DEVELOPMENT OF A GAS CHROMATOGRAPHIC METHOD FOR PROFILING NEUTRAL LIPIDS IN MARINE SAMPLES

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DEVELOPMENT OF A GAS CHROMATOGRAPHIC METHOD FOR PROFILING NEUTRAL LIPIDS IN MARINE SAMPLES

By

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ABSTRACT

Profiling neutral lipids is one of more difficult applications of gas chromatography (GC), but it can contribute important information on unrevealed neutral lipid suites in their entirety from marine environments. Thin layer chromatography with flame ionization detection (TLC-FID) is extensively used today but has many inherent limitations. In this study, a high temperature GC lipid profiling method was optimized for use with neutral lipids in highly unsaturated samples from a subpolar marine environment. Chemometrics was employed to optimize autoinjection procedures and column temperature programs. Other components in the GC, including the selection of carrier gas types, injection techniques, length of the column, septum types and retention gap, were investigated. A hydrogenation step was required to avoid discrimination of the FID responses in highly unsaturated samples. Analyses of fatty acids, moieties of the neutral lipids, supported the neutral lipid profile data. The identification of major sterols was confirmed by GC coupled to mass spectrometry (GC-MS). Summed molecular species from each neutral lipid class determined gas chromatographically were linearly correlated with neutral lipid subclasses determined by Chromarod TLC-FID. The developed method was applied to a range of marine samples, including algae, bivalves, polychaetes, fish eggs and fish larvae, revealing for the first time, neutral lipid carbon number distributions in marine species from cold oceans.

DEDICATION

This thesis is dedicated to my mother, Yu-hua Yuan, my wife Wei and my son Si-cun.

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GLOSSARY

CRV	chromatographic response value
FAME	fatty acid methyl esters
FID	flame ionization detection
f_w	weight correction factor for flame ionization detector
GC	gas chromatography
GC-MS	gas chromatography with mass spectrometry
HPLC	high performance liquid chromatography
m/z	mass to charge ratio
PUFA	polyunsaturated fatty acids
SFC	supercritical fluid chromatography
SPI	septum-equipped temperature-programmable injection
TAG	triacylglycerols
TLC-FID	thin layer chromatography-flame ionization detection
TMS	trimethylsilylation

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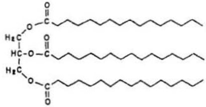
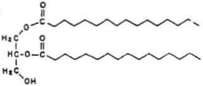
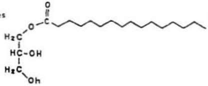
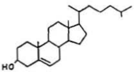
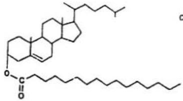
1 INTRODUCTION

Neutral lipids, including tri-, di- and monoacylglycerols, sterols, steryl esters, waxes, wax esters, free fatty acids, free aliphatic alcohols and hydrocarbons (Figure 1.1), are important components of marine food webs (Parrish, 1988). Of them, triacylglycerols (TAG) constitute a major part of energy reservoirs and act as thermal insulators for marine organisms. Certain neutral lipid classes have other important physiological functions. For example, some polyunsaturated fatty acids (PUFA) esterified in acyl lipid classes are essential for the normal metabolism and growth of organisms (Bell *et al*, 1986). Lipids are hydrophobic and are potential solvents for lipophilic pollutants. Neutral lipids in marine species reflect physiological and environmental variations so that accurately profiling neutral lipids will provide a key to understanding physiological responses to environmental variability.

1.1 Lipid Nomenclature

Acylglycerols can be defined as glycerol esters in which one or more hydroxyl groups have been combined with fatty acids. A serial shorthand notation was employed in this thesis. Carbon numbers in TAG indicate summed acyl carbon numbers only, while in sterols, steryl esters, wax esters and hydrocarbons, the carbon numbers represent the total in the molecule.

L:Bn-X was used for fatty acid identification, while L is the carbon chain length,

Lipid class	Structure	Name
Triglycerides		Tripalmitin
Diglycerides		1,2-Dipalmitin
Monoglycerides		1-Monopalmitin
Sterols		Cholesterol
Sterol esters		Cholesteryl palmitate

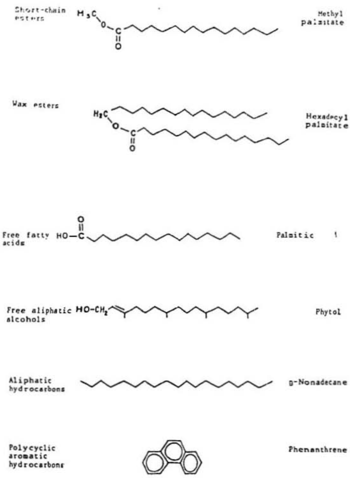


Figure 1.1. Structures of typical neutral lipid compounds

B is the number of double bonds, and n-X is the position of the double bond closest to the terminal methyl group. For example, n-3, represents the fatty acids with the first double bond located at the third carbon from the methyl end.

1.2 Sources of Marine Lipids

Marine environments are populated with numerous and diverse plants and animals. The environmental matrix basically consists of sediments, colloidal material and water.

1.2.1 Plants

Marine plants are mainly comprised of microalgae, seaweeds and seagrasses. Most species of phytoplankton have an essential role in the marine ecosystem as primary producers of energy and various types of organic matter through photosynthesis (Kayama *et al*, 1989). Evidence has accumulated to show that the quality, as well as the quantity, of microalgal lipids is very important in the nutrition of marine organisms (Klungsoyr *et al*, 1989). Some PUFA synthesized by algae are essential for growth and development of marine fish larvae, shrimps and molluscs (Sukenik *et al*, 1991).

Generally, planktonic inputs lead to a fatty acid mixture of 14:0 (tetradecanoic acid), 16:0 (hexadecanoic acid), 16:1n-7 (9-hexadecenoic acid), 18:0 (octadecanoic acid), 18:1n-9 (9-octadecenoic acid), 20:5n-3 (cis-5,8,11,14,17-eicosapentaenoic acid) and 22:6n-3 (cis-4,7,10,13,16,19-docosahexaenoic acid). High values of the $\Sigma C16/\Sigma C18$ and C16:1/C16:0 ratios are characteristic of diatoms (Scribe *et al*, 1991).

One alga, *Isochrysis galbana* (Clone T-iso) with high levels of 22:6n-3, is being increasingly used in the mariculture industry. However, *Chaetoceros* seems to be superior to *I. galbana* for increasing oyster growth (Enright *et al*, 1986).

The effects of environmental conditions, such as temperature, light intensity and nutrient availability on lipid amounts and composition (Roessler, 1990; Renaud *et al*, 1991), and related algal culture techniques (Wangersky *et al*, 1989; Parrish *et al*, 1990) have been extensively studied. Decreasing temperature, for instance, tends to increase the concentration of (n-3) PUFA in cellular total lipids in algae (Seto *et al*, 1984). Algae grown under conditions of nitrogen limitation continue to photosynthesize and may accumulate their end products of photosynthesis as lipids, mostly TAG (Ben-Amotz *et al*, 1985). High-light-grown phytoplankton cells are nutritionally superior to low-light-grown cells in terms of the fatty acid compositions (Thompson *et al*, 1994).

1.2.2 Invertebrates

Invertebrates are important in the marine food web since they serve as the link in the transfer of energy from phytoplankton to the upper trophic levels. Usually food chains can include up to about five trophic levels (Fenchel, 1988). For example, zooplankton transfer PUFA, such as 18:4n-3, from phytoplankton to herring larvae (Fraser *et al*, 1989). The ratios of 16:1n-7/16:0 and 18:1n-7/18:1n-9 were highlighted as biomarkers to provide information on food sources and possible feeding grounds of Antarctic krill (Virtue *et al*, 1993).

The world-wide commercial importance of bivalves, such as scallops, oysters and mussels, has focused interest in lipid research because neutral lipids are the major reserve accumulated in maturing larvae and more precisely reflect the nutritional conditions of the larvae than either protein or carbohydrate (Whyte *et al*, 1992).

1.2.3 Fish

Lipids rather than protein or carbohydrates are also the favoured source of metabolic energy in most species of fish (Sargent, 1995). Fish lipids differ from TAG in other terrestrial animals as the former contains a wider range of longer chain fatty acids, and a large proportion of PUFA. Variations in both amount and composition of lipids in fish in relation to environmental and dietary factors have been thoroughly investigated.

Fish continuously alter the fluidity of their membranes through changes in the relative amounts of different unsaturated acids in response to temperature. Maintaining a certain proportion of 22:6n-3 in relation to 20:5n-3 in particular plays a special role in both the control of membrane fluidity and temperature adaptation processes in fish (Farkas *et al*, 1980). The larger, long-lived species, such as cod, appear to accumulate more 22:6n-3 than 20:5n-3 in their lipids. However, fish such as capelin (shorter-living species) accumulate 20:5n-3 in their depot fat.

Clearly, fish have a dietary requirement for the long chain PUFA which they are unable to fulfil by chain extension of shorter chain acids in their diet (Morris *et al*, 1989). Studies have shown that the contents of 20:5n-3 and 22:6n-3 in fish are similar to those found in some marine algae from the same areas (Ackman, 1994). 22:6n-3 may be superior to 20:5n-3 in terms of essential fatty acid value in a range of marine larval fish (Watanabe, 1993). But amounts of 22:6n-3 in total fatty acids vary greatly in different fish species from cold oceans (Sigurgisladottir *et al*, 1993).

The composition of neutral lipid compounds, especially TAG, in marine animals can easily change through diet and other environmental stresses. Certain ratios of neutral lipid subclasses, such as the ratio of TAG to sterols, may be useful as indices of physiological conditions in marine animals (Fraser, 1989).

1.2.4 Sediments and Seawater

Lipid compositions of ocean sediments may serve as a tool to determine their probable origin because they often contain the basic skeletal structures and even functional groups of the original sources (Venkatesan *et al*, 1987). Vertical profiles of lipids can also provide information on the location and sources of these high-energy reduced organic compounds (Parrish *et al*, 1992).

Finally, lipids are present in detritus, and in the operationally defined "dissolved" and particulate fractions of seawater at the µg/L level (Parrish, 1988). Lipid class data from the extracts of these fractions are useful as indicators of pollution, certain types of organisms, or of anabolic or catabolic processes (Delmas *et al*, 1984), while certain fatty acids may be used as markers or signature compounds for specific organisms.

In summary, neutral lipid amounts and composition are extremely important in monitoring physiological condition in a variety of marine species. Quantitative evaluation of neutral lipid profiles has specific significance in terms of biomarkers and indices. To acquire such data in meaningful numbers necessitates accurate, precise and rapid analytical procedures.

1.3 Methodology for Analyses of Neutral Lipids

Analyses of marine lipids have long been an important aspect of marine chemistry research. Development of lipid methodology yielding more detailed information will promote the identification of key lipid compounds in aquatic ecosystems. Simplification of the procedures will greatly benefit the routine determination of lipids as well as decrease experimental cost.

The extremely complex nature and the large numbers of molecular species with similar physio-chemical properties make chromatographic methods the exclusive way to profile lipids (Kuksis, 1984).

1.3.1 Thin Layer Chromatography-Flame Ionization Detection

Thin layer chromatography-flame ionization detection (TLC-FID) has been widely used in the analysis of simple and complex lipids from the early 1980s (Shantha, 1992). Evidence shows that Iatroscan TLC-FID is not a simple combination of plate TLC and FID. For example, the partial scanning and redevelopment have no parallels in any other analytical technique. TLC-FID is an analytical system in its own right, requiring careful consideration of the parameters affecting both separation and quantification (Delmas *et al*, 1984). Kaitaranta (1981) investigated the response and linearity of different lipid components; Parrish *et al* (1983) examined the effect of developing solvents; Indrasena *et al* (1991) compared different Chromarod adsorbents. It is obvious that the TLC-FID approach, having only a decade of development, is now in a phase of rapid improvement in quantitative data output (Ackman, 1991).

