.

SERIES	STAGE	GROUP	FORMATION	MEMBER	Age of stage boundarys, Williams et al. (1988) Age (Ma)	Beu & Edwards (1984) Stage	Pillans (1983) Age (Ma)	Pillans (1994) Stage	Pillans (1994) Age (Ma)	Walter (1992) Age (Ma)	Sampling location	New Zealand Topographical Map Edition 1, 1985 Co-ordinates
	Oturian		Rapanui							0.20		
				Hauriri Terrace			0.06	5	0.1		Waipipi beach	Q22/482520
			Inaha				0.08	5	0.1			
Hawera			Rapanui				0.12	5	0.1			
			Ngarino	Ngarino Terrace			0.19	7			Wilson's bluff, Kaitoke	R22/862317
			Brunswick	Brunswick Terrace			0.2	9	0.3			
	Terangian			Brunswick Terrace				9	0.3			
			Kaiatea III									
			Kaiatea II									
		Porukai	Kaiatea I									
			Landguard Formation	Landguard Fm.(top)		9?		9	0.3		Landguard, Wanganui river	R22/831368
				Landguard Sand		9?		9	0.3	0.35	Landguard, Wanganui river	R22/831368
				Landguard Fm.(base)	9/10 0.359	9?		9	0.3		Landguard, Wanganui river	R22/831368
			Putiki Shell bed			11 up.		11	0.4			
				Rangitawa Shellbed		11 up.	0.4	11	0.4			
				Windsor Shellbed		11 up.						
				Unnamed siltstone	0.37 / 0.39	11 up.						
			Mowhanau Sand	Basal conglomerate		11 up.						
			Kamba Siltstone	Tawera lenticles		11 up.		13	0.5			
			Upper Castlecliff Shell Bed			11 up.		13	0.5	0.45		
						11 up.				0.45		
			Shakespeare Cliff Sand			11 up.		13	0.5			
				Shakespeare Cliff Sand (base)		11 up.						
			Shakespeare Cliff Siltstone			11 lr.		15	0.6		Castlecliff beach, above Tainui	R22/771413
			Tainui Shell Bed			11 lr.		15	0.6	0.45	Castlecliff beach	R22/771413
						11 lr.						
			Pinnacle Sand			11 lr.		15	0.6	0.45		
	Castlecliffian		Lower Castle Cliff Shellbed			11 lr.		15	0.6		Castlecliff beach	R22/756426
						11 lr.						
		Shakespeare	Seafield Sand	Toms conglomerate	11/12 0.430	11 lr.		15	0.6			
			Upper Kai-iwi Siltstone			13		17	0.7		Castlecliff beach	R22/752431
			Kupe Formation			13		17	0.7	0.50	valley	S22/028365
						13		17	0.7	0.50	Rockfall, Kai-iwi beach	R22/738440
				Pecten layer		13		17	0.7	0.50	Kaimangarara road, Whangaehu river valley	S22/028366
				Pelecypod shellbed		13		17	0.7	0.50		
				Mactra tristis layer		13		17	0.7	0.50		
				Gastropod shellbed member		13		17	0.7	0.50		
				Cross-bedded sand member	13/14 0.521	13		17	0.7	0.50		
			Upper Westmere Siltstone	Kaikokopu Shell Grit	15/16 0.589	15		19	0.8			
			Lower Westmere Siltstone	Basal Sand Member		17		21				
			Omapu Shellbed			17		21				
			Lower Kai-iwi Siltstone		17/18 0.658	17		21				
		Kai-iwi	Kaimatira Pumice Sand	(local member)		19-20??		25/27		0.73	Rockfall, Okehu stream mouth	R22/707458
			Upper Okehu Siltstone	Basal Shell Conglomerate		21		29				
	Okehuan		Okehu Shell Grit		21/22 0.786	21				1.00	Okehu stream mouth	R22/707458
			Lower Okehu Siltstone	Basal shell Conglomerate	23/24 0.831	23						
			Butlers Shell Conglomerate	Ototoka Siltstone Tongue	25/26 0.877	25						
		Okehu	Makirikiri Tuff	Conglomerate Members								
			Upper Maxwell formation	Lignite Members		Mn?						
Wanganui			Mangahou Siltstone			Mh?						
			Middle Maxwell formation	Lignite Members		Mh?						
			Pukekiwi Shell Sand			Mc?						
			Lower Maxwell formation	Lignite Members		Mc?						
		Maxwell	Tewkesbury formation	Shell Sand & Cong. Lenticles		Md				1.67	Turakina valley road	S22/157437
	Nukumaruan		Waipuru Shellbed			Md				1.78	Turakina valley road, Kaikawarra farm land	S22/166466
			Undifferentiated shell bed	Upper Nukumaru Group		Md				1.80		
			Nukumaru Brown Sand			Md				1.85	Nukumaru beach, Nr. stream	R22/628482
			Mangamako Shellbed									
			Nukumaru Formation	Nukumaru Limestone		Mf?					Fishing rocks, Nukumaru	R22/621479
		Nukumaru	Ohingaiti Sand			Mn?						
				Upper Okiwa Group?		Mf				2.15		
			Undifferentiated formations			Mf?						
	Hautawan		Kuranui Limestone									
			Hautawa Shellbed			Mf				2.20		
		Okiwa	Te Rama Shellbed							2.30		
			Parihauhau Shellbed									
			Undifferentiated formations									
			Te Rimu Sand									
	Waitotaran	Lower Okiwa	Te Rimu Sand									
			Wilkies Shell Bed									
			Makokako Sand									
			Mangaweka Mudstone	Mangaweka Cong. Member								
		Paparangi	Paparangi Sandstone	Mangamanui Shell conglomerate							Waitotara valley road, Nr. Puao	R21/662604
			Waverley Formation									
			Waipipi Formation	Upper Waipipi Shellbed						2.50	Waipipi beach, Waverley	Q22/484519
				Middle Waipipi Shellbed						2.60	Mouth of Wairoa stream, Waipipi beach, Waverley	Q22/478521
				Snapper Point Shellbed								
				Snapper Point Conglomerate								
			Rangikura Sandstone	Bremer Shell Grit								
				Tuha Shell Sand								
				Basal Sand Member								
	Waipipian	Whenuakura	Pepper Shell Sand									

APPENDIX 13

Data set of reported soft tissue fatty acid compositions

Tissue	Hepatopancreas	Hepatopancreas	Hepatopancreas	Muscle	Muscle	Muscle	Shell	Shell	Shell	Shell	Shell
Lipid fraction	Phospholipid	Steryl esters	Triglyceride	Phospholipid	Steryl esters	Triglyceride	Intercrystalline bound acidic	Intracrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound acidic	Intracrystalline bound alkaline
Species	*Artica islandica*	*Artica islandica*	*Artica islandica*	*Artica islandica*	*Artica islandica*	*Artica islandica*	*Artica islandica 1*	*Artica islandica 1*	*Artica islandica 2*	*Artica islandica 2*	*Artica islandica 2*
Reference	1	1	1	1	1	1					
Total % (sic.)	100	100	101	100	100	101	100	100	100	100	100
Total saturated %	39	42	32	22	33	48	100	100	100	100	100
Total unsaturated %	54	48	65	76	58	50	0	0	0	0	0
Iso., Branched & others %	7	10	4	2	9	3					
Cmax saturated	16	16	16	16	16	16	16	18	16	16	18
Cmax unsaturated	20	18	20	20	20	20	-	-	-	-	-
10 saturated											
11 saturated											
12 saturated						1					
13 saturated											
14 saturated	1	8	5	0	4	4	8		25		
15 saturated	1	2	0	0	1	1	6		18		
16 saturated	22	20	20	13	19	32	48		48	74	
17 saturated	2	1	2	2	1	2	3	0		11	
18 saturated	12	8	5	6	8	8	35	100	5	15	100
19 saturated		2	0	0	1	0					
20 saturated	0	1	0			1			4		
21 saturated											
22 saturated											
23 saturated											
24 saturated											
14 unsaturated	0	2	0			0					
15 unsaturated	0	1	0		0						
16 unsaturated	1	9	15	2	5	13					
17 unsaturated			0	0							
18 unsaturated	13	17	20	9	15	16					
19 unsaturated		0	0	0		0					
20 unsaturated	30	15	28	37	36	17					
21 unsaturated	0	0	0	1		0					
22 unsaturated	10	3	0	27	2	3					
23 unsaturated											
24 unsaturated											