1.3.2 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is especially useful for nonvolatile and thermally sensitive lipids, such as phospholipids and conjugated lipid classes (Shukla 1988). Reversed phase HPLC has been employed to separate individual TAG according to their degrees of unsaturation, and, to a lesser extent, by molecular weight. The combination of reversed phase HPLC with a mass spectrometer can provide additional value for reliable identification of natural acylglycerols (Kuksis *et al*, 1984).

1.3.3 Gas Chromatography

Use of GC for determination of lipid classes was mainly developed by Kuksis and co-workers. They did the first application with packed columns for lipid profiles of human blood plasma (Kuksis *et al*, 1967), and then in 1984 they initially applied fused silica capillary columns in the same field (Myher *et al*, 1984a). However, this method has not yet been applied to neutral lipids in cold ocean samples with a high degree of unsaturation.

The analysis of fatty acid moieties by GC does not yield information on the original combination of the compounds in the marine lipid molecules, as much of the specificity is lost by combining all the esterified compounds in one analysis (Lee *et al*, 1984).

The tremendous progress in the adoption of high temperature nonpolar and polar capillary columns, and less discriminative injection devices made the applicability of capillary GC extend to molecular weights up to 1500 daltons (Sandra *et al*, 1988). In high temperature GC, a potential tool to profile marine lipids, the mode of injection of samples, the column, and the derivatization of samples are recognized as critical for effective neutral lipid profiling. Other refinements based on such factors as sample loads, solvents, the nature of the carrier gas and its flow rate are also important factors in optimizing the lipid profiling (Kuksis *et al*, 1989).

1.3.3.1 Injection Techniques

The conventional technique of hot injection has a number of shortcomings such as discrimination of low volatility compounds and contamination by nonvolatile impurities because solutions often start partially evaporating within a hot syringe needle during injection. These problems are solved by replacing hot injection with cold on-column injection where a cold injector directly deposits the sample liquid into column or pre-column inlet. Thus the flow of carrier gas redistributes the inserted plug of liquid into a film on the tubing wall (Grob, 1979, 1984). Efficient sample purification is achieved by placing a short piece of empty fused silica deactivated tubing, or pre-column, before the analytical column. This avoids contamination of the analytical column with on-column injection. Besides a deposition site for low volatility substances, a pre-column provides a "retention gap" which has the function of refocusing the injected sample in order to avoid peak splitting which can result from cold on-column injection. The septum-equipped temperature-programmable injection (SPI), a new type of injection, allows programmed temperature vaporizing direct injection as well as the on-column injection.

The critical steps in modern injection techniques are of separation of sample liquid from the needle, elimination of sample evaporation in the needle and suitable evaporation speeds for both solvents and solutes in the top of columns (Grob, 1994). To obtain consistent, quantitative results the only approach for both fatty acid methyl esters (FAME) and TAG is to adopt splitless, on-column or SPI using a "retention gap" (Hammond, 1989).

1.3.3.2 Columns

Until now, fused silica capillary columns of 5 to 8 meter length with chemically bonded nonpolar stationary phases (DB-5 or OV-1) have yielded the best results in lipid profiles (Kuksis *et al*, 1986). For example, high temperature DB-5 (methylsilicones, with 5 % phenyl content) capillary columns can work at temperature higher than 340°C without detectable bleeding, and the neutral lipid compounds from C_{16} free fatty acid to

C_{56} TAG are eluted within half an hour and with reasonable separation in terms of carbon numbers (Myher *et al*, 1984; Kuksis *et al*, 1986). High temperature, more polar (phenylmethylsilicones, with 50-65 % phenyl content) stationary phases resolve various lipid classes into molecular species according to chain length and number of double bonds (Myher *et al*, 1982, 1990; Kuksis *et al*, 1990). But a higher column bleeding of these polar phases leads to TAG with high carbon numbers giving a lower response on them than on dimethylsilicone columns (Termonia *et al*, 1987). Mares *et al* (1979, 1985) recommended conditioning columns by repeated injection of a large amount of TAG prior to analysis of samples to stabilize the recovery of higher carbon number TAG.

Ackman (1986) considered the polyglycol phase in a bonded or cross-linked form as the universal polar stationary phase, which may be best for the fatty acid profiles of marine samples (Napolitano *et al*, 1988; Whyte *et al*, 1990). The main advantage of using polyglycol wall coated open tubular columns for profiling of fatty acids is that the fatty acids elute in a fixed order.

Capillary columns are more and more widely used in lipid analyses because they have greater resolving power and have lower background noise (Ackman, 1986). As well, the dependence of FID weight correction factor (f_w) on the amounts of analyzed lipids is less in capillary columns than in packed columns (Mares, 1987). For lipid profiles, wide-bore (0.32 mm i.d.) columns are preferred over narrow-bore (0.21 mm i.d.) columns since the former has higher sample capacities and elution temperature can be kept lower because of higher flow rate in wide-bore columns (Kuksis *et al*, 1989). Column lengths over 10 meters may lead to greater losses, especially of high molecular weight and highly unsaturated lipids (Myher *et al*, 1984). The mechanisms of TAG loss may include the following: 1) For saturated TAG, losses probably occur through a mechanism of reversible saturation of the stationary phase by the solute; 2) For unsaturated substances, losses may occur primarily through thermal degradation or polymerization. The correction factors (f_w) for compensation of losses of higher carbon number and unsaturated TAG are affected by the following factors: 1) the quality and length of the column, 2) the type of stationary phase, 3) injection technique, 4) temperature programs, and 5) the chemical nature and molecular weight of the analytes (Mares, 1988).

1.3.3.3 Carrier gases

Hydrogen may be considered to be the carrier gas of choice for all chromatographic analyses on capillary columns (Ackman, 1981; Decker *et al*, 1993). Grob *et al* (1990) demonstrated that both hydrogen and helium at low gas velocity (< 4 cm/s) at either 60°C or 370°C showed almost the same plate heights, but at high gas flow and high temperature, hydrogen was preferable to helium. However, hydrogen as the carrier gas may result in a hydrogenation of cholesteryl esters (Smith, 1982), and carries the risk of explosion. The flow rates of carrier gases in capillary columns in the analysis of intact lipids mainly depend on the column diameter and carrier gas type. In general, rates around 10 ml/min have been widely employed.

1.3.3.4 Other Considerations

The most common GC detector used for the detection of lipids is the FID which is relatively insensitive to flow and temperature change (Lee *et al*, 1984). However, the mass spectrometer is one of the most powerful detectors for lipid profiles by GC (Kuksis *et al*, 1984, 1985a, b). Unseparated peaks can easily be resolved by single ion chromatography and other mass spectrometric techniques (Kuksis *et al*, 1991). The mass spectrum may provide a fragmentation pattern which is usually characteristic of a particular compound. GC-MS analysis of wax esters, steryl esters and triacylglycerols showed that methane-chemical ionization spectra provided maximal structural information regarding the fatty acid and alcohol moieties for the first two types of compounds, whereas electron impact spectra were most useful in interpreting triacylglycerol structures (Wakeham *et al*, 1982). Typical characteristic fragment ions in the case of FAME are the McLafferty fragment at m/z 74 as well as the ion at m/z 87 (Traitler, 1987).

The internal standard method in quantitative GC analysis is widely used in order to compensate for losses during the isolation of intact lipids and to offset fluctuations in GC conditions and uncertainties introduced by sample injection. For lipid profiles, tricaprin (30:0 TAG) is usually selected as the internal standard (Kuksis *et al*, 1975). In special cases, trioctanoylglycerol or trilauroylglycerol may be better suited as an internal standard. Maximum accuracy is obtained when the internal standard yields a peak area similar to that of the sample peaks. However, workable proportions may also be obtained by adding the internal standard at a level of 10 % of the concentration of the analyte

(Kuksis, 1976).

1.3.4 Comparison of Chromatographic Methods

The comparison of different methods for routine determination of marine lipids depend on both technical merits, and practical and economic considerations.

TLC-FID, with a high sample capacity and simultaneous determination of both neutral and polar lipids, has been widely used to profile marine lipids (Volkman *et al*, 1989; Goutx *et al*, 1990). But the variability of the results obtained by TLC-FID has been generally found to be greater than that observed by GC on a packed column (Crane *et al* 1983; Mares *et al*, 1983a). Also, TLC-FID does not lead itself readily to automation, it has non-linear calibration curves and there are slight variations among individual Chromarods (Tvrzicka *et al*, 1990). Another shortcoming of TLC-FID is that this method, after several steps of development in different solvent systems, separates lipids only into approximately ten subclasses. In contrast, GC or HPLC can resolve each subclass into individual compounds (Kuksis *et al*, 1975; Rezanka *et al*, 1991). Unlike TLC, GC can be readily connected to the mass spectrometer and is readily automated. Due to these inherent limitations of TLC-FID, more precise, sensitive and readily automated methods for profiles of marine lipids need to be explored.

The greatest advantage of HPLC over GC is again the elimination of loss of sensitive unsaturated substances and higher molecular weight homologues. This advantage is, however, compensated by other problems including less sensitive detection, more difficulties in identification and longer analytical times (Mares, 1988).

Recently, supercritical fluid chromatography (SFC) has received a lot of attention for lipid analyses because SFC can provide a wide range of operating conditions from gas-like separations similar to GC, to liquid-like separations more comparable to HPLC (Laakso *et al*, 1992). But analysis time with SFC is only shorter than those of HPLC, and co-elution of some important neutral lipid compounds is still unavoidable (Staby *et al*, 1994).

In conclusion, the current methods of profiling marine lipids need improving for both technical reasons and for operational convenience. GC provides, by far, the highest resolution of TAG based on carbon number in the shortest analytical time among the techniques mentioned above (Sandra *et al*, 1988). Moreover, GC is relatively sensitive and is readily automated. But its application is partially restricted to the more saturated low molecular weight species (Kuksis, 1994). It needs to be developed for the very long chain and highly unsaturated marine oils (Holmer, 1989), because effects of the degree of unsaturation and losses of high molecular weight neutral lipids in high temperature GC still pose problems. The purpose of this study was to resolve these problems.

1.4 Optimization of Experiments and Data Treatment

To develop an analytical method, the region of optimal analytical performance and stability in a multidimensional experimental variable space has to be located. The ultimate goal of the experimentation is the production of systematic information from raw experimental data. Chemometrics, defined as the discipline that uses mathematics and statistical methods to 1) design or select optimal measurement procedures and experiments and 2) provide maximum chemical information by analyzing chemical data, can be attempted in all stages of experimentation (Kaufmann, 1992).

The steps usually are: 1. Choose factors for the design: Plackett-Burman designs and Latin Square designs are excellent for screening the variables. 2. Choose the experimental domain to pick a suitable range where intervals are large enough to show appreciable changes. 3. Define the response: This study mainly concerned itself with recovery of compounds of high molecular weight and resolution between major individual components. 4. Generate the design by running the experimental points, and then redefine the domain if necessary. 5. Ensure model validity through the use of proper statistical tests (t-tests, normal probability plots). 6. Locate the optimum by using a polynomial model to generate a response surface over the experimental domain.

These statistical techniques have been applied in marine hydrocarbons and fatty acids (Gomez-Belinchon *et al*, 1988; Sicre *et al*, 1988; Mayzaud *et al*, 1990).

1.5 Objectives

The overall objective of the proposed research is to develop a relatively precise, sensitive and readily automated GC method to measure neutral lipid profiles, including hydrocarbons, free fatty acids, sterols, wax esters, sterol esters and TAG in marine samples. This technique basically involves lipid extraction, fractionation, hydrogenation and analysis with capillary GC at temperatures up to 340°C on a short, bonded nonpolar DB-5 liquid phase column.

The specific objectives were as follows:

1) to find a complete column chromatographic method to fractionate neutral lipids;

2) to investigate a simple and effective hydrogenation method;

3) to optimize the conditions of high temperature nonpolar capillary GC;

4) to analyze fatty acids, neutral lipid moieties, to support neutral lipid profile data;

5) to compare the results obtained from the proposed method with those from TLC-FID, and

6) to verify the usefulness of the developed procedure by applying it to various marine samples.

2 EXPERIMENTAL

2.1 Materials

2.1.1 Glassware and Chemicals

Any containers, in part or whole, which may have come into contact with solvent, were made of glass or were Teflon coated. All glassware was made from Pyrex.

All chemicals and solvents employed in extraction and characterization of lipids were either of analytical or chromatographic grade. The authentic neutral lipid mixtures employed in this study were prepared from chromatographically pure materials (at least 99% purity) supplied by Sigma (St. Louis, MO, USA).

2.1.2 Marine Samples

One species of bivalve, *Yoldia hyperborea,* and two species of polychaetes, *Nephthys ciliata* and *Artacoma proboscidea*, were taken from the bottom of Conception Bay, Newfoundland, Canada (Parrish *et al*, 1995). Larvae of cod (*Gadus morhua*) were obtained originally from the broodstock of Dalhousie University (Scotian Shelf Cod). The remaining marine samples were cultured in the Ocean Sciences Centre, Memorial University of Newfoundland. The flagellate, *Isochrysis galbana* (Clone T-iso), was grown in cage culture turbidostats. Fertilized eggs were obtained from two captive Atlantic halibut (*Hippoglossus hippoglossus*). Juvenile sea scallops (*Placopecten magellanicus*), fed differently cultured phytoplankton diets, were obtained from a pilot scale hatchery.

2.1.3 Sample Blanks

Blanks, which involve all operational procedures but without a sample, were carried out during the analysis of each marine species. The amounts of the background peaks associated with analyte peaks were subtracted from those of certain compounds.

2.2 Methods

The entire experimental design is outlined in Figure 2.1.

2.2.1 Extraction

To an aliquot of homogenized marine samples, a measured amount of one or other of the internal standards, tricaprin for neutral lipid profiles or n-hexadecan-3-one for TLC-FID analysis, was added. The samples were extracted with a mixture of chloroform and methanol following the procedure of Bligh and Dyer (1959). The chloroform layer containing extracted total lipids was transferred into a 10-ml glass tube and was stored under nitrogen at -20°C prior to analysis.

2.2.2 Fractionation by Column Chromatography

The Florisil (Fisher Scientific, NJ, USA) column was prepared by placing a small amount of pre-combusted glass wool into the tapered seat of disposable pasteur pipet

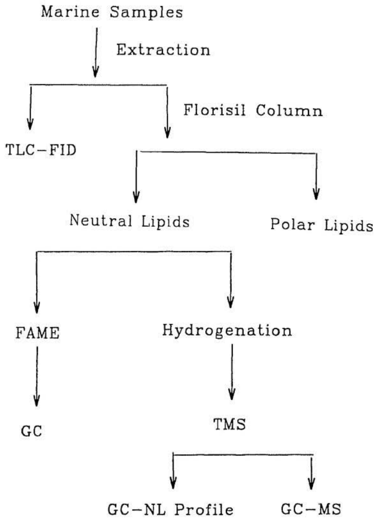


Figure 2.1. Flow chart of entire experimental design

(Kimble, borosilicate glass, 5.75 in.), and then by packing Florisil to a depth of 3 cm. The column was washed with a bed volume of methanol and then with the same volume of chloroform.

An aliquot of total lipid chloroform extract from marine samples was passed through this column. Neutral lipids were fractionated with 8 ml of chloroform:methanol: formic acid (99:1:1 by vol.), polar lipids were then eluted from the Florisil using 6 ml of methanol.

2.2.3 Hydrogenation

An aliquot of each neutral lipid (3 mg or lower) in 5 ml chloroform and 5 mg fresh platinum oxide (Sigma, St. Louis, MO, USA) were placed in a 10 ml vial, and bubbled with a gentle flow of hydrogen gas for 20 minutes without stirring. The hydrogen-filled vial was capped and sealed, and then stirred vigorously on a magnetic stirrer for two hours. The hydrogenated sample was then filtered with a No. 4 Whatman filter paper to remove the catalyst.

In addition, most samples were also hydrogenated by means of an atmospheric pressure hydrogenator in semi-micro scale (Vogel, 1958) to check the efficiency of the simpler method.

2.2.4 Derivatization

2.2.4.1 Trimethylsilylation

An aliquot of dried neutral lipid fraction was mixed with one drop of N,O-bis(trimethylsilyl)-acetamide and two drops of N,O-bis(trimethylsilyl)-trifluoroacetamide (Pierce, IL, USA), then heated at 70°C for 15 minutes. The trimethylsilylation (TMS) derivatives were diluted with chloroform prior to GC profiling.

2.2.4.2 Fatty acid methyl esters

An aliquot of the neutral lipid fraction (3 mg or lower) was dried and derivatized to fatty acid methyl esters (FAME) using 14 % of BF_3/ methanol solution (Pierce, IL, USA). The sample and 2 ml of methylating reagent were heated at 80°C for one hour. One ml of doubly distilled water was added, followed by 4 ml of hexane. The FAME were obtained by withdrawing the organic phase after centrifuging the mixture at 1000 rpm for 5 minutes.

2.2.5 Chromatographic Methods

2.2.5.1 Gas Chromatography

Neutral Lipid Profiles:

Neutral lipids were investigated using a Model 3400 Varian GC equipped with a

Model 8100 autosampler, a Model 1093 septum-equipped programmable injector with a high performance insert and a FID (Varian, CA, USA). The analytical column was a flexible fused-silica column (0.32 mm ID and 0.25 μm film thickness) coated with cross-linked 5% phenylmethyl silicone (Supelco, Bellefonte, USA). The carrier gas (helium) flow was set at 10 psi of column head pressure, and the total flow of carrier gas and make up gas (helium) to the FID was kept at 30 ml/min. The flow of fuel gas (hydrogen) was adjusted to 30 ml/min and the air flow was set at 300 ml/min. The temperature of the FID was 345°C. Column temperature was programmed to rise from the initial temperature to 115°C at 40°C/min, to 225°C at 25°C/min, to 280°C at 15°C/min, and to the final temperature, 340°C, at 5°C/min, and then to hold for 4 minutes. Data acquisition, baseline subtraction, quantification and chromatogram re-plotting were processed with Varian GC Star Workstation software.

The parameters for autoinjection, the injection temperature program, initial column temperature, type of carrier gas used, injection technique, length of the column and septum types in the GC were optimized in this study.

In order to obtain good quantitative data in the broad acyl carbon number range of marine neutral lipids, a wide carbon number range of standard TAG, including 30:0, 42:0, 48:0, 54:0 and 60:0, was used. Intermediate points were calculated by mathematical interpolation (Christie, 1989). During analyses of samples, GC performance, especially f_w, was checked daily using the standard mixture of 14 neutral lipid compounds (as listed in Table 3.4). A same temperature program run with 1 μl solvent injection was inserted between two sample analyses to purge retained components from previous analysis so as to minimize their interference. Maintenance for keeping relatively constant f_w values was performed regularly, such as cleaning the flame tip, the collector tube and the insulator in the FID, and injecting 1 μl of mixed TMS reagents to minimize active sites in the GC system.

The use of a retention gap was tried and was made as follows. One 1 m x 0.53 mm ID deactivated empty fused silica column (Supleco, PA, USA) and an analytical column were cut with a silica cutter (Supleco, PA, USA) so that the ends were square and even, and ensuring that there was undamaged polyimide coating right to the tips of the columns. The tips were moistened with methanol and the parts near the tips were coated with a little polyimide resin (J&W, CA, USA). Then the column ends were gently pushed into the tapered seat of the all-glass connector (Supleco, PA, USA). Finally, the assembled retention gap was cured under a low carrier gas flow and a slow oven temperature program up to 250°C for four hours.

Analysis of Fatty Acid Methyl Esters

FAME analyses were carried out with the same GC model using a flexible fused silica column (30 m x 0.32 mm ID) coated with Omegawax™ 320 (Supelco, PA, USA). The gases used and the flow rates were the same as those for neutral lipid profiles. Injection temperature was programmed to rise from 65°C to 250°C at 200°C/min, and then to hold for 0.10 minute at 250°C. Column temperature was programmed to rise from

70°C to 195°C at 40°C/min, to hold for 8 minutes at 195°C, then to rise to 240°C at 3°C/min, and finally kept constant for 5.4 minutes at 240°C. The temperature of the FID was kept at 260°C. Peaks were identified by comparing their retention times with those of FAME standards: menhaden oil, PUFA-1 and PUFA-2 FAME mixtures (Supelco Canada, ON, Canada), and 13:0-21:0 and 20:0-20:4 pure FAME (Alltech, IL, USA).

2.2.5.2 Gas Chromatography-Mass Spectrometry

Some of the identities of the methylsilylated neutral lipid compounds were confirmed with a Hewlett-Packard 5890/5971A gas chromatograph-mass spectrometer (GC-MS) using 70-eV electron impact ionization over the scanning range of 35-650 m/z. The mass detector temperature was 260°C. Neutral lipid compounds were separated on the same column as that for the above neutral lipid profiles except that a 0.25 mm ID column was used. Following manual on-column injection at 150°C, the column temperature was programmed to rise to 275°C at 10°C/min, to 310°C at 4°C/min, to 340°C at 2°C/min and then to hold for 3 minutes at 340°C.

2.2.5.3 Thin Layer Chromatography-Flame Ionization Detection

Lipid classes in aliquots of the extracts were directly separated on silica gel-coated Chromarods-SIII using four different solvent systems and measured in an Iatroscan MK V after development with each solvent system (Parrish, 1987).

2.2.6 Multivariate Method

A two-level multivariate analysis, Latin Squrre L_8 (2^7), was used to screen a number of potentially important variables in the autosampler, as well as in the injection temperature program and the initial column temperature. As shown in Table 2.1, L_8 (2^7) means that in 8 runs 7 variables were scanned and each at two levels. The factor values at low and high levels were selected based on the literature, or 0.3 of full-range of the variables was selected as the low level, and 0.7 of full-range as the high level. The changes of factors were run independently, thus allowing uncorrelated parameter estimation. Compared to conventional one-variable-at-a-time approach, Latin Square method is multivariate, faster (rapid reduction) and much objective. Method 9 in Table 2.1 uses the parameters recommended by the manufacturer, Varian.

Two responses were selected to measure chromatographic behaviour. The primary one was the relative recovery of 54:0 TAG which was a predominant acyl carbon number in most marine samples. A second factor was the resolution between cholesteryl arachidate and tripalmitin (48:0 TAG), two closely eluted compounds in the chromatogram. A higher recovery of long chain TAG is usually taken to be more important than the separation efficiency in analysis of natural lipid mixtures. The prime response values were evaluated with the following equation:

$$\text{Chromatographic response values (CRV)} = \Sigma(L_i) - \Sigma(H_i) \qquad (2.1)$$

Where L_i is the recovery of 54:0 TAG at the low level of the variable$_i$ and H_i is the same recovery at the high level of the same variable$_i$. Positive values meant that the

Table 2.1. Reduced factorial design, L_8 (2^7), in the first experimental block[1]

Method No.	Plug Size (µl)	Injection time (min.)	Viscosity factor	Injection rate (µl/sec)	Injection ramp (°C/min)	T_{Max} Injection (°C)	T initial column (°C)	Results (n=2)	
								Recovery[2] (M±SD, %)	Resolution[3] (%)
1	0.5	0	2	1	100	340	62	21.8±3.4	93
2	0.5	0	2	8	250	355	82	39.9±2.9	83
3	0.5	1	4	1	100	355	82	33.8±2.3	95
4	0.5	1	4	8	250	340	62	40.4±1.5	53
5	2	0	4	1	250	340	82	37.6±1.6	80
6	2	0	4	8	100	355	62	65.4±3.8	50
7	2	1	2	1	250	355	62	45.1±4.2	80
8	2	1	2	8	100	340	82	57.8±6.0	75
9[4]	1	0	1	5	150	360	80	43.9±4.3	90
CRV[5]	-1.40	-0.247	-0.253	-1.303	0.319	-0.533	0.319		

[1]: each compound at 5 ng/injection, helium carrier gas a 5.5 m length of DB-5 column and a "Thermolite" septum; remaining conditions as in the Section 2.2.5.1

[2]: recovery of 54:0 TAG compared with the internal standard, 30:0 TAG

[3]: resolution between cholesteryl arachidate and 48:0 TAG (100% = complete baseline separation)

[4]: manufacturer recommended method

[5]: Chromatographic response values

low level was better, if negative, the high level was better. Large absolute CRV suggested that this variable was more important and significantly affected the recovery of 54:0 TAG.