Tissue	Whole animal	Whole animal	Whole animal	Whole animal	Shell	Shell
Lipid fraction	Phospholipid	Steryl esters	Triglycerides	Total lipids	Intercrystalline bound acidic	Intracrystalline bound acidic
Species	*Crepidula fornicata*	*Crepidula fornicata*	*Crepidula fornicata*	*Crepidula fornicata*	*Crepidula fornicata*	*Crepidula fornicata*
Reference	2	2	2	3		
Total % (sic.)	107	99	111	106	100	100
Total saturated %	27	25	23	51	100	100
Total unsaturated %	72	69	79	56	0	0
Iso., Branched & others %	9	4	9			
Cmax saturated	16	16	16	10	16	16
Cmax unsaturated	16	20	20	20	-	-
10 saturated				19		
11 saturated						
12 saturated	1	3	1	0	7	
13 saturated	1	1	0		2	
14 saturated	2	1	2	13	15	
15 saturated	1		1		7	
16 saturated	12	11	11	13	52	81
17 saturated	1	1	2		2	
18 saturated	6	6	6	5	16	19
19 saturated			0			
20 saturated						
21 saturated		3				
22 saturated	1					
23 saturated						
24 saturated	1					
14 unsaturated						
15 unsaturated	0					
16 unsaturated	23	17	21	4		
17 unsaturated						
18 unsaturated	18	18	17	14		
19 unsaturated			2			
20 unsaturated	21	22	24	30		
21 unsaturated						
22 unsaturated	9	13	16	8		
23 unsaturated						
24 unsaturated						

Tissue	Whole animal	Whole animal	Whole animal	Whole animal	Whole animal	Whole animal	Whole animal	Whole animal	Shell	Shell	Shell	Shell	Shell	Shell	Shell
Lipid fraction	December total hydrogenated	May total hydrogenated	May total lipids	May Triglyceride	May Polar lipids	Triglyceride 1	Triglyceride 2	Triglyceride 3	Intercrystalline bound acidic	Intracrystalline bound alkaline	Intracrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound alkaline
Species	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea*	*Littorina littorea 1*	*Littorina littorea 1*	*Littorina littorea 1*	*Littorina littorea 2*	*Littorina littorea 2*	*Littorina littorea 3*	*Littorina littorea 3*
Reference	4	4	4	4	4	5	5	5							
Total % (sic.)	100	98	99	99	100	95	94	88	100	100	100	100	100	100	100
Total saturated %	95	94	20	20	22	19	27	8	100	100	100	100	100	100	100
Total unsaturated %	0	0	79	79	79	76	67	80	0	0	0	0	0	0	0
Iso., Branched & others %	5	4		0											
Cmax saturated	20	20	16	16	16	16	16	16	18	16	18	18	18	18	16
Cmax unsaturated	-	-	20	20	20	18	20	18	-	-	-	-	-	-	-
10 saturated															
11 saturated															
12 saturated	0		0	0	0				0		1	8	1	2	
13 saturated	0		0		0				0			1	0	1	
14 saturated	7	3	5	5	4	2	9	3	1		3	9	3	5	
15 saturated	1	0	0	0	0				0			2	2	2	0
16 saturated	18	15	10	10	9	13	17	4	17	74	27	24	26	31	87
17 saturated	1	1	0	0	0				4	6	4	6	6	5	0
18 saturated	26	25	4	4	7	4	1	1	74	21	60	49	62	53	13
19 saturated	1	0							1		4	0	0		
20 saturated	33	39										2	0		
21 saturated		0							0						
22 saturated	10	10							3						
23 saturated									0						
24 saturated									0						
14 unsaturated			0	1	0										
15 unsaturated															
16 unsaturated			9	11	6	6	9	10							
17 unsaturated			0	0	0										
18 unsaturated			23	29	13	32	17	26							
19 unsaturated															
20 unsaturated			39	33	48	24	29	24							
21 unsaturated			0	0											
22 unsaturated			7	4	11	14	12	20							
23 unsaturated															
24 unsaturated			0	0	0										

Tissue	Whole animal	Whole animal	Whole animal	Shell	Shell	Shell	Shell
Lipid fraction	Phospholipids	Steryl esters	Triglyceride	Intercrystalline bound acidic	Intracrystalline bound acidic	Intercrystalline bound acidic	Intracrystalline bound alkaline
Species	*Patella vulgata*	*Patella vulgata*	*Patella vulgata*	*Patella vulgata 1*	*Patella vulgata 1*	*Patella vulgata 2*	*Patella vulgata 2*
Reference	2	2	2				
Total % (sic.)	95	91	92	100	100	100	100
Total saturated %	20	17	17	100	100	100	100
Total unsaturated %	75	74	75	0	0	0	0
Iso., Branched & others %							
Cmax saturated	16	16	16	16	16	16	18
Cmax unsaturated	20	18	18	-	-	-	-
10 saturated							
11 saturated							
12 saturated					0		
13 saturated				1	0		
14 saturated	2	2	2	7	6	11	
15 saturated	0	1	1	4		4	
16 saturated	14	12	11	77	74	85	
17 saturated	0	1	1		3		
18 saturated	3	2	3	10	16		100
19 saturated							
20 saturated							
21 saturated							
22 saturated					1		
23 saturated							
24 saturated							
14 unsaturated							
15 unsaturated							
16 unsaturated	4	12	8				
17 unsaturated							
18 unsaturated	14	26	35				
19 unsaturated							
20 unsaturated	52	21	25				
21 unsaturated							
22 unsaturated	4	15	7				
23 unsaturated							
24 unsaturated							

APPENDIX 14

Examination of freeze drying methodology

A standard mixture of C_{11} *n*-alcohol, C_{19} *n*-alkane, C_{16} fatty acid, C_{27} FAME, cholesterol and lupeol in DCM was added to 50 g of inorganic $CaCO_3$ and a control containing no carbonate. The solvent was left to evaporate. The carbonate was decalcified, with the same volume of acid added to the blank control. The resulting acidic solutions were neutralised by the addition of KOH, frozen at -70°C and freeze dried (Edwards Model EF2 freeze drier). Two sessions of 40 and 48 h. were required. The solids were broken up with difficulty, and solvent extracted with 100 ml methanol, then two aliquots of 100 ml DCM with 5 min sonic extraction combining.

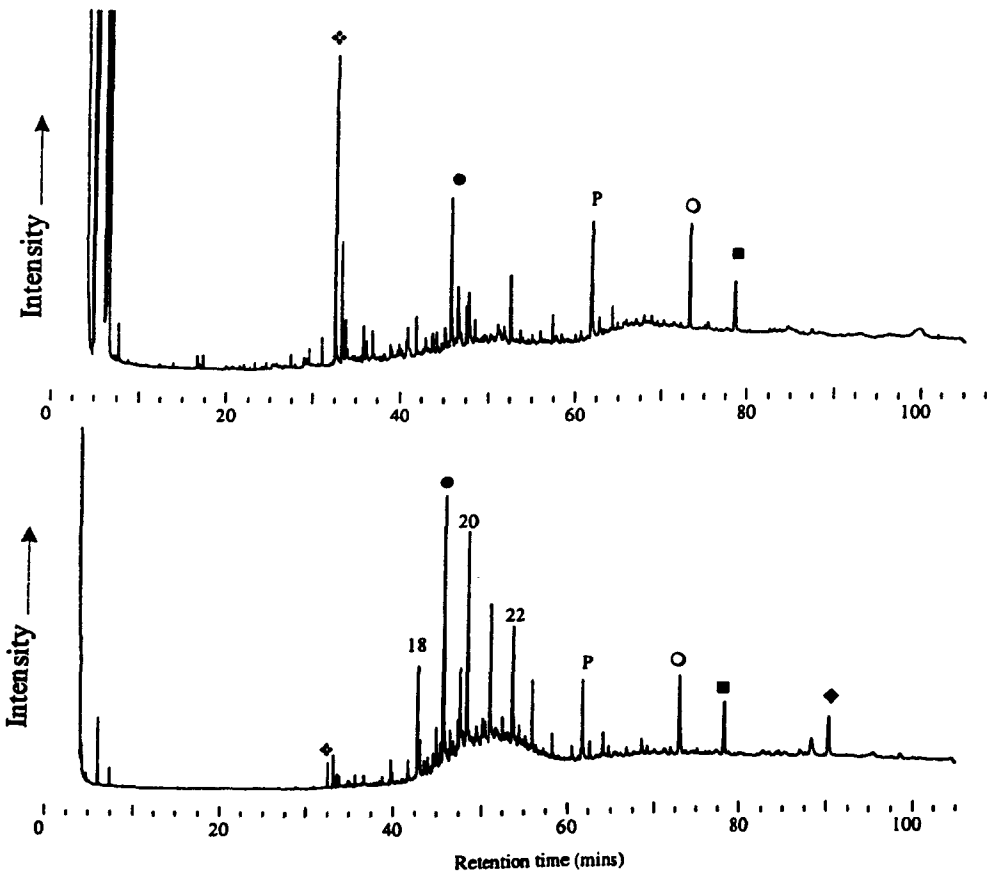


Figure 1. Gas chromatogram of extracts from freeze dried samples. Upper trace is neutralised decalcified $CaCO_3$. Lower trace, blank control. Standards and peaks labelled as: ❖ = C_{11} *n*-alcohol, ● = C_{19} *n*-alkane, O = C_{27} FAME, ■ = cholesterol, ◆ = lupeol, P = phthalate plasticiser, numbers indicate *n*-alkane chain length.