In the second experiment (Table 2.2), the better points of each variable in the previous run served as the central value. New values of the two-levels were selected from the middle points between the better points in last run and the limits of these variables, and the middle points between the better and bad points in last run. The response-surface method was employed to locate the optimum over the major experimental variables.

Table 2.2. Reduced factorial design of the second experimental block[1]

Method No.	Plug Size (μl)	Injection time (min.)	Viscosity factor	Injection rate (μl/sec)	Injection ramp (C°/min)	T_{Max} Injection (C°)	Recovery (mean±SD, %)[2]
1	1	0	1	1	100	340	67.6±2.1
2	1	0	1	5	200	360	75.8±4.2
3	1	0.5	3	1	100	360	68.2±3.6
4	1	0.5	3	5	200	340	72.7±1.6
5	3	0	3	1	200	340	74.4±1.6
6	3	0	3	5	100	360	88.1±2.2
7	3	0.5	1	1	200	360	66.0±0.4
8	3	0.5	1	5	100	340	87.4±4.9
CRV	-0.635	0.235	-0.129	-0.959	0.449	0.079	

[1]: same conditions as those in footnote 1 in Table 2.1

[2]: triplicate recovery of 54:0 TAG compared with the internal standard, 30:0 TAG, in a random injection sequence

3 OPTIMIZATION OF THE GAS CHROMATOGRAPHY CONDITIONS FOR NEUTRAL LIPID PROFILES

3.1 Optimization of Autoinjection

The transfer of the liquid sample from the syringe needle to the internal wall of the column is a crucial step in obtaining a high degree of accuracy and precision due to the high molecular weight of TAG and the corresponding high temperatures required for GC elution. Factorial design offers a very effective coverage of the experimental domain in comparatively few experiments. By varying all of the experimental factors simultaneously, the main effects of the variables and the potential interactions can be determined (Olsson *et al*, 1990).

Absolute CRV calculated in Table 2.1 and 2.2 show that the order of importance is: injection rate ≥ plug size >> injection ramp > maximum temperature of injector > injection time ≈ solvent viscosity factor ≥ initial temperature of the column.

The autosampler injecting rate and the solvent plug size appeared to be critical to increase the recovery of 54:0 TAG. The optimum, the apex of the response surface of recovery of 54:0 TAG (the recovery rather than CRV was used for a direct comparison) over these two experimental domains, was located at 3 µl plug size and 5 µl/sec injection rate, as illustrated in Figure 3.1. A rapid injection pushes most of the sample into the column or the insert of the GC and thus eliminates the possibility of sample "coating" the outside of the needle, while slow injection leaves some liquid hanging at the needle tip.

As a consequence, a greater proportion of high boiling compounds remains on the withdrawn needle, causing discrimination against high molecular weight TAG (Grob, 1994). A similar result was obtained by Grob *et al* (1979) with alkanes. Large solvent plugs probably flush most of the remaining viscous neutral lipid compounds from the needle to the column, as well as serve as a wall to protect against possible backflushing of the sample in the column or insert during the rapid increase of column temperature. Large plug sizes also enlarge the peak area of 54:0 TAG with smaller standard deviations thus improving the precision of the analysis. As shown in Figure 3.2, the peak area of 54:0 TAG almost doubles with a change in plug size from 0 to 3 μl. But this advantage is accompanied by a decrease in the resolution between cholesteryl arachidate and 48:0 TAG.

It should be noted that interactions between most variables are insignificant. Only the injection rate and plug size showed a definite co-effect on the recovery of 54:0 TAG. CRV of summed same levels in both predominant variables (HxH plus LxL) were greater than that of summed different levels (HxL plus LxH). The entire sequence for these two variables from the most significant to insignificant was HxH > HxL or LxH > LxL. This was quite predictable. A rapid injection not only eliminates sample filming the outside of the needle but also forces the solvent plug behind the air gap and the sample segment to spray from the syringe to the column in order to flush out any lipids remaining in the syringe. Both functions enhance recovery of high molecular weight compounds.

The septum-equipped temperature-programmable injector (SPI) allows the injector

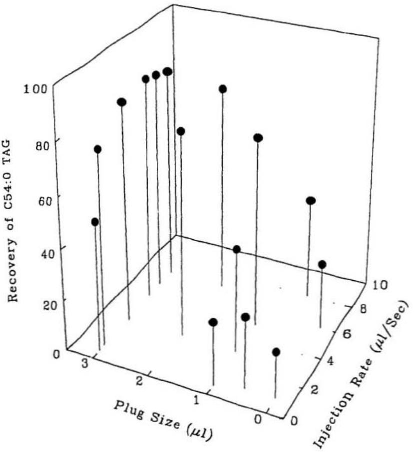


Figure 3.1. Response-surface plot of recovery of tristearin (54:0 TAG) versus solvent plug size and injection rate (helium carrier gas, a 5.5 m length of DB-5 and "Thermolite" septum, for the remaining conditions see the Section 2.2.5.1)

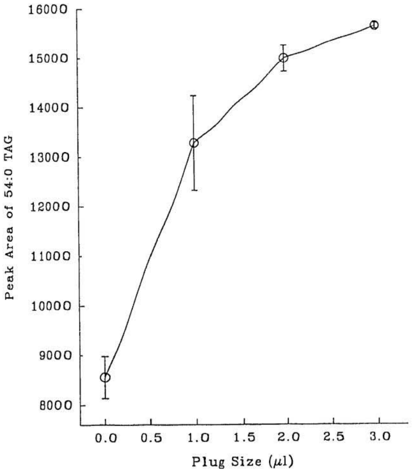


Figure 3.2. Effect of solvent plug size on peak area of tristearin (helium carrier gas, a 5.5 m length of DB-5, "Thermolite" septum, chloroform solvent plug, 5 μl/sec injection rate, 0.0 min injection time, viscosity factor 2, 100 °C/min injection ramp, 355 °C maximum injection temperature, 61°C initial column temperature, for the remaining conditions see the Section 2.2.5.1)

temperature to change independently from the column oven temperature. Other parameters also improved chromatographic performance at certain levels. The rate of the injector temperature program seemed to have quite an influence on CRV next only to the effects of the solvent plug and injection rate (Table 2.1 and 2.2). The rate at 100°C/min achieved much better results than that at either 200 or 250°C/min since this rate created a speed of evaporation that was faster than the column ramp to transfer sharp peaks to the column but not so fast as to yield wild evaporation and to produce a flashback from the column.

The initial injector temperature, like the initial column temperature, was set close to the boiling point of the solvent to let the entire sample deposit on the column in liquid form. The final injection temperature was set at 15°C above the maximum column temperature. This achieved higher 54:0 TAG recovery because the top of the injector which includes the septum is cooler than the main body of the injector. A higher temperature for the injector prevents high boiling components from recondensing in a relatively cool injector head.

The initial temperature of the column was tested to determine if solvent or solute focusing techniques were better. Setting the temperature slightly below the solvent boiling point allowed higher recovery of 54:0 TAG, shortened the interval between two analyses and avoided possible phase stripping in the front coils of the column as compared to the solvent focusing technique in which the initial column temperature is usually set at 10-20 C° below the boiling point of the solvent. After a rapid injection which deposits samples onto the column or the insert of the injector, a column temperature near the boiling point of the solvent produces sufficiently slow evaporation to avoid violent backflow. On the other hand, solute focusing, with rapid solvent evaporation, cannot transport all the vapour through the carrier gas flow because the initial column temperature is far above the boiling point of the solvent. Too high sampling temperatures at both the injector and the column cause difficulties with fast and complete transfer of samples into the column thus decrease the column resolution, as well as the precision and accuracy of quantitative analyses (Kuksis *et al*, 1989).

Reducing the dwelling time of the syringe needle in the injector inlet from 1 minute to 0.5 minute, then to 0 minute slightly increased 54:0 TAG recovery. The short dwell time prevents needle heating and associated needle discrimination problems where a partial evaporation in a hot needle results in some high molecular weight compounds remained in the withdrawn needle. Similarly, Klee (1990) found that longer needle dwell times were associated with smaller peak areas as carbon numbers of n-alkanes increased. These observations can also be explained by assuming that some time is needed in order to build up an effective back-flow (Grob *et al*, 1980).

Injection volume is also important to receive reproducible, undiscriminated data. Too small injection volumes not only yield large standard deviations between injections but also deplete the speed of the liquid at the needle tip. On the other hand, back-flow through the column entrance and the possibility of shortening column lifetimes may be problems with large injection volumes (Grob *et al*, 1980). Comparison of the different injection volumes showed that a volume of 1 μl was the most suitable.

3.2 Carrier Gases

Sorts and flow rates of carrier gas, make up gas, combustion gas and fuel gas paly an essential role on the FID output. Based on the GC manual, the optimum flow ratio of carrier gas and make up gas : fuel gas (hydrogen) : combustion gas (air) was set at 30:30:300 ml/min for the 0.01-inch flame tip in the FID. Total flow rate of carrier gas and make up gas was kept constantly during optimization of the types and the flow rates of carrier gases due to flame instable at the higher flow rates (Simon, 1985).

Hydrogen is the least viscous gas, thus compounds have higher diffusion rates in it. In comparison with helium, hydrogen carrier gas provides superior efficiency over a larger linear velocity range and its optimum linear velocity is much higher (Table 3.1). At the same column head pressure, the retention times of high molecular weight TAG, such as 54:0 and 60:0 TAG, decreased 20-30 % with hydrogen carrier gas as compared to helium so that unstable high molecular weight lipids have less chance to decompose during GC analysis. Furthermore, the higher optimum flow rate of hydrogen maintained similar resolution that achieved by helium at a lower flow rate. So, overall, analysis time using hydrogen as carrier gas can be shortened and column lifetime prolonged.

The flow rate also has a considerable effect in optimizing GC performance. The data with hydrogen carrier gas set at 12 and 4 psi of column head pressure showed that there was no difference at the low TAG carbon number range, C_{30} to C_{48}, in both the FID response and peak sizes, but higher flow rates definitely minimize discrimination in the FID responses for higher carbon number TAG (Figure 3.3). This observation confirms

Table 3.1. Comparison of carrier gas flow rates and retention times for 54:0 and 60:0 TAG on a DB-5 column[1]

Gas Pressure (psi)[2]	Flow Rate (ml/min)[3]		Retention Time (min)				Resolution[4]	
	H_2	He	H_2		He		H_2	He
			54:0	60:0	54:0	60:0		
12	23.1	16.7	17.81±0.04	21.54±0.08			90	
10	21.0	12.5	18.41±0.04	22.20±0.06	22.20	28.90	95	70
8	17.6	9.38	19.51±0.08	23.63±0.08			95	
6	14.0	6.25	20.43±0.04	-[5]			90	
4	8.63	3.45	22.02±0.09	-[5]			80	

[1]: each compound at 5 ng/injection and at optimized conditions: column length (5.5m x 0.32mm ID), 3 µl chloroform solvent plug, 5 µl/sec injection rate, 0.0 min injection time, viscosity factor 2, 100 °C/min injection ramp, 355 °C maximum injection temperature, 61°C initial column temperature and "Thermolite" septum, n ≥ 2

[2]: carrier gas pressure at the column head

[3]: measured at ambient temperature

[4]: resolution between cholesteryl arachidate and 48:0 TAG (100% = complete baseline separation)

[5]: retention time of 60:0 TAG > 25 minutes

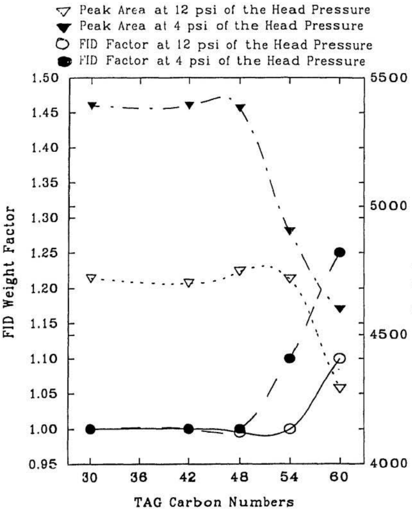


Figure 3.3. Dependence of the FID weight correction factor (f_w) and the peak areas of TAG with different carbon numbers on the flow rates of hydrogen carrier gas (at optimized conditions: a 5.5 m length of DB-5 column, 3 µl chloroform solvent plug, 5 µl/sec injection rate, 0.0 min injection time, viscosity factor 2, 100 °C/min injection ramp, 355 °C maximum injection temperature, 61°C initial column temperature and "Thermolite" septum)

those of Mares *et al* (1985) with hydrogen carrier gas and Hinshaw *et al* (1989) with helium.

The possibility of an explosion is a major concern using hydrogen carrier gas, especially if the GC is left running automatically overnight. As the explosive threshold for hydrogen in air is 4 %, keeping the fume hood fan working, and regularly checking for leaks should prevent any potential accidents. However, for reasons of safety, helium carrier gas was used initially in this methodology study as well as for the analyses of most marine samples.

3.3 Injection Techniques

Manual injection is required for the analysis of trace amounts of lipids when there is not sufficient material for an autosampler to remove a sample from the vial (minium volume is 0.4 ml/sample). Compared to autoinjection, manual injection methods exhibited slightly higher recoveries of 54:0 TAG (Figure 3.4). However, resolution of cholesteryl arachidate and 48:0 TAG was very different. The reason may be that manual solvent flush injection (method 3 in figure 3.4) cannot inject as fast as the autoinjecter, thus allowing the solute to migrate in a relatively greater solvent matrix and making the peaks wider. Manual injection without the solvent plug (method 2 in Figure 3.4) gave the best resolution. However, as mentioned above, no solvent plug led to the smallest peak size and to the highest standard deviation.

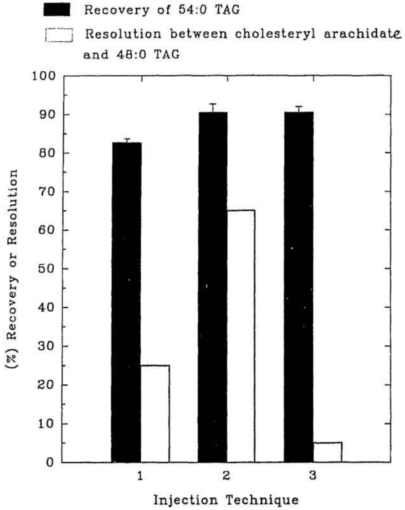


Figure 3.4. Influence of injection technique on recovery and resolution at optimized GC conditions (1, autoinjection, 2, manual with lower air gap, and 3, manual solvent flush injection, mean±S.D, n≥3, under optimized conditions as listed in Figure 3.3 and helium as the carrier gas)

3.4 Length of the Capillary Column

The suitable column length was tested under optimized conditions and using helium as the carrier gas. In reference to recovery of 54:0 TAG, durability of the column and resolution in chromatograms, a column length of 5.5 meters turned out to be best (Table 3.2). Higher elution temperatures and increased residence time in the chromatographic system lead to possible polymerization and/or decomposition of high molecular weight neutral lipid components and result in their disappearance or lowered recoveries (Mares, 1988). Both factors increase with column length so that the shortest column which still provides acceptable resolution of sample components is best.

3.5 Septum Types

Differences in recovery of 54:0 TAG and the backgrounds of chromatograms with different septum types in the injector may result from different sealing status in subsequent injections (Table 3.3). The higher the temperature limit of the septum, the harder is the septum material, which leads to weaker sealing ability. The septum "Thermolite" (Pestek, PA, USA) displayed the best performance among the six kinds of tested septa.

The septum needs to be changed approximately every one hundred injections because small septum fragments frequently pass to the inlet of the column and produce ghost peaks, while the sealing condition progressively deteriorates.

Table 3.2. Recovery and retention time of tristearin (54:0 TAG) with respect to the column length[1]

Length of DB-5 Column (m)	8.6	8.1	7.5	5.5
Recovery of 54:0 TAG (mean ± SD, %)	-	71.9±3.9	89.2±2.1	90.7±2.5
Retention Time of 54:0 TAG (min)	not eluted after holding column at 340°C for 14 min.	32.1	25.7	22.2

[1]: minimum of 3 injections each of 10 ng under optimized conditions (as in footnote 1 in Table 3.1) for each length of the column; helium as the carrier gas

Table 3.3. Evaluation of six kinds of septa used in the injection port[1]

Septa	T_{max} (°C)	Relative Recovery of 54:0 TAG (mean±SD, %)	Nature of Chromatogram
Thermolite[2]	340	89.1 ± 3.1	clean baseline
77 HT[3]	400	75.9 ± 8.4	clean baseline
Red HT[3]	400	75.6 ± 4.2	clean baseline
Blue[3]	350	74.4 ± 7.0	clean baseline
Thermogreen[4]	300	89.1 ± 2.5	rough baseline at beginning
White5[5]	?		peaks split; "ghost" peaks present

[1]: helium as the carrier gas and minimum of three injections under optimized method with each kind of septum
[2]: septum from Restek, Bellefonte, PA, USA
[3]: septa from Chromatographic Specialties Inc., Brockille, ON, Canada
[4]: septum from Supelco Inc., Bellefonte, PA, USA
[5]: septum from Varian Inc., Sugar Land, TX, USA

3.6 Retention Gap

Use of a 1 m x 0.53 mm ID retention gap consisting of a deactivated empty fused silica column generally offered sharper peaks, minimized peak tailing and splitting, especially at higher carrier gas flow rates. Connecting this pre-column and the analytical column by an all-glass press-fit connector was superior to that using a metallic connector since the latter had a larger dead volume thus producing a very sloping initial baseline in the chromatograms. However, the durability of the connection between the retention gap and the column was poor because of the large temperature changes used in the column oven. Slight leaking occurred within a few days, even with the connection coated with polyimide resin to help make it gas tight. Bemgard *et al* (1992) recommends replacing polyimide resin with epoxy resin glue to effect all-glass connections for both high temperature and high pressure. It would be worthwhile trying this glue in future work.

3.7 Solvents

The results of using different solvents in the solvent plug of the autoinjector and in the sample solution, including chloroform, hexane, petroleum ether and pyridine, showed chloroform to be the best with respect to solubility for all ranges of neutral lipid compounds and recoveries for high molecular weight TAG.

3.8 Influence of Sample Composition on the Chromatograms

Interferences or overlap of individual neutral lipid compounds have been carefully studied. TAG are a main group of neutral lipids in most marine samples. TAG with acyl carbon numbers over 49 usually are the sole components at the high temperature region during GC analysis. However, the C_{48} TAG peak may overlap with that of cholesteryl arachidate if both are major compounds as is the case for lipids isolated from marine species. Their resolution has been selected as a critical objective in evaluating GC performance. Generally, hydrogen carrier gas achieved better resolution than helium for this pair of compounds.

A small proportion of TAG may have carbon numbers below 48, which are commonly found to have even carbon numbers down to 34 or so, but do not reach C_{10} in most samples. These minor compounds may interfere in the determination of cholesteryl esters with carbon numbers 47 or 45. In most cases, the concentrations of these low carbon number TAG are sufficiently low, so that their effect on the determination of cholesteryl esters is negligible. But a few marine samples are rich in low carbon number TAG (Litchfield *et al*, 1971). Such an significant interference for cholesteryl esters can be resolved by prehydrogenation of samples, which enables good separation of C_{47} cholesteryl ester from C_{46} TAG (Mares *et al*, 1979).

After establishing that there were no detectable TAG with carbon numbers over 62 in marine samples analyzed, the upper temperature limit of the GC analyses was set at the retention time of C_{63} TAG to prolong the column life.

Wax esters, found in abundance in some marine species (Falk-Petersen *et al*, 1987), may cause overlaps at the low temperature end of the chromatogram. For instance, the internal standard, tricaprin, may be interfered with by palmitic acid stearyl ester. This problem can be avoided by scanning for potential interferences with tricaprin.

3.9 Precision and Linear Ranges of Saturated Neutral Lipid Standards

Quantitative analysis requires not only independence of the f_w values on the compositions and size of neutral lipid compounds, but also reproducibility of the measurement over wide ranges of concentration.

Precision of the optimized method can be seen from a repeated sequence of measurements with a mainly saturated synthetic mixture which represented most neutral lipid subclasses in marine samples (Table 3.4). With helium carrier gas and at 5 ng/compound, the relative standard deviation of peak areas for most compounds was below 5 %. Only cholesteryl arachidate showed a somewhat larger variation (8.5 %) due to partial overlap with 48:0 TAG. FID weight correction factors (f_w) ranged from 0.6 for most sterols to 1.2 for 54:0 TAG compared to an internal standard, 30:0 TAG. These were comparable with published data (Kuksis *et al*, 1975). With a 28-minute run and using an autosampler, over 40 injections could be accomplished daily. Thus this method is relatively precise and rapid.

Values of f_w depend on the sample size and on the absolute amounts of the neutral lipid compounds in the injected samples. Table 3.5 shows that the linear ranges of each

Table 3.4. Reproducibility of relative retention times and peak areas, and measurements of FID weight correction factor (f_w)[1]

Lipid Compounds	Carbon Number[2]	Retention Time (min)	Peak Area	C. V. of Peak Area (%)	f_w
octodecane	C18 HC	3.236±0.007	3735±43	1.151	0.7756
nonadecane	C19 HC	3.634±0.006	3913±133	3.399	0.7222
palmitic acid	C16 FA	4.214±0.007	3870±101	2.610	0.6952
stearic acid	C18 FA	4.919±0.006	3663±154	4.204	0.7185
cholesterol	C27 ST	7.968±0.009	4219±20	0.4740	0.6518
campesterol	C28 ST	8.366±0.009	3558±17	0.4778	0.6234
sitosterol	C29 ST	8.701±0.010	4564±69	1.502	0.6030
tricaprin	30:0 TAG	9.450±0.012	2661±69	2.602	1.000
stearic acid stearyl ester	C36 WE	10.45±0.014	2804±148	5.293	0.9358
cholesteryl palmitate	C43 SE	16.95±0.026	2923±69	2.360	0.9260
cholesteryl stearate	C45 SE	18.54±0.026	2663±44	1.646	0.9380
cholesteryl arachidate	C47 SE	20.11±0.027	2320±198	8.527	1.100
tripalmitin	48:0 TAG	20.36±0.027	2775±47	1.707	0.9131
tristearin	54:0 TAG	25.52±0.045	2159±80	3.703	1.226

[1]: 5 consecutive runs at the optimized conditions (as those in footnote 1 in Table 3.1) compared with an internal standard, 30:0 TAG, each compound at 5 ng/injection, and helium as the carrier gas
[2]: carbon numbers in hydrocarbon (HC), fatty acids (FA), sterols (ST), wax esters (WE) and steryl esters (SE) are summed carbon numbers; in triacylglycerols (TAG) summed acyl carbon numbers are used.

neutral lipid compound under optimized conditions encompassed three orders of magnitude. The detection limits of most compounds reached 0.01 ng/compound, about three orders of magnitude lower than those of TLC-FID. However, linear ranges only reach 1 ng/compound for most tested neutral lipid subclasses. At 0.1 ng/compound, their f_w change from 25 % to 75 % from the values in their linear curves as the tiny compound peaks are near the size of background noise. Maximum loading amount was up to 400-500 ng/compound. Above that, peak tailing seriously decreased the resolution between adjacent peaks.