After freeze drying no C_{16} fatty acid is extracted. The yields are also variable for lupeol and the C_{11} *n*-alcohol. The variation in extracted yield of the low molecular weight alcohol is probably due to loss by volatilisation in the freeze drying chamber at low pressures. The difficulty of breaking up the solid produced by the freeze drying process can hinder solvent extraction, and the bonding between polar standards and the calcium chloride solid residue after freeze drying, may explain the variation in the yields of the other standards. The reformation of a solid after decalcification also presents the possibility of intracrystalline components being re-trapped and therefore not being accessible for solvent extraction.

The control contains a relatively large amount of *n*-alkane contaminants in a homologous series from nC_{18} to nC_{31}. The levels of contamination are different in the two samples although they were treated identically and freeze dried together. The most probable source for the contamination is the lubricating oil in the vacuum pump which becomes heated and the volatised components transferred into the drying chamber. These problems make this method of extraction impracticable.

APPENDIX 15

GC peak areas for experimental batch 1

Batch 1	*Patella vulgata* 1	*Modiolus modiolus*	*Littorina littorea* 1	CaCO3 blank 1	*Patella vulgata* 1	*Modiolus modiolus*	*Littorina littorea* 1	CaCO3 blank 1	*Patella vulgata* 1	*Modiolus modiolus*	*Littorina littorea* 1	CaCO3 blank 1	*Patella vulgata* 1	*Modiolus modiolus*	*Littorina littorea* 1	CaCO3 blank 1
Extract	1	1	1	1	2	2	2	2	3	3	3	3	4	4	4	4
Dilution (ul)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Injected (ul)	0.6	0.7	0.7	0.6	0.6	0.7	0.6	0.6	0.6	0.7	0.7	0.6	0.7	0.6	0.7	0.7
Material extracted (g)	74.7	73.2	71.0	42.0	69.2	67.6	65.4	39.8	69.2	67.6	65.4	39.8	69.2	67.6	65.4	39.8
C27 FAME																
C30 n-alcohol																
Lupeol																
C36 n-alkane																
Cholesterol		1956	1963	1824	9680		144826	2147	5240		34914336	143524				
Plasticiser A	31165	29836	12496	28214	*c*	9775		8500					41016			30602
Plasticiser B																
Plasticiser C	43093	54723	26410	59666	69568	70441		121024	8798	35541			66719	39264		
Plasticiser D					16107											
Plasticiser E	215288	176206	35594	99571	71736	40309		37043	*3779*	6533		*27680*	17358	9548		
Plasticiser F	55076	55999	10901	34691				7844								
Pristane	*c*	*c*	937		4036	tr	13664	544		514						
Phytane	6075	1278	1403		*c*	552	13333	*c*		1226			16918			
C17 n-alkane	*c*	*c*	6871	tr	17487	tr	57629	2016		1132						
C18 n-alkane	5725	1636	7856	tr	*c*	3441	65964	1079		2816			30514			
C19 n-alkane	5160	*3982*	*c*	1227	*c*	*c*	*c*	*c*	7782	*c*		540592	*c*			
C20 n-alkane	26555	2003	*c*	*c*	7193	2076	38545	1701	1823	2211		233465				
C21 n-alkane	5457	7361	8357	1804	17197	7119	35471	2523	*c*	*c*			*13015*			
C22 n-alkane	5426	2436	5059	987	6724	1772	44871	2146	4745	1731			*25664*			
C23 n-alkane	5463	2277	3849	2207	7659	2114	29790	2449	*7522*	1637						
C24 n-alkane	6830	2225	*3626*	5861	7940	*c*	*c*	*c*	9913	1992		45479		18982		
C25 n-alkane	8977	3286	3311	12609	*10354*	3199	21904	4003	14780	2436		12239		3722		
C26 n-alkane	9416	3918	2904	21200	*10602*	*10856*	14884	*c*	18339	3666		19951		28724		
C27 n-alkane	8489	5154	2728	25658	7664	3946	13765	5735	20010	4533		19807		6514		
C28 n-alkane	10659	5379	2374	27118	*c*	4237	10866	6390	21348	5597		23867		2771		
C29 n-alkane	6062	4946	3200	23487	10281	5426	14882	6787	21288	6326		25789		3231		
C30 n-alkane	*c*	3543	2689	17133	6813	3835	10681	5414	16679	5102		18920		tr		
C31 n-alkane		3670	3637	12683	*c*	4219	16875	5933	13730	4504		11733		tr		
C32 n-alkane		1788	1651	8543	2894	2220	*c*	2856	7600	2597						
C33 n-alkane		1644	894	4965			28396	2328	4869	1832						
C34 n-alkane				3033												
C35 n-alkane																
C12 n-alcohol																
C13 n-alcohol																
C14 n-alcohol																
C15 n-alcohol																
C16 n-alcohol																
C17 n-alcohol							tr									
C18 n-alcohol							tr									
C19 n-alcohol																
C20 n-alcohol																
C21 n-alcohol																
C22 n-alcohol												c				
C23 n-alcohol																
C24 n-alcohol																
C25 n-alcohol																
C26 n-alcohol																
C27 n-alcohol																
C28 n-alcohol																
C12 TMS acid																
C13 TMS acid																
C14 TMS acid																
C15TMS acid																
C16 TMS acid																
C17 TMS acid																
C18 TMS acid																
C12 FAME														*c*	*28729*	
C13 FAME													19144	8029	28905	
C14 FAME													125559	62482	144701	
C15 FAME													75403	36302	102963	
C16 FAME											1752000		1293777	256481	4519947	
C17 FAME											131610		*c*	15489	1062149	36920
C18 FAME											489128		166607	69019	19949584	
C19 FAME															156728	54009
C20 FAME														tr	c	
C21 FAME															tr	
C22 FAME														tr	932642	
C23 FAME															tr	
C24 FAME															tr	
C25 FAME															tr	
C26 FAME																

Patella vulgata 1	*Modiolus modiolus*	*Littorina littorea 1*	CaCO3 blank 1	*Patella vulgata 1*	*Modiolus modiolus*	*Littorina littorea 1*	CaCO3 blank 1	*Patella vulgata 1*	*Modiolus modiolus*	*Littorina littorea 1*	CaCO3 blank 1	*Patella vulgata 1*	*Modiolus modiolus*	*Littorina littorea 1*	CaCO3 blank 1	*Patella vulgata 1*	*Modiolus modiolus*	*Littorina littorea 1*	CaCO3 blank 1
5	5	5	5	6	6	6	6	7	7	7	7	8	8	8	8	9	9	9	9
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.6	0.6	0.6	0.7	0.6	0.7	0.7	0.6	0.6	0.7	0.7	0.7	0.7	0.5	0.7	0.6	0.6	0.6	0.6	0.6
69.2	67.6	65.4	39.8	68.5	67.0	65.1	39.0	68.5	67.0	65.1	39.0	68.5	67.0	65.1	39.0	68.5	67.0	65.1	39.0
1087		8350	1091	207900	24728	1499984	2704			6172		21120	594	95617	10239				
1782	826		2670	78630	18963		13179		17337	16548	1725			12230				62559	
10841	29272	25122	39050	144195	45265	88520	20633	6467	33675	50174	14639	*10980*	5331	9376	9387	6869	17403	128165	14187
				95776											2880	1906			
7256	13347	5277	14981	144581	106461	*c*	24163	1625	54128	604107	21064	4560	1697	58336	*c*	11688	19705	121306	9204
1655	1771	1861	2754	36851	9104		3610		3055	7586	2100						366		
953	328	932	614		*c*									1249		tr	566		tr
1189	787	1233	887		*c*							tr	tr	*c*		4203	404		536
1706	759	2125	556		*c*							775		1433		tr	822		412
2212	1756	2164	1052		40432		1422					656	tr	*c*	273	2852	1112		*732*
2044	1897	1876	1227	tr	*c*		*3448*					*c*		*c*	585	978	*c*		1165
2020	2084	1686	1412	60196	*c*		*c*		tr		402	*716*	*c*		499	*c*	926		1267
1769	1930	1740	1819		*c*		889		542	1076	228	*c*	tr		*c*	2012	*c*		*945*
1570	1991	1279	1605	*c*	1956		918		668	948	348	279	tr		523	698	1099		1415
1154	1759	1126	1478	10493	2071		1058		637	1338	489	282	338	2587	481	*c*	1085	*154409*	1414
1178	2057	986	1762	18581	1546		*c*		*c*	*c*	518	343	470	3408	795	971	1253	21549	1623
995	2277	796	1406	13698	2440		1123		828	2232	437	469	700	6735	624	*c*	1451	44403	2063
1518	2893	872	1847	16422	2984		1955		1506	4169	625	1050	987	10825	1496	1500	2579	85293	3519
1876	3231	1216	2449	21862	3467		1520		1642	4579	722	1448	1173	13697	1776	2332	2863	113522	3530
2054	3302	1392	2735	24371	3426		1725		1796	5496	936	1570	1024	17804	1589	3141	2736	113951	3242
2212	3301	1777	2922	26396	4795		1878		2046	5594	1064	1579	1046	17171	1316	3891	2681	118359	3505
2008	2568	1473	2599	19968	3328		1765		1908	5497	889	1331	781	14448	1241	2821	2221	88274	2899
1576	1935	1319	2990	13696	2624		1410		1452	3523	628	1303	515	10512	823	2651	1625	79685	2078
1040	1122	829	1052	13772	tr		801		764	1870	313	697	282	5727	537	1124	*635*	43398	1273
639	429	tr	tr		tr		tr		440	1171	317	tr	tr	4919	*c*	540	551	27840	521
							1300												
												c			3142				
															234				
					3877		850					4470			5697				
															305				
							c					*c*			*2880*				
												590			606				
												c			c				
												706			207				
												2240			1408				
																tr		28173	
																tr			
																3626		61924	
																		c	
																42466	10658	603014	
																1621		89333	
																8984	*c*	1322688	
																		92389	
																c			
																766	426		
																	1250		
																	1884		
																	689		