3.10 The Role of Hydrogenation

3.10.1 Problems without Hydrogenation

Model systems using mainly saturated neutral lipids are unable to match all molecular species in real samples, particularly when samples contain a high percentage of long chain PUFA which are common in samples from subpolar marine environments. The chromatographic behaviour of highly unsaturated neutral lipids in high temperature GC could be inconsistent with those of saturated or monounsaturated homologues. Most systematic GC studies have omitted the effect of unsaturation. Mares *et al* (1983b, 1985) optimized GC conditions without examining hydrogenation in the analysis of intact long chain TAG. They used both short nonpolar capillary and packed columns and optimized a range of parameters. However, only saturated TAG with different chain lengths were

Table 3.5. FID weight correction factor (f_w) of typical neutral lipid subclasses versus individual amounts per injection[1]

Neutral Lipid Compounds	Amount (ng, n=3)				
	0.1	1	10	100	500
octadecane	1.601±0.899	0.961±0.094	0.757±0.035	0.716±0.029	0.704±0.002
palmitic acid	0.931±0.024	0.842±0.069	0.749±0.032	0.718±0.031	0.713±0.013
monopalmitin	1.101±0.123	0.629±0.168	0.729±0.033	0.716±0.030	0.724±0.013
cholesterol	0.667±0.254	0.753±0.172	0.666±0.030	0.679±0.028	0.677±0.014
tricaprin (30:0 TAG)	1.000±0.149	1.000±0.025	1.00±0.043	1.000±0.043	1.000±0.022
stearic acid stearyl ester	1.426±0.490	0.986±0.248	0.922±0.097	0.845±0.043	0.836±0.018
dipalmitin	0.453±0.095	0.741±0.036	0.915±0.045	0.883±0.083	0.851±0.019
cholesteryl stearate	0.706±0.264	0.846±0.088	0.959±0.048	0.932±0.042	0.903±0.021
tripalmitin	0.663±0.290	0.969±0.232	1.061±0.045	1.013±0.042	0.993±0.023
tristearin	1.000±0.199	1.255±0.106	1.283±0.057	1.187±0.046	1.152±0.028

[1]: as optimized conditions (as in footnote 1 in Table 3.1) and helium as the carrier gas

employed in their model mixtures probably because of the small proportions of long chain PUFA in their biological samples. Subsequently, Mares (1987) explained that higher f_w values were used to compensate for losses of unsaturated lipids. However, the diverse levels of unsaturation in neutral lipids with the same carbon numbers suggests there could be a large range in differences in their f_w.

Discrimination according to the degree of unsaturation on the recoveries of TAG was observed with standards using either hydrogen or helium as the carrier gas (Figure 3.5). An increase in the number of double bonds accompanied a decline in recoveries of TAG with the same carbon number, C_{54}. Highly unsaturated TAG with helium as carrier gas stayed in a high temperature for a longer time (Table 3.1) so that they gave poor precision. This suggests TAG rich in PUFA are susceptible to adverse effects at high temperature. Gilkison (1989) observed a similar result with helium carrier gas and a polar stationary phase, 65% phenylmethyl silicone. Losses of unsaturated TAG may not be reproducible when there are over six double bonds in a TAG molecule or the number of double bonds in an individual acyl group is greater than three (Mares, 1988).

This recovery problem was even more serious in lipids from actual cold water samples. In Figure 3.6, the Y axis gives the ratios of hydrogenated over unhydrogenated samples, each divided by the peak area of the internal standard, 30:0 TAG. At the lower carbon number range, 50 to 56, the ratios were near one, but at the higher carbon number region, a steep increase of ratios with larger standard deviations was observed. Mares *et al* (1979) found that the amounts of TAG in higher carbon numbers, such as 56 and 58,

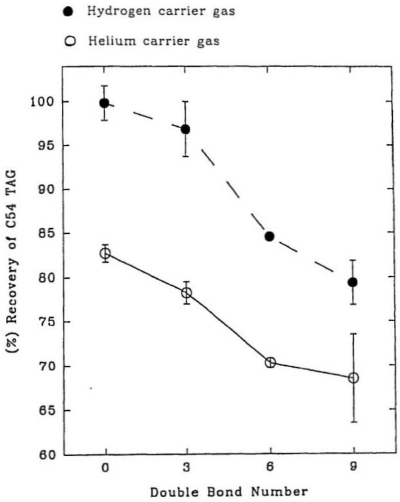


Figure 3.5. Relative recovery of synthetic C_{54} triacylglycerols in relation to the degree of unsaturation (mean±S.D., n=3, the flow rate of both carrier gases at 10 psi of column head pressure and under optimized conditions as listed in Figure 3.3)

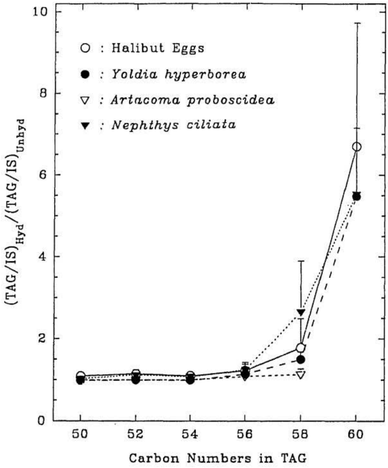


Figure 3.6. Effect of hydrogenation on recovery for different acyl carbon numbers in TAG from four kinds of cold water marine animals (mean±S.D., n≥3, under optimized conditions as listed in Figure 3.3 and with helium as the carrier gas)

were significantly different before and after hydrogenation. Their biological samples did not contain TAG with carbon numbers of 60 or higher and their mean carbon numbers were lower compared to these samples from a cold ocean environment. They concluded that differences in recoveries of high molecular weight TAG with respect to hydrogenation had only a small influence on the recovery of the total TAG. Here, the mean carbon numbers of TAG in the cold ocean animals ranged from 52 to 56, which indicated about 50% or more TAG with carbon numbers greater than 54. This implied that they probably accumulated quite lot of long chain PUFA. Compared to the results of hydrogenated marine samples, carbon number distributions of unhydrogenated TAG were shifted to the lower carbon number side, thus leading to biased carbon number patterns.

The bias in TAG carbon number distributions was confirmed by comparison with the mean chain length per fatty acid provided by FAME analysis. The mean chain length per fatty acid determined by neutral lipid profile of unhydrogenated samples (1/3 of mean acyl carbon number in TAG in mole %) was significantly lower than that determined by fatty acid analysis ($P < 0.05$). Samples with higher ratios in Figure 3.6 usually showed higher mean double bonds per fatty acid but no linear correlation was found between them. The importance of polyunsaturation is shown by the large percent of long chain PUFA in these samples (Figure 3.7), especially of the essential fatty acids, 20:5n-3 and 22:6n-3 in the neutral lipids. The highest ratio of hydrogenated to unhydrogenated neutral lipids in halibut eggs in Figure 3.6 coincides with their having the highest proportion of 22:6n-3 in fatty acids in the neutral lipid fraction (Figure 3.7). The high ratios in *Y.*

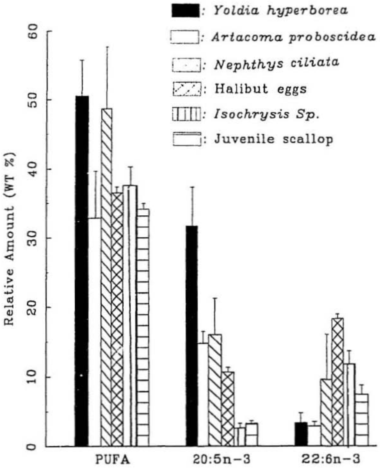


Figure 3.7. Percentage of total PUFA and major representatives in six kinds of marine species (mean±S.D., n≥3, FAME analysis conditions as in the Section 2.2.5.1)

hyperborea and *N. ciliata* (Figure 3.6) were related to them having the largest proportion of PUFA, around 50% of their fatty acid composition, and the largest percentage of 20:5n-3 in *Y. hyperborea* (Figure 3.7). Shantha *et al* (1990) showed similar data in TLC-FID analysis that responses of FID for PUFA-rich marine samples increased 33-45 % after hydrogenation of them.

One explanation for the high proportion of long chain PUFA in those cold ocean species is that a greater amount of intracellular molecular oxygen is available at lower temperatures. This would be required by oxygen-dependent enzymes catalyzing the desaturation of long chain PUFA (Brown *et al*, 1969). Also, unsaturated TAG may be more easily mobilized at subzero temperatures.

TAG with higher carbon numbers often have a greater degree of unsaturation and elute in a higher temperature region with a longer retention time and greater risk of decomposition and/or polymerization during GC analysis. Therefore, hydrogenating samples prior to GC analysis should avoid discrimination with respect to diverse degrees of unsaturation.

Figure 3.8 shows that hydrogenation efficiencies of two kinds of hydrogenators for the most highly unsaturated marine samples, *Y. hyperborea*, were almost identical, at approximately 90 %, ($P > 0.05$). Long chain PUFA containing up to six double bonds per fatty acid may be only partially hydrogenated (Patterson, 1983). In contrast to the complicated single-sample operation of the commercial apparatus, the simple hydrogenation set up in our laboratory is rapid and allows processing of several samples

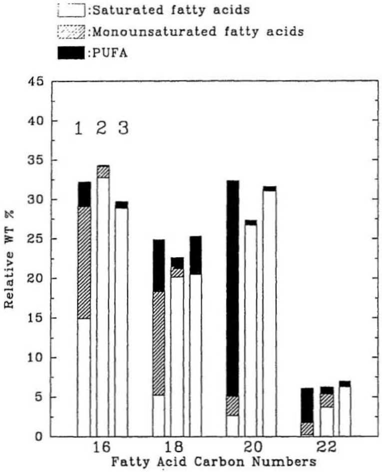


Figure 3.8. Comparison of hydrogenation efficiency in two hydrogenators (1, without prehydrogenation, 2, hydrogenated with a simple home-made hydrogenator, and 3, with a commercial, atmospheric pressure hydrogenator, [sample: *Yoldia hyperborea*, n=2, FAME analysis conditions as in the Section 2.2.5.1])

simultaneously.

3.10.2 Chromatographic Improvement in Hydrogenated Triacylglycerols

In addition to improving separations and ensuring sample stability, hydrogenation simplifies samples with different degrees of unsaturation and locations of double bonds to identical saturated ones so that a single f_w for each carbon number becomes practical (Christie, 1989).