FID response (μV.s) for lipid components identified by gas chromatographic analysis for the sample indicated. Dilution and the amount injected are shown. c indicates coelution, tr indicates trace recovered amounts.

APPENDIX 16

GC peak areas for experimental batch 2

Batch 2	*Nucella lapillus* 1	*Littorina littorea* 2	*Artica islandica* 1	*Artica islandica* 2	*Sepia officinalis*	CaCO3 blank 2	*Nucella lapillus* 1	*Littorina littorea* 2	*Artica islandica* 1	*Artica islandica* 2	*Sepia officinalis*	CaCO3 blank 2	*Nucella lapillus* 1	*Littorina littorea* 2	*Artica islandica* 1	*Artica islandica* 2
Extract	1	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3
Dilution (ul)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Injected (ul)	0.5	0.6	1	0.6	0.6	0.6	0.6	0.5	0.9	0.6	0.6	1	0.4	0.8	0.6	0.6
Material extracted (g)	109	98	125	98	38	29	102	91	117	91	33	29	102	91	117	91.4
C27 FAME																
C30 n-alcohol																
Lupeol																
C36 n-alkane																
Cholesterol													33032928	2544036	12489	91430
Plasticiser A	*c*	5271	39812	185529	24555	34913	*c*	10913	28079	796763	9152	tr				
Plasticiser B																
Plasticiser C	214447	104138	131214	159452	84455	97038	116447	43183	86211	4558591	148930	15116				
Plasticiser D		tr	5690	60614	3453		8759		3670	tr	1834					
Plasticiser E	99920	42284	34392	60160	22389	41093	26931	17304	54699	121552	29169	16287			730	
Plasticiser F	*c*	7253	14211	16828	7162	25917			39312		*c*	*c*			tr	
Pristane	30282	12727		6399	tr		17240	6812	8328	91322						33515
Phytane	25262	*c*		13081			*c*	3147	11680	95449						65223
C17 n-alkane	100110	*18812*		*2867*			*122597*	*12467*	9338	104240						67752
C18 n-alkane	116631	12578		16816	tr		40851	2595	19265	214669						98956
C19 n-alkane	*114860*	7619	1417	49895	1520		*c*		22673	469651		tr		tr		267237
C20 n-alkane	*99737*	15395	2129	88107	3960		34622	7755	24977	721041	1813	441		tr		449497
C21 n-alkane	*55237*	12234	3576	121992	3416		*c*	tr	27687	1030194	2508	510			251	712567
C22 n-alkane	*26588*	*c*	4508	127241	2787		*c*	tr	24635	1157874	2798	557			370	759269
C23 n-alkane	*9528*	6553	4792	88954	1776		*c*	2229	20155	869339	2376	667			565	552241
C24 n-alkane	*8590*	8551	4877	7238	2058		7385	4027	17311	562859	3262	3286			369	334355
C25 n-alkane	11738	6484	3744	44513	2149		4935	3729	17557	378876	3465	3916			465	199475
C26 n-alkane	*c*	tr	2927	23704	2563		*c*	5453	15277	301975	*18057*	5758			487	152346
C27 n-alkane	15684	6024	2569	31344	3935		4911	4599	20263	220662	4820	7604			867	119604
C28 n-alkane	3991	1842	2374	36647	4456		4210	2690	13712	189179	4516	8167			540	94381
C29 n-alkane	9502	6088	2592	32665	6327		8806	5947	18840	151750	5613	7628			514	70655
C30 n-alkane	4536	2000	2049	29151	4143		4886	*c*	12092	102382	3665	6816			313	49622
C31 n-alkane	7465	4289	1995	26074	11617		7045	4631	13852	82383	3722	5535			294	38973
C32 n-alkane			tr	13580	2246				6955	40108	1903	tr				18952
C33 n-alkane			tr	9916	2518				5280	22404		tr			tr	tr
C34 n-alkane									3589							
C35 n-alkane																
C12 n-alcohol													28294			66748
C13 n-alcohol													tr			62780
C14 n-alcohol													158596	tr		229961
C15 n-alcohol							*c*						176103			80797
C16 n-alcohol							83353						*401649*	*51727*		*565048*
C17 n-alcohol							21215						152416	11941		tr
C18 n-alcohol							16528						187529	20786		261860
C19 n-alcohol																48337
C20 n-alcohol																160858
C21 n-alcohol																21822
C22 n-alcohol																89468
C23 n-alcohol																28237
C24 n-alcohol																62643
C25 n-alcohol																
C26 n-alcohol																
C27 n-alcohol																
C28 n-alcohol																
C12 TMS acid																
C13 TMS acid																
C14 TMS acid																
C15TMS acid																
C16 TMS acid																
C17 TMS acid													157262			
C18 TMS acid																
C12 FAME																
C13 FAME																
C14 FAME																
C15 FAME																
C16 FAME																
C17 FAME																
C18 FAME																
C19 FAME																
C20 FAME																
C21 FAME																
C22 FAME																
C23 FAME																
C24 FAME																
C25 FAME																
C26 FAME																

Sepia officinalis	CaCO3 blank 2	*Nucella lapillus* 1	*Littorina littorea* 2	*Artica islandica* 1	*Artica islandica* 2	*Sepia officinalis*	CaCO3 blank 2	*Nucella lapillus* 1	*Littorina littorea* 2	*Artica islandica* 1	*Artica islandica* 2	*Sepia officinalis*	CaCO3 blank 2	*Nucella lapillus* 1	*Littorina littorea* 2	*Artica islandica* 1	*Artica islandica* 2	*Sepia officinalis*	CaCO3 blank 2
3	3	4	4	4	4	4	4	5	5	5	5	5	5	6	6	6	6	6	6
10	10	20	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.6	0.8	0.5	0.6	0.6	0.8	0.6	0.4	0.5	0.6	0.6	0.85	1	0.8	0.6	0.6	0.5	0.7	0.6	0.5
33.4	29	102.2	91	117	91.4	33.4	29	99.8	*88*	114.3	*93*	30.5	*27*	98.1	86.9	114.3	91.7	29.5	26
68605	67418							853441				4769		76905	254595	*20527*		20001	
	tr							8695	2718	7325		tr	*5785*	*c*	28802	42111	37412	16278	54429
																tr			1303
							tr	30118		33380	33113	8957	*16057*	100868	66956	119821	71054	24065	161501
							tr								tr	17286			
					2591		tr		1811	8133	4473	9670	tr	48647	28717	42932	22663	17759	18129
										2830		1962	tr	33814	12566	21814	7476	3699	6402
	tr									3238	*c*	2313							
	tr									4521	*c*	2573							
	tr									3783	1181	1761							
	tr									7333	4793	2559							
	tr									5588	*c*	2082							
	tr									4149	*c*	1300		75631			3290	673	
	tr				4725					3099	*c*	868	tr			1284	5923	584	
	tr									2976	52139	468	tr			2101	6816	912	
	tr									1797	48129	302	tr		tr	2240	5840		
	tr									1531	41342	1155	tr	c	tr	2712	1397	475	
	tr							7980		*1449*	28519	1670	tr	*4539*	2140	4302	3269	1429	tr
	tr							5089		1918	23055	1955		4443	2377	7858	4968	2581	tr
	tr							7264		2019	19824	2104		4989	2238	9441	3352		
	16400							6572		1994	15308	2274	tr	7500	2024	11609	3386	2291	tr
	20819							6665		2088	11231	2388	tr	8733	2476	13494	3664	*c*	tr
	15196							6794		1671	7952	1548	tr	8079	tr	11875	2989	*c*	tr
	17213							5893		1603	5801	1526	tr	14224		10761	2924	*c*	tr
	6001							10159		976	2635			3640		6953	tr	*c*	
	tr									632				*5773*		4952	tr		
																2901			
	tr																		
	2252																		
	8932							10970											
	3143																		
933	*23407*																		
	4984																		
1304	28539																		
	tr																		
791	22713																		
	tr																		
	14505																		
	tr																		
	tr																		
		45487	1572188																
		138753	156878																
		420648	1689223	350922	80196	68549													
		767092	313671	241547	*58690*	49470													
		8547003	4377743	2034429	153011	761877	49460							86082				8932	
		1953869	1020372	131257		73325	tr							*c*				1733	
		11245971	9146822	1468229	14919	394685	26625							140317				9413	
		2060677	289627		12918									61483					
		931019																	

Nucella lapillus 1	*Littorina littorea 2*	*Artica islandica 1*	*Artica islandica 2*	*Sepia officinalis*	*CaCO3 blank 2*	*Nucella lapillus 1*	*Littorina littorea 2*	*Artica islandica 1*	*Artica islandica 2*	*Sepia officinalis*	*CaCO3 blank 2*	*Nucella lapillus 1*	*Littorina littorea 2*	*Artica islandica 1*	*Artica islandica 2*	*Sepia officinalis*	*CaCO3 blank 2*
7	7	7	7	7	7	@	@	@	@	@	@	@	@	@	@	@	@
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.6	0.6	0.6	0.7	0.6	0.5	0.9	0.9	1	0.7	0.9	0.9	0.5	0.6	0.6	0.8	0.7	0.7
98.1	86.9	114.3	91.7	29.5	26	98.1	86.9	114.3	91.7	29.5	26	98.1	86.9	114.3	91.7	29.5	26
27448	36434			2569	2384	5736564	211731	214997	25037	5799	14230	11496145	525797	1133	63735		2778
18806	27491	13296	*22840*	26913	tr		1553	3102	10473					*c*		tr	1284
	2928	1468															
39689	74578	23613	*124208*	37669	1990		14628	*231090*	299258				*159485*	86583	*521071*	*c*	4873
c	7221	tr		tr	3017												
22186	51088	17956	3439	25631	2753		6282	6269	*c*					6495	*c*	19056	4166
5995	16694	7698	598	5508				*4331*						557	4526	4311	1578
		257															
			823														
		380	843								1533						
	tr	426	899	*1097*													
	tr	433	868	tr					7227						43036		
886	1805	658	909	267				3361	12929		1909			tr	76501		tr
1243	2615	985	1015	371				tr	13564					813	98577		559
881	3349	972	854	*1030*			tr	tr	11282					1592	85511	tr	460
c	*c*	1116	1183	*c*			tr	3058	7854					717	60882	tr	484
1395	3731	1688	715	*3078*			3109	2500	5082					840	43680	1755	615
2222	5743	2685	1083	*4809*			4943	tr	4591					1461	38401	6074	1177
2629	3777	3156	822	5125			6934	6508	4417					1911	31669	4453	1203
3445	3635	3639	748	5390			6537	6348	3750					2178	27076	3595	1099
	4712	4484	588	4787			7583	7459	3713					2291	23018	4799	1246
2696		3706	519	4355			5769	6238	2730					1752	18376	5720	898
3915		3018		3525			7271	5630	2257					1328	15320	3921	805
		2254						7169	1452					889	7349		376
		1406												tr	tr		tr
		753															
						26936											
						26828											
						61837						*c*	*c*				
						26923		*c*	1179								
						28075		29241	19459		11743				27888		
									2462								
								11289	11107						20783		
									2904						6133		
								c									
															10199		
										3264							
							6721										
													116290				
													45086				
												213837	303778				
												363898	167219				2062
												4496150	2724393		48119	*1326*	3733
												1104717	647153	tr	6690		
									3201			8835369	6619899	2813	9144	*c*	3092
													tr				
												2708426	tr				

APPENDIX 17

GC peak areas for experimental batch 3

Batch 3	*Crepidula fornicata, Recent*	*Crepidula "radiata", Waipuru*	*Pecten benedictus marwicki, Kupe*	*Tiostrea chilensis, Kupe*	*Tiostrea chilensis, Waipuru*	CaCO3 blank 3	*Crepidula fornicata, Recent*	*Crepidula "radiata", Waipuru*	*Pecten benedictus marwicki, Kupe*	*Tiostrea chilensis, Kupe*	*Tiostrea chilensis, Waipuru*	CaCO3 blank 3	*Crepidula fornicata, Recent*	*Crepidula "radiata", Waipuru*	*Pecten benedictus marwicki, Kupe*
Extract	1	1	1	1	1	1	2	2	2	2	2	2	3	3	3
Dilution (ul)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
injected (ul)	1.0	0.9	0.9	0.9	1.0	1.0	0.8	0.9	0.9	0.9	1.0	1.1	0.7	1.0	1.1
Material extracted (g)	87.3	86.5	113.4	112.7	103.3	136.1	87.3	86.5	113.4	112.7	103.3	136.1	87.3	86.5	113.4
C27 FAME															
C30 n-alcohol															
Lupeol															
C36 n-alkane															
Cholesterol													11334	63890	368918
Plasticiser A	813084	215389	883515	404519	*401736*	195853	140517	34069	*11381*	*51174*	17268	*68437*			
Plasticiser B															
Plasticiser C	2057487	624727	2399477	1228578	1491697	557035	1129058	160271	86108	467398	103386	268468	c		c
Plasticiser D	176423	*c*	214276	98241		49645	9058	19826				9149			
Plasticiser E	483468	168542	608775	371523	349807	174760	*75462*	41133	20785	59379	16083	47822	c	*c*	
Plasticiser F	372334	120647	510861	278803	271955	136259	21215	*c*	4747	23919	3162	31353			
Pristane		15791		*c*	5451			12010	1340	3022	879				
Phytane	*c*	*c*		*c*	9312		c	*c*	1521	3057	3031	tr			
C17 n-alkane		64211		10798	41808			41933	2374	4881	10016				
C18 n-alkane	*c*	32317		12337	47714		6160	*25148*	3496	8887	11018	tr			
C19 n-alkane	46650	34699	28739	15107	129805			*c*	2978	7512	6210	2619	2665		
C20 n-alkane	55842		27848	14463	27293	*c*	7188	8501	1906	5925	1957	1775	2409		c
C21 n-alkane	62316	27963	32183	17195	28559	3038	8569	10333	2123	8395	2398	3007	1886		5549
C22 n-alkane	69478	26738	44180	23308	28179	*1743*	8551	6643	2673	11416	1385	3214	2136		7355
C23 n-alkane	56131	28108	42603	19380		4514	6831	9601	2685	8756	2005	2886	1894		5886
C24 n-alkane	*25567*	*14983*	*17867*	*19279*	*c*	*2492*	*3446*		3957	*8545*	*1731*	*1191*	2292	12389	13071
C25 n-alkane	36505	65285	37069	20311	49614	7469	6068	22389	4050	6916	4187	3099	3681	29396	16531
C26 n-alkane	27330	13500	42710	23717	23167	17080	*6632*	*12074*	7018	*c*	*2925*	6838	8395	49639	24999
C27 n-alkane	28712	83085	32958	20086	38178	13701	6315	*c*	5213	7782	4985	5194	7362	62475	30345
C28 n-alkane	25682	*c*	29332	16590	21886	14680	5929	*c*	5143	7310	2498	5793	8662	58411	30587
C29 n-alkane	32679	88228	40913	22654	43109	18000	6290	37141	5868	7666	6061	7848	6792	67267	29416
C30 n-alkane	20981	12818	25761	14114	20419	13655	4590	7240	4944	5749	2538	6512	6026	50237	22396
C31 n-alkane	24751	78440	30163	17956	38284	14035	3595	37365	5264	5051	6463	7193	3549	43289	23931
C32 n-alkane	14262	10172	17702	9169	12982	8890		4431	3298	3151	1771	5209	2840	37448	24720
C33 n-alkane	10294	27077	tr	8070	17177	6648		14619	2841		2547	4245	2111	21756	17682
C34 n-alkane															6561
C35 n-alkane															
C12 n-alcohol														tr	
C13 n-alcohol													1142	tr	
C14 n-alcohol													3369	29648	
C15 n-alcohol															
C16 n-alcohol													*4376*	35868	*84129*
C17 n-alcohol													tr		c
C18 n-alcohol													7620	81753	153064
C19 n-alcohol															23172
C20 n-alcohol													4759	31655	114612
C21 n-alcohol													717	4763	
C22 n-alcohol													*2942*	*19493*	*62477*
C23 n-alcohol															
C24 n-alcohol													2813	14042	60572
C25 n-alcohol															
C26 n-alcohol															
C27 n-alcohol															
C28 n-alcohol															
C12 TMS acid															
C13 TMS acid															
C14 TMS acid															
C15TMS acid															
C16 TMS acid															
C17 TMS acid															
C18 TMS acid															
C12 FAME															
C13 FAME															
C14 FAME															
C15 FAME															
C16 FAME															
C17 FAME															
C18 FAME															
C19 FAME															
C20 FAME															
C21 FAME															
C22 FAME															
C23 FAME															
C24 FAME															
C25 FAME															
C26 FAME															