Comparison of typical chromatograms of a bivalve, *Y. hyperborea*, with and without hydrogenation shows that hydrogenated sample peaks are sharper and narrower, and resolution is greatly improved (Figure 3.9). Small TAG peaks with odd and higher carbon number were readily distinguished from large peaks with even carbon numbers (Figure 3.9, B). This is important for marine species since their neutral lipids seem to have wider carbon number distributions as compared to land-based animals, and some marine species contain moderate amounts of odd carbon numbered TAG (Litchfield *et al*, 1967). Peak broadening of natural TAG usually occurs on nonpolar columns due to a relatively early elution of the unsaturated compounds within a given carbon number. This does not allow true resolution of saturated and unsaturated species, but may result in overlap of different carbon numbers, especially if odd carbon number and/or branched-chain compounds are also present. Without hydrogenation, TAG containing high proportion of PUFA exhibited peak shape distortion and tailing (Figure 3.9, A), and small TAG peaks with odd or high carbon numbers were totally covered by major peaks with

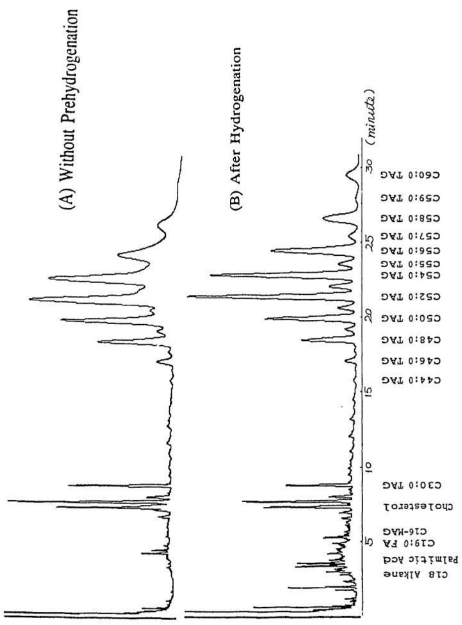


Figure 3.9. Chromatograms of neutral lipids from *Yoldia hyperborea*. (at optimized conditions as listed in Figure 3.3 and with helium as carrier gas)

even carbon numbers.

3.10.3 Chromatographic Improvement in Hydrogenated Sterols

At the lower temperature zone of chromatograms, a better resolution of hydrogenated sterols in terms of carbon number was achieved in sea scallops which have a complex sterol composition (Figure 3.10). In humans, terrestrial animals and fish, cholesterol is the principal sterol compound (> 95% of the total). For this reason most investigators may have focused on TAG in the high temperature area in lipid profiles. However, 20 or more kinds of sterols with wide variations in concentration exist in some marine invertebrates. Complete separation of them requires a column length of up to 60 meters and it takes two hours or more (Napolitano *et al*, 1992).

Unhydrogenated sterols showed overlapping peaks with the adjacent carbon numbers because of different side-chains, the various double bond locations, and the diverse degrees of unsaturation (Figure 3.10, A). For example, C_{27} sterols, 22-dehydro-cholesterol and desmosterol, and C_{28} sterol, brassicasterol, eluted at almost the same retention time between two standards, cholesterol and campesterol, even with a 30 meter DB-5 column (Albro *et al*, 1992). In between these two saturated standards cold water samples contained several sterols which could be identified as either C_{27} or C_{28} depending on fluctuations in GC conditions. This indicates that carbon number profiles for scallop sterols were unreliable without hydrogenation. After hydrogenation scallop sterols were grouped into three major peaks based on carbon numbers. Scallops probably contain small

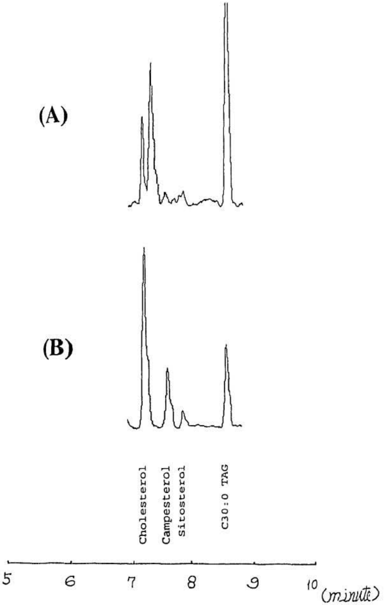


Figure 3.10. Effect of hydrogenation on sterol carbon number distributions in sea scallops (*Placopecten magellanicus*). (A, unhydrogenated, and B, hydrogenated, at optimized conditions as listed in Figure 3.3 and with helium as carrier gas)

proportions of C_{26} and C_{30} sterols as well (Idler *et al.* 1972).

The overlapping adjacent carbon number sterols in sea scallops, 22-dehydrocholesterol and brassicasterol, were hydrogenated to their saturated homologues, the former hydrogenated to the C_{27} standard, cholesterol, and the later became campesterol. Three saturated standards, cholesterol, campesterol and sitosterol, eluted at identical distances with baseline resolution as displayed in Figure 3.10B. Thus, correct evaluation of sterol and TAG carbon number distributions in sea scallops were simultaneously achieved in a 28-minute run.

The carbon numbers of sterols in real samples were also confirmed by GC-MS using a standard mass spectrum library. As shown in Figure 3.11, the mass spectra of the three TMS derivatized sterols contain a few characteristic fragment ions, e.g. m/z 73 which is a TMS ion, and m/z 129 which is a rearrangement ion of the TMS derivative of Δ^5-3β-hydroxy sterols (Middleditch, 1979). The m/z 329, 343, and 357 ions in the three spectra are also due to rearrangement of other sterol fragments which differ by m/z 14 as each additional methylene group.

3.11 Column Chromatography on Florisil

The addition of one percent of formic acid in the solvent system plays an essential role in eluting free fatty acids. Otherwise, they are firmly retained by the Florisil. It is also important to have sufficient volume of eluent. Four mL of optimized eluent containing 1 % formic acid did not elute free fatty acids from the bed. By increasing the

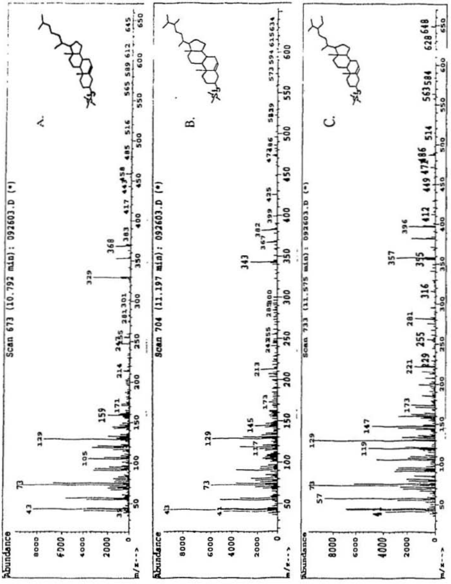


Figure 3.11. Mass spectra of three major sterol TMS derivatives (A. cholesterol, B. campesterol and C. sitosterol, GC-MS conditions as listed in the Section 2.2.5.2)

eluent from 6 to 8 ml, the recovery of 19:0 fatty acid rose from 76 % to 99 %. The relative recoveries of individual synthetic neutral lipid compounds ranged from 91 % for wax ester to 106 % for cholesterol, compared to the internal standard, 30:0 TAG. The only exception was monopalmitin with 73 % recovery due to its relatively high polarity.

3.12 Comparison Between Gas Chromatography and Thin Layer Chromatography-Flame Ionization Determination

Quantitative standards for complex lipids are normally not available. Therefore, the analytical results of the complex lipid mixtures in the cold ocean samples were validated by another accepted chromatographic method, TLC-FID, which is a basic lipid profiling method used in our laboratory (Parrish, 1987). Since these two methods have different separation abilities, the sum of molecular species estimated by GC can be compared to the estimate for each neutral lipid subclass in TLC-FID analysis. In three marine invertebrates, summed molecular species from each major neutral lipid class were linearly correlated to corresponding subclasses determined by TLC-FID (Figure 3.12). A log-log plot was used for the TAG data as it ranged over two orders of magnitude. The error bars for TLC-FID of sterols are slightly larger. This was similar to the comparison for plasma (Mares *et al*, 1983a).

Although complete hydrogenation causes the loss of original double bonds in neutral lipid molecules, in terms of lipid profiling, no information is lost since the GC method achieves separations based on carbon numbers. Furthermore, many marine species possess characteristic lipid compositions that can be recognized without complete resolution and determination of individual molecular species. Quantitative estimation of partially resolved lipid structures can be sufficient for establishing the origin of a neutral lipid extract and for evaluating physiological conditions of marine species.

Some examples that employed the optimized high temperature GC method follow.

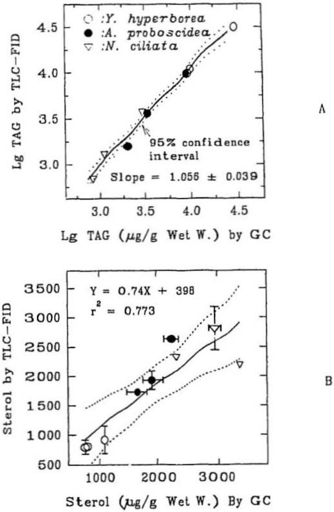


Figure 3.12. Correlation of neutral lipid amounts in three marine invertebrates analyzed by GC-FID and TLC-FID (A. triacylglycerols, and B, sterols, mean±S.D., n=2, at optimized conditions as in Figure 3.3 and with helium as carrier gas)

4 APPLICATIONS

4.1 Laboratory Grown Marine Plants

The carbon number distributions in the neutral lipids of *Isochrysis galbana* (T-iso) were distinctly different under different growth conditions (Figure 4.1). *I. galbana* grown under conditions of nitrogen limitation, had a maximum at C_{50}, while *I. galbana* grown under nutrient-replete conditions had an apex at C_{54}. However, the *I. galbana* grown in the continuous culture had a higher proportion of higher carbon number TAG than did *I. galbana* grown in nutrient-replete semi-continuous (batch) culture.

These differences are supported by the TLC-FID data where the twice amount of more saturated TAG (including TAG with monoenic acyls) are in the nitrogen-limited *I. galbana* as compared to that in the other two kinds of cultures (Parrish *et al*, 1993a). This indicates there are more relatively shorter fatty acids, such as 14:0, 16:0 and 18:0, rather than longer chain length PUFA in the nitrogen-limited *I. galbana*. The major fatty acids in the neutral lipids, 16:0 and 18:1n-9, follow this trend with the level of 16:0 shifting from 19 % in the nitrogen-limited *I. galbana* to 13 % in the batch *I. galbana* and then to about half the amount (9 %) in the continuous culture. But a reverse trend is found in 14:0 in these three kinds of cultures (Table 4.1). It should be noted that these data were based on only three cultures under each growth condition, and that there can be considerably biological variation for algae even when cultured under identical conditions (Alonso *et al*, 1992) or when harvested at different growth stages (Fernandez-Reiriz *et al*,

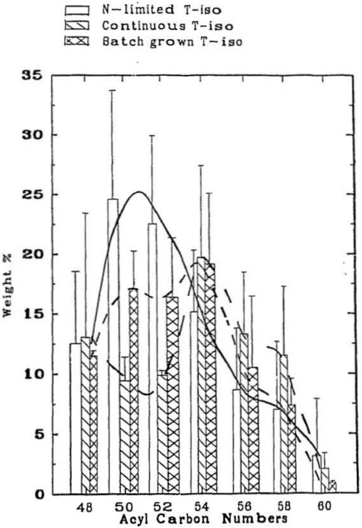


Figure 4.1. Acyl carbon number distributions in neutral lipids in differently cultured *Isochrysis galbana* (Clone T-Iso) (mean±S.D., n=3, under optimized conditions as listed in Figure 3.3)

Table 4.1. Fatty acids composition (wt %) of neutral lipids in three differently cultured *I. galbana* (Clone T-Iso) and their predators, sea scallops (*P. magellanicus*)