Tiostrea chiliensis, Kupe	*Tiostrea chiliensis*, Waipuru	CaCO3 blank 3	*Crepidula fornicata*, Recent	*Crepidula "radiata"*, Waipuru	*Pecten benedictus marwicki*, Kupe	*Tiostrea chiliensis*, Kupe	*Tiostrea chiliensis*, Waipuru	CaCO3 blank 3	*Crepidula fornicata*, Recent	*Crepidula "radiata"*, Waipuru	*Pecten benedictus marwicki*, Kupe	*Tiostrea chiliensis*, Kupe	*Tiostrea chiliensis*, Waipuru	CaCO3 blank 3	*Crepidula fornicata*, Recent	*Crepidula "radiata"*, Waipuru	*Pecten benedictus marwicki*, Kupe	*Tiostrea chiliensis*, Kupe
3	3	3	4	4	4	4	4	4	5	5	5	5	5	5	6	6	6	6
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
1.0	1.1	0.8	1.0	1.1	1.1	0.9	0.4	0.8	0.9	1.2	1.1	1.0	1.0	1.0	1.2	1.4	1.3	1.6
112.7	103.3	136.1	87.3	86.5	113.4	112.7	103.3	136.1	87.3	86.5	113.4	112.7	103.3	136.1	77.9	85.7	113.9	96.8
															tr		*5042*	2820
30261	190820	2131									1181				2675		1212	
		1617			tr			tr			*c*		610		56033	43693	92504	116567
															c			
c		877			*c*			3304	tr		7170		2583		79024	117758	*107134*	312867
											409				*3022*			
c		2761			5069			3656	tr		5265		2270		50755	22230	38613	41657
		742			2767			2070			1100		tr		16374	7436	7018	16867
									5540		tr				1592	2052	543	tr
					1390			519	2674		586				2591	1895	3845	tr
									7515		tr				7786	7356	1647	tr
					3974			1085	3999		674				5322	6364	7114	45892
	56663	tr							tr		684				6679	6498	7949	9382
		661			tr			tr	tr		369				4077	4428	5935	4548
	c	tr			7968			tr			487				3678	7040	4669	5131
		450						tr			552				4678	4791	5178	4879
1713		357						tr			524				4299	3234	4202	3961
3229		755						tr			*483*				4006	2206	3221	4340
4838	25846	918			tr			tr			500				5681	2574	3317	6343
6881	35579	2065			724			1344	tr		681				6576	4203	5875	12004
7992	46729	2520			270			1439	tr		632				7019	3649	5236	15592
7636	41661	3355			352			1105			355				7748	3883	4943	20452
6889	48350	3204			479			1304			537				7621	4146	5161	22948
6525	29441	2935						1027			435				6385	3688	4604	21381
5278	24352	2528						882			tr				5018	3465	5004	20722
4064	*c*	1404						tr			tr				3444	7263	3009	16536
2701	17004														2617	2114	1973	12798
															2089		1018	7881
5972																		
16623																		
19541	*77066*																	
2259																		
33707	169564										*778*							
5033																		
23981	91233																	
2536	12759																	
10969	*44917*																	
1632	6685																	
4483	42300																	
	38215																	
	21143																	
			358016			511279												
			94660			73418												
			775576	104005		459950	73556											
			362109	27880		178150												
	71524		2746321	771195	66466	1149349	953267											
			122281	7755			16891											
			819421	*c*	13573	232637	77060											

Tiostrea chiliensis, Waipuru	CaCO3 blank 3	*Crepidula fornicata*, Recent	*Crepidula "radiata"*, Waipuru	*Pecten benedictus marwicki*, Kupe	*Tiostrea chiliensis*, Kupe	*Tiostrea chiliensis*, Waipuru	CaCO3 blank 3	*Crepidula fornicata*, Recent	*Crepidula "radiata"*, Waipuru	*Pecten benedictus marwicki*, Kupe	*Tiostrea chiliensis*, Kupe	*Tiostrea chiliensis*, Waipuru	CaCO3 blank 3	*Crepidula fornicata*, Recent	*Crepidula "radiata"*, Waipuru	*Pecten benedictus marwicki*, Kupe	*Tiostrea chiliensis*, Kupe	*Tiostrea chiliensis*, Waipuru	CaCO3 blank 3
6	6	7	7	7	7	7	7	8	8	8	8	8	8	9	9	9	9	9	9
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
1.4	0.6	1.1	1.2	1.6	1.4	1.2	0.6	0.5	1.0	1.7	0.9	1.0	0.7	1.5	1.6	0.9	1.5	1.6	0.4
101.7	128.7	77.9	85.7	113.9	96.8	101.7	128.7	77.9	85.7	113.9	96.8	101.7	128.7	77.9	85.7	113.9	96.8	101.7	128.7
1670		192686	36488	8004	31133			191175	261742	238780	tr	206373		88556	33690	863		72407	
54		8016		736		489	292	28133	50608	25215		28366	4354	1690	2039			10097	796
5494	35513	80879	55139	51188	29963	*11695*	30636		20557		c	9822	3874	6454	15999	2855	44384	32193	12463
		4878		*3912*			2712												
10328	46309	59448	90854	*35307*	33478	15017	29358		9562		4910	c	3564	11205	*11742*	3750	*61023*	26389	12055
												32850			7928			4308	
12348	13681	51497	29964	26283	17075	17404	14295	c	17630	c	2121	14258	6733	26166	34616	6196	55944	16954	22209
4146	3356	13842	7823	4597	4814	4257	3113		4092	1679	1169	2880	1229	9326	9023	1322	9851	4063	5262
947		1122	1494		1651	1784		tr		2011		2647	1068	2384	1830	*436*	26631	2895	1841
1246		1496	2004	1137	2466	1563		tr		c		c	528	2592	3779	1178	30001	tr	2257
3720		2198	2069		3088	3766	378	tr		5287		4789	1323	4131	3118	*299*	49402	5863	3826
5051		2977	3969	1800	3815	3758		c		c		5503	1185	3623	7767	1215	64739	tr	4135
5545		2785	3243	3189	2782	3454	284	tr	tr	3811		*4578*	c	3380	7162	1694	45638		4400
3916	tr	2838	2518	2834	1882	2294	390	2099	5121	3099	tr	7116	852	2368	5681	1281	31428	2263	2991
3158	1257	3005	2538	2761	1434	3302	683	1663	c	2670	100	tr	*991*	2443	5086	1434	20122		3153
2948	1310	3876	3235	2981	1585	2379	876	2223	4275	2253	197	c	1211	3078	5708	1431	18750	2618	3572
2730	1221	3375	2211	2503	1181	2083	793	2342	2672	2538	190	*3061*	1237	3148	5433	1380	12998	4849	3390
2003	1064	2817	1902	1947	1059	1991	711	2420	4001	3050	232	1889	1403	3517	5255	1609	10921	2153	3246
1646	1188	3314	2152	2339	1120	1907	804	2362	5818	2570	255	1819	2155	4488	7205	2316	12657	c	4322
2967	1363	4096	2362	2609	*1247*	2804	995	3114	6528	4578	1035	2545	2908	4994	8297	3676	19684	5379	7892
2185	1439	3634	2699	3015	1496	3078	1092	4160	7077	6757	396	1748	3319	5999	8629	5574	14920	3341	6381
2119	1441	4230	2929	3193	1694	2680	1126	3854	6506	7780	417	3156	3249	6227	7906	7218	15511	2807	6843
2346	1377	4568	2996	3656	2117	3507	1187	4398	6818	9919	550	3916	3333	6854	7987	8495	18664	4421	6996
2160	1294	*3883*	2275	3314	1935	3183	1027	3881	5298	9518	288	2332	2644	5848	6899	8338	15472	3099	6117
1954	1206	*2547*	2183	3624	2035	2428	1137	3367	4724	9554	911	tr	2317	5939	6818	8138	16895	3509	5881
1941	783	3043	1288	2237	1544	1872	687	2845	c	8270		0	1608	4195	4646	6903	11705	2083	3959
967		1838	931	1538	1019	1156	516	1799	tr	5241	tr		831	3052	2786	5285	10537		2846
570		tr		3717		653	263	1872		7660			598	2122	1669	3407	6239		1787
																2491	c		
										10557									
										3415									
								11223	*24499*	11386		c							
								c											
								15422	18677	11684		9208							
								9162	c	20170									
								1495											
								c		c									
								tr											
								4280		3418		3154							
																		44537	
																		28301	
																		4999	
										c		37064						263152	
										c									
																	24429		
																	25629		
tr						tr						13896		22003	95575	*1291*	101792	45595	
						1300									2845		c		
tr						tr						2697		5109	24195	c	34901	11580	
																	4138		
																	7795		
																	2136		
																	7108		
																	3277		
																	8327		
																	6749		
																	5087		

APPENDIX 18

GC peak areas for experimental batch 4

Batch 4	Patella vulgata 2	Nucella lapillus 2	Littorina littorea 3	CaCO3 blank 4	Patella vulgata 2	Nucella lapillus 2	Littorina littorea 3	CaCO3 blank 4	Patella vulgata 2	Nucella lapillus 2	Littorina littorea 3	CaCO3 blank 4	Patella vulgata 2	Nucella lapillus 2	Littorina littorea 3	CaCO3 blank 4	Patella vulgata 2
Extract	1	1	1	1	2	2	2	2	3	3	3	3	4	4	4	4	5
Dilution (ul)	20	10	10	10	20	100	50	10	100	100	50	100	200	200	200	10	10
injected (ul)	0.7	0.7	0.7	0.7	0.7	0.7	0.9	0.8	0.9	0.8	0.8	0.7	0.8	0.7	0.7	0.7	0.8
Material extracted (g)	115.1	68.2	85.8	76.7	115.1	68.2	85.8	76.7	115.1	68.2	85.8	76.7	115.1	68.2	85.8	76.7	115.1
C27 FAME									tr				41336	30607	21479	442977	
C30 n-alcohol									67619	80489	294597	14688	5713			585208	6555
Lupeol	174013	6460	144783	548943	244499	40304	138801	405089	97961	125713	c	34898	25163		19062	734243	4661
C36 n-alkane									72443	59575	149614	18761	48469	40613	24561	772842	13533
Cholesterol		66146	102856	2956	856975	4775274	9106058	175995	354577	5850743	15058509	15782				7275	108802
Plasticiser A		13119			6728	tr		10523									99757
Plasticiser B		29289			c			122986	6333				126322				
Plasticiser C				tr	c	tr											c
Plasticiser D					21172												
Plasticiser E	175967	15690		tr	12961			71348	3620							6901	c
Plasticiser F		8161						7963								3721	1648
Pristane																	
Phytane																	
C17 n-alkane																	
C18 n-alkane																	
C19 n-alkane																	
C20 n-alkane																	
C21 n-alkane																	
C22 n-alkane					2110			3070									1030
C23 n-alkane					1565			4333	3136								1431
C24 n-alkane					c			9946									1528
C25 n-alkane					6661			8288	24630							6831	8809
C26 n-alkane					4441			19287	1864							3933	2764
C27 n-alkane					2661			17030	5846							2347	2100
C28 n-alkane	6255				6318			15546								1913	1662
C29 n-alkane	6967				tr			19105	1259							2663	1814
C30 n-alkane	4126				tr			18536	tr							2957	1262
C31 n-alkane	5631							13924	tr							c	1567
C32 n-alkane									2146								4982
C33 n-alkane																	
C34 n-alkane																	
C36 n-alkane																	
C12 n-alcohol							17700				55490						
C13 n-alcohol																	
C14 n-alcohol							17414				45274						
C15 n-alcohol							22475				54212						
C16 n-alcohol							167461				257231						
C17 n-alcohol							35692				58826						
C18 n-alcohol							42760				117299						
C19 n-alcohol							26148				56939						
C20 n-alcohol							16077				39837						
C21 n-alcohol																	
C22 n-alcohol																	
C23 n-alcohol																	
C24 n-alcohol																	
C25 n-alcohol																	
C26 n-alcohol																	
C27 n-alcohol																	
C28 n-alcohol																	
C12 TMS acid																	
C13 TMS acid																	
C14 TMS acid		tr															
C15TMS acid	4596582	tr															
C16 TMS acid	3356635	tr															
C17 TMS acid			c														
C18 TMS acid			1173099														
C12 FAME														59625	31226		
C13 FAME										5003				61195	16303		
C14 FAME										31679			12016	137720	72367		
C15 FAME										26793			4290	292384	33011		
C16 FAME										84230			92026	2684701	438146		
C17 FAME										24163				524479	76445		
C18 FAME										28034				2256897	748752		
C19 FAME																	
C20 FAME																	
C21 FAME																	
C22 FAME																	
C23 FAME																	
C24 FAME																	
C25 FAME																	
C26 FAME																	

Nucella lapillus 2	*Littorina littorea* 3	CaCO3 blank 4	*Patella vulgata* 2	*Nucella lapillus* 2	*Littorina littorea* 3	CaCO3 blank 4	*Patella vulgata* 2	*Nucella lapillus* 2	*Littorina littorea* 3	CaCO3 blank 4	*Patella vulgata* 2	*Nucella lapillus* 2	*Littorina littorea* 3	CaCO3 blank 4	*Patella vulgata* 2	*Nucella lapillus* 2	*Littorina littorea* 3	CaCO3 blank 4
5	5	5	6	6	6	6	7	7	7	7	8	8	8	8	9	9	9	9
10	10	10	100	20	100	10	10	10	100	10	10	10	200	10	100	10	200	10
0.7	0.8	0.8	0.8	0.7	0.9	1	0.7	?	0.9	0.7	0.8	0.8	0.8	0.9	0.8	0.8	0.7	0.9
68.2	85.8	76.7	112.5	61	80.9	88.5	112.5	61	80.9	88.5	112.5	61	80.9	88.5	112.5	61	80.9	88.5
	5034		63764	104374	82001	2777	859622	86587	19736	339845	431617	16277	3429	507521	76165	1020840	32426	885410
	4871		14616			492	1293407	42806	12194	266132	689274	70408	13666	224307	46080	224757	33506	681980
	4689	14104	53321		102662	523	1176757	104186	27446	*167966*	851956	*123340*	14155	*487087*	71627	1096735	35559	114530
			88783	188093	109170	3336	1231010	113811	41220	304787	782686	305018	8593	454486	105467	854532	42910	904890
1563964	1387962	30108	99740		2617321	2434	452013	448454	80167	13739	1517964	284004	143316	20230	46665	615043	147171	18291
		1185	19711					c		8109	18096							
		3079	119753							2032	c							
										24033								
	11560		26237															
		7064	204198			290	554272	65886	6392	2765	53390	5021		7169	2973			c
		1220	51857			378	*109494*		11419			5118		2336	680			5233
									1023									
									945									tr
	3341		19093						1078	474	5930			1683				4054
	10090	562	24960				62974		1338	1299	7225			1648	c			3495
	2454	1386	*24136*				66431	14731	1796	1420	8566	14053		2423	325			4900
	3699	1416	44564				92575	*4525*	5828	2866	16262	c		3966	900			5057
	17186	2314	69113				190245	11035	2068	5392	31054	10247		7049	1613			9951
	9766	1611	28064				86108	11958	1881	4883	35428	7413		8377	1404			8293
		1718	32124				83493	13421	1926	5114	39386	9892		9062	2260			7605
		1979	23399				71036	9308	1723	5413	39655	4254		9871	2019			8168
	6499	1879	25687				76152	10097	2158	6060	34212	6948		9289	1734			7851
	8620	1830	28011				65733	8044	2073	4247	30554	6004		6878	1709			5157
	3220		13753						697	2819				3988	734			4713
					4992324													
					578292													
					15034284													
													tr					
													54402					
													tr					
		6408								13534	23785		8364	4895				8346

APPENDIX 19

GC peak areas for experimental batch 5

Batch 5	*Patella vulgata, 3 "Braer"*	*Pecten tainui, Landguard*	*Tiostrea chiliensis, Landguard*	*Tiostrea chilliensis, Waipipi*	*Neothyris lenticularis, Waipipi*	CaCO3 blank 5	*Patella vulgata, 3 "Braer"*	*Pecten tainui, Landguard*	*Tiostrea chilliensis, Landguard*	*Tiostrea chilliensis, Waipipi*	*Neothyris lenticularis, Waipipi*	CaCO3 blank 5	*Patella vulgata, 3 "Braer"*	*Pecten tainui, Landguard*	*Tiostrea chilliensis, Landguard*
Extract	1	1	1	1	1	1	2	2	2	2	2	2	3	3	3
Dilution (ul)	20	10	50	10	20	10	10	10	10	10	10	10	50	10	10
Injected (ul)	0.7	0.6	0.7	0.8	0.9	0.7	0.8	0.8	0.8	0.7	0.9	0.3	0.8	0.8	0.6
Material extracted (g)	31.2	121.6	78.6	149.5	73	114.2	31.2	121.6	78.6	149.5	73	114.2	31.2	121.6	78.6
C27 FAME															1124
C30 n-alcohol													75664	100843	112561
Lupeol	236320	376112	64427	187255	143988	13636	720721	463683	309592	572063	501684	82973	133737	680299	777612
C36 n-alkane													126959	344942	285482
Cholesterol	246408	0	0	4390	0	5398	1525037	103757	41197	137815	41699	8803	264686	62666	162250
Plasticiser A		89431	14905	81254	30584	c	13206	7291	2277	12764	3933	27398		13363	5448
Plasticiser B			tr	13280	321941		21066	c		7744					
Plasticiser C		432852	120169	364828	452206	337793	c	108696	87116	149001	41449	102277		2502	9892
Plasticiser D		14450			c	17727	27226	c	1340	c					9382
Plasticiser E		42242	6758	23780	18886	33341	20151	24909	*10242*	62039	18462	15131			c
Plasticiser F		17264	2040	10524	6120	26731	5304	4332	1733	7124		7881			
Pristane		27744													
Phytane															
C17 n-alkane		13633													
C18 n-alkane		20148													
C19 n-alkane		14918						2917	1876	5239	1774				
C20 n-alkane		11385						c	1012	4587	1516				
C21 n-alkane		7822	tr	3928				4494	c	3604	1398				
C22 n-alkane		c					5924	2461	1116	4019	1141				
C23 n-alkane		c					c	2197	1458	4027	1563	c			
C24 n-alkane		3069					2252	*1477*	1094	*2960*	2094	1082			
C25 n-alkane		5182		2572			3757	2910	1721	5887	2084	1771			
C26 n-alkane		20645		4726			5538	5349	2805	11777	4365	4949			
C27 n-alkane		5229		2075			4212	4601	2706	9011	3251	3371		2971	
C28 n-alkane		c	tr	993			5889	c	2129	8061	3737	2954		2101	
C29 n-alkane		4712	tr	2258			4747	5571	2532	11445	4550	3308		3515	
C30 n-alkane		1489					4053	4954	2052	9133	3335	2538		4339	tr
C31 n-alkane		3459					3992	5194	2060	11523	3988	2747		4417	tr
C32 n-alkane								c	3536	c	c	1699			
C33 n-alkane															
C34 n-alkane															
C35 n-alkane															
C12 n-alcohol															
C13 n-alcohol															
C14 n-alcohol															
C15 n-alcohol															
C16 n-alcohol															
C17 n-alcohol															
C18 n-alcohol															
C19 n-alcohol															
C20 n-alcohol															
C21 n-alcohol															
C22 n-alcohol															
C23 n-alcohol															
C24 n-alcohol															
C25 n-alcohol															
C26 n-alcohol															
C27 n-alcohol															
C28 n-alcohol															
C12 TMS acid															
C13 TMS acid															
C14 TMS acid		tr	c												
C15TMS acid			tr												
C16 TMS acid	12079289	295312	137304		452206										
C17 TMS acid	118873														
C18 TMS acid	70116		5284												
C12 FAME															
C13 FAME															
C14 FAME															
C15 FAME															
C16 FAME															
C17 FAME															
C18 FAME															
C19 FAME															
C20 FAME															
C21 FAME															
C22 FAME															
C23 FAME															
C24 FAME															
C25 FAME															
C26 FAME															

Tiostrea chiliensis, Waipipi	*Neothyris lenticularis*, Waipipi	CaCO3 blank 5	*Patella vulgata*, 3 "Braer"	*Pecten tainui*, Landguard	*Tiostrea chiliensis*, Landguard	*Tiostrea chiliensis*, Waipipi	*Neothyris lenticularis*, Waipipi	CaCO3 blank 5	*Patella vulgata*, 3 "Braer"	*Pecten tainui*, Landguard	*Tiostrea chiliensis*, Landguard	*Tiostrea chiliensis*, Waipipi	*Neothyris lenticularis*, Waipipi	CaCO3 blank 5	*Patella vulgata*, 3 "Braer"	*Pecten tainui*, Landguard	*Tiostrea chiliensis*, Landguard	*Tiostrea chiliensis*, Waipipi	*Neothyris lenticularis*, Waipipi
3	3	3	4	4	4	4	4	4	5	5	5	5	5	5	6	6	6	6	6
10	10	10	50	10	10	10	50	10	10	10	10	10	10	10	10	10	10	20	10
0.7	0.8	0.6	0.7	1	0.6	0.8	0.7	0.6	0.8	0.7	0.7	0.8	0.7	0.7	0.8	0.8	0.8	0.7	1.4
149.5	73	114.2	31.2	121.6	78.6	149.5	73	114.2	31.2	122	78.6	150	73	114	28.9	137.6	76.5	156.9	71.2
71031	28054	*10475*	130911	121697	288761	698466	158968	403138							54576	151972	488254	140902	174088
83512	1092528	90614	142690	11354											*46159*	*32602*	85435		c
c	1121541	76793	139574	45021	12120	72948	128752		tr						208195	7309	66628		
180836	1132830	99677	155208	117746	214940	361977	209249	284125							192481	184911	653322	198877	*328160*
c	98156	2235	10681	2965	1677	39022			27126	1313		7162	1936		49829	3278	16416	3913	3629
	126482			*5963*		26081			14349								c	8053	18928
	66010			*49018*		390579		*2055*	11644								50524	110521	c
	71601								c						8617				2297
	30474			7072	1941	7474			5139				1752		1848	6673	24289	6316	6336
				1393		3798									1051	6259	10998	2725	3664
																			3805
																			1251
																1088			811
																4907			373
	c					2848										993		512	843
	23292					4534										c		752	610
	28225					4190										1325		859	1005
	28273					3503										886		964	942
	26948					2728										c		1051	1081
	22630					3910										1451		986	1130
	17484					2003										1340		1148	710
																629			
				49422		14317													
						6115													
			28851	62019		51963	12057												
			15506	31889		28329	4865												
			86304	517786	112682	247370	18348	1839											
						10470													
			19872	36302		47641	2380												

CaCO3 blank 5	*Patella vulgata*, 3 "Braer"	*Pecten tainui*, Landguard	*Tiostrea chiliensis*, Landguard	*Tiostrea chiliensis*, Waipipi	*Neothyris lenticularis*, Waipipi	CaCO3 blank 5	*Patella vulgata*, 3 "Braer"	*Pecten tainui*, Landguard	*Tiostrea chiliensis*, Landguard	*Tiostrea chiliensis*, Waipipi	*Neothyris lenticularis*, Waipipi	CaCO3 blank 5	*Patella vulgata*, 3 "Braer"	*Pecten tainui*, Landguard	*Tiostrea chiliensis*, Landguard	*Tiostrea chiliensis*, Waipipi	*Neothyris lenticularis*, Waipipi	CaCO3 blank 5
6	7	7	7	7	7	7	8	8	8	8	8	8	9	9	9	9	9	9
10	10	10	10	5	10	10	10	10	10	10	10	10	10	10	10	10	10	10
0.8	0.8	0.7	0.9	0.5	0.7	0.8	1	0.8	0.8	0.8	0.5	0.7	0.8	0.7	0.9	0.4	0.7	0.8
161.9	*28.9*	*137.6*	*76.5*	*157*	*71.2*	*161.9*	*28.9*	*137.6*	*76.5*	*156.9*	*71.2*	*161.9*	*28.9*	*137.6*	*76.5*	*156.9*	*71.2*	*161.9*
c	*47779*	11843	1301736		*177382*	*65307*	247790	100868	9988	101910	101738	*71159*	301328	319650	496171	66807	c	606087
185699	1090186	43498	1893557	1189	576121	286117	388611	231641	51219	71028	242873	120317	178128	98197	87378	3694	146010	6220
2302	258529	23856	2014494	2321	987364	919248	167346	317396	164667	180101	179806	429771	195778	17193	3410	853	48279	33115
446985	213516	51125	1788991	5020	429141	655282	554048	307986	150120	134378	224252	366038	198082	310905	718367	89752	484095	669227
9085	1060	2597	56420		9415	10644	251017	12939	5714	*5834*	*20156*	*2699*	*79652*	*4672*	*8305*	*2100*	*12361*	*8562*
c			94225		8220	18359								tr	54587	8136	6338	*21189*
															11440			
24905			80650		8981	c								tr	c	32636	16607	c
						74471					3295					4981		
13001			74478		6372	11852	6845	9040		3801	1847	2711	7210	c	15609	*8662*	8766	*36135*
			19641							688		c	1588		4617	2337		
						13222									27484		1565	3950
c						25684						1196			2297	651	c	3236
10825						*8318*					817	1338			2505	336	c	2843
20630						25462					1388	1258			2081	296	c	3465
19871						28662					*1872*	*1945*			*5449*	*464*	*c*	4894
27042						28866	6941				2517	3265			10291	*587*	c	7380
26257			4661			28574	*3976*			437	2887	c			11318	727	c	6568
20885			c			27674	1842			523	3575	3264			10015	888	c	5134
18612			3754		1043	29168	2282			318	3159	3645			10820	779	c	6210
15710			7341		2419	18592	1686			862	3233	2791			*9775*	*487*	c	*5715*
18861			3171		1184	18969	1286			368	1904	2763			7122	365	c	3783
11386			2661				c				681				3313		c	2580
																959		
														c				
														c				
														c				
c																		
62952																		