	A). T-iso growing in 3 different kinds of cultures						B). Scallops fed T-iso growing in 4 different kinds of cultures							
Fatty	1).N-limit		2).Continuous		3).Batch		1).N-limit		2).Continuous		3).Batch		4).Control	
Acids	(N=3)	S.D.	(n=3)	S.D.	(n=3)	S.D.	(N=3)	S.D.	(n=3)	S.D.	(n=3)	S.D.	(n=3)	S.D.
14:0	7.13	3.04	15.95	2.16	13.06	6.10	5.83	0.37	4.00	0.28	6.75	4.08	4.48	0.95
15:0	0.61	0.55	0.56	0.22	0.29	0.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
16:0	19.57	10.07	9.16	1.34	12.93	1.00	17.84	5.87	16.79	1.61	20.20	5.49	16.56	2.92
17:0	0.41	0.34	0.47	0.22	0.00	0.00	0.63	0.51	0.43	0.52	1.20	1.25	1.13	0.98
18:0	1.68	2.59	0.66	0.42	5.92	4.13	5.05	4.17	16.27	0.74	16.43	3 48	11.46	1.52
20:0	0.77	0.36	0.62	0.55	0.55	0.51	0.67	1.16	1.77	2.31	0.45	0.40	0.33	0.12
22:0	0.78	0.41	0.33	0.15	0.00	0.00	0.08	0.14	0.61	0.52	1.78	2.22	0.12	0.21
Total Saturated FA	32.36	14.15	29.64	3.05	33.83	13.41	31.51	8.60	41.44	2.65	49.69	4.30	34.51	6.26
14:1n-9 ?	0.64	0.55	0.67	0.52	0.09	0.16	0.55	0.81	0.56	0.97	0.00	0.00	0.00	0.00
16:1n-9 ?	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
16:1n-7	4.93	0.96	5.61	1.46	9.91	8.42	10.23	1.88	5.23	0.82	5.96	4.30	8.11	1.87
18:1n-9	22.96	5.83	17.74	3.82	21.11	7.85	17.72	3.83	15.21	0.95	6.31	4.29	10.03	0.75
18:1n-7	0.96	1.67	0.10	0.17	2.19	1.95	5.60	2.57	1.06	1.84	4.04	1.50	4.15	2.93
18:1n-5 ?	0.00	0.00	0.00	0.00	0.04	0.06	0.07	0.11	0.00	0.00	0.00	0.00	0.00	0.00
20:1n-9	0.00	0.00	0.00	0.00	0.00	0.00	0.38	0.66	2.04	2.23	0.17	0.30	0.99	0.86
20:1n-7 ?	0.28	0.48	0.06	0.11	1.51	2.62	0.62	0.78	0.10	0.18	3.01	1.58	1.70	1.70
22:1n-9	0.14	0.24	0.41	0.27	0.73	0.25	0.08	0.14	1.26	1.00	0.58	1.00	1.18	1.18
24:1	0.00	0.00	0.00	0.00	0.25	0.44	0.00	0.00	0.02	0.04	0.18	0.32	0.84	1.13
Total Monoenic FA	30.02	3.99	24.94	2.24	36.56	23.02	35.38	7.61	26.06	6.36	17.99	6.97	27.11	0.67
16:2n-6 ?	0.50	0.46	2.20	0.15	1.64	0.64	0.50	0.33	0.86	0.54	0.62	1.07	0.19	0.32
16:2n-4 ??	0.00	0.00	0.00	0.00	0.26	0.24	0.07	0.13	0.00	0.00	0.00	0.00	0.21	0.36
18:2n-6	6.55	3.11	5.88	0.51	7.93	4.78	3.96	3.36	15.20	6.22	5.66	2.73	7.96	1.34
18:2n-4 ?	0.22	0.37	0.13	0.22	0.21	0.10	0.31	0.08	0.28	0.29	0.89	1.53	0.43	0.39
20:2n-6	0.00	0.00	0.04	0.07	0.33	0.30	0.38	0.14	0.70	1.15	2.69	2.10	0.55	0.56
Total Dienoic FA	7.27	2.97	8.25	0.55	10.37	6.06	5.23	3.29	17.04	4.28	9.85	2.54	9.33	1.55
18:3n-6	0.00	0.00	0.05	0.09	0.10	0.18	0.14	0.23	0.56	0.52	0.00	0.00	0.46	0.46
18:3n-3	3.09	0.76	3.53	4.24	4.47	2.38	1.17	0.97	1.60	0.97	1.26	1.45	3.21	1.97
20:3n-6	0.00	0.00	0.40	0.69	0.01	0.01	0.00	0.00	0.18	0.31	0.00	0.00	0.00	0.00
Total Trienoic FA	3.09	0.76	3.99	3.75	4.58	2.57	1.31	1.17	2.35	0.42	1.26	1.45	3.67	1.56
16:4n-3 ?	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.45	0.00	0.00	0.00	0.00
16:4n-1 ?	0.00	0.00	0.00	0.00	0.00	0.00	1.29	2.24	0.00	0.00	0.00	0.00	0.00	0.00
18:4n-3	8.89	4.52	11.39	12.52	2.02	1.34	4.65	2.23	3.21	1.31	2.58	2.82	3.32	2.14
18:4n-1 ?	1.47	0.66	0.76	0.63	1.43	0.87	0.74	0.42	3.39	1.83	0.32	0.56	9.28	1.88
20:4n-6	0.53	0.92	0.41	0.36	0.29	0.11	0.96	1.02	0.14	0.12	3.67	2.16	0.90	0.41
20:4n-3 ?	0.00	0.00	0.00	0.00	0.28	0.31	0.00	0.00	0.59	0.39	0.37	0.64	0.00	0.00
22:4n-6	0.07	0.12	0.00	0.00	0.50	0.52	0.04	0.07	0.30	0.53	0.00	0.00	0.00	0.00
224n-3 ?	0.95	0.99	0.65	0.07	1.15	0.66	1.32	0.75	0.06	0.11	0.84	1.12	0.94	0.92
Total Tetraenoic FA	11.91	6.01	13.21	11.51	5.69	3.81	9.01	5.06	7.96	0.59	7.78	2.57	14.44	1.98
20:5n-3	4.98	7.25	1.61	0.96	1.36	0.88	4.10	2.28	1.28	0.48	3.96	1.11	3.75	1.63
21:5n-3	0.11	0.19	0.10	0.17	0.11	0.11	0.27	0.27	0.80	0.10	0.85	1.47	0.43	0.37
22:5n-3	0.60	0.52	0.09	0.08	0.10	0.10	0.05	0.09	0.93	0.77	0.89	0.95	0.00	0.00
Total Pentaenoic FA	5.69	7.74	1.80	0.77	1.57	1.09	4.43	2.46	3.02	1.32	5.70	3.39	4.18	1.27
22:6n-3	9.66	4.92	18.17	9.96	7.41	4.84	13.13	4.35	2.14	0.88	7.73	2.78	6.76	1.56
P/S	1.64	1.66	1.56	0.34	0.90	0.24	1.11	0.31	0.79	0.16	0.65	0.08	1.16	0.43
Sum PUFA	37.62	17.48	45.42	4.98	29.61	4.81	33.11	1.04	32.50	5.88	32.32	4.12	38.38	6.44
Sum(n-3)	28.28	17.34	35.54	6.19	16.91	5.24	24.70	1.24	10.88	1.34	18.48	3.49	18.40	6.03
22:6/20:5	6.13	4.32	11.81	1.33	8.06	6.38	5.05	5.25	1.87	1.23	2.01	0.80	1.95	0.71
Mean FA C.L.	17.53	0.72	17.45	0.29	17.22	0.44	17.66	0.04	17.53	0.02	17.76	0.63	17.71	0.19
Mean D.B./FA	1.76	0.82	2.04	0.03	1.09	0.23	1.72	0.12	1.22	0.11	1.35	0.16	1.67	0.24

1989).

Preliminary fatty acid results also show that 18:2n-6 and 18:3n-3 stayed at similar levels regardless of culture difference. Compared to the continuous counterpart, the nitrogen-limited *I. galbana* has relatively low 18:4n-3 and 22:6n-3 in its neutral lipids, in agreement with the observation by Sukenik *et al* (1991) for fatty acids from total lipids. The entire fatty acid profiles of neutral lipids for the three cultured *I. galbana* show that the *I. galbana* grown under nutrient-replete (continuous and batch) cultures share the same distributions as those for the total lipids of *I. galbana* grown at similar temperatures in other laboratories (Dunstan *et al*, 1993; Delaunay *et al*, 1993). Temperature is always a major environmental stress which significantly influences fatty acid composition. For instance, the *I. galbana* grown in batch at 25°C exhibits quite a different ratio of PUFA to saturated fatty acids compared to *I. galbana* grown at 15°C, cultured by Napolitano *et al* (1990), but shows a similar profile in summed neutral and polar lipids to that for total lipids of *I. galbana* grown at the same temperature (Thompson *et al*, 1992).

Specific fatty acids in the fatty acid suites of the most important prey organisms can be used as indicators of the dietary lipid input to marine predators. As showed in Table 4.1, 18:4n-3 is depleted 2-4 times more in the scallops fed the nitrogen-limited *I. galbana* as compared to that in the scallops fed the other kinds of diets. 20:5n-3 remains the same level in the prey and the predators in the above conditions. The scallops fed the nitrogen-limited *I. galbana* display possible elongation and desaturation in fatty acids from 18:4n-3 until 22:6n-3. The scallops fed the batch grown *I. galbana* do not show the above metabolic procedures, while strange proportions of 18:4n-3 and 22:6n-3 in the scallops fed the *I. galbana* grown under continuous culture may need data from greater sample numbers to make the biological significance clearer.

4.2 Marine Benthic Invertebrates

4.2.1 Laboratory Fed Bivalves

In contrast to diverse TAG carbon number distributions in their diets (Figure 4.1), the neutral lipids in sea scallops (*P. magellanicus*) show similar carbon number patterns (Figure 4.2) with different abundances. The most abundant TAG, C_{54}, in the scallops fed four kinds of *I. galbana* ranged from 25% in the scallops fed the nitrogen-limited *I. galbana* to near 60% in those fed the control diet (mixed microalgae). The different carbon number distributions between the prey and the predators may suggest certain elongations of dietary lipids by scallops.

Fatty acids, the moieties of the neutral lipids, show that there are similar levels of 14:0 and 16:0 in sea scallops fed all four kinds of cultured *I. galbana* (Table 4.1). Scallops fed the nitrogen-limited *I. galbana* show the lowest levels of 18:0 and 18:2n-6, but the highest of 16:1n-7, 18:1n-9, 18:1n-7, 18:4n-3 and 22:6n-3. Scallops fed the continuous *I. galbana* had the highest amount of 18:2n-6, at 2-4 times the amount in the others but the lowest of 16:1n-7, 20:5n-3, 22:6n-3. As a consequence, they have the lowest summed (n-3) PUFA and mean double bond per fatty acid.

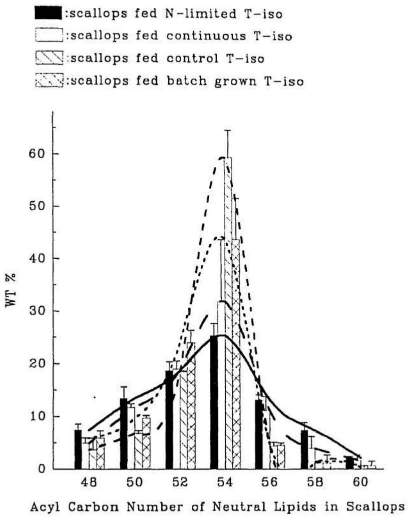


Figure 4.2. Acyl carbon number distributions in neutral lipids of sea scallops (*Placopecten magellanicus*) fed differently cultured *Isochrysis galbana* (Clone T-Iso) (mean±S.D., n=3, at optimized conditions as in Figure 3.3 and with helium as carrier gas)

4.2.2 Field Sampled Bivalves and Polychaetes

Cold water bivalves and polychaetes are part of the food web leading to cod and other predators. However, very little is known about the lipid composition of *Y. hyperborea* and the polychaetes, *N. ciliata* and *A. proboscidea*.

Abundances and proportions of three major sterols, C_{27}, C_{28} and C_{29}, in three types of invertebrates were very different (Figure 4.3, B). C_{27} sterol was the most abundant in *N. ciliata*, followed by *A. proboscidea* and *Y. hyperborea*, respectively. Sterols regulate membrane function, such as maintaining membrane fluidity, and act as precursors for a range of metabolically active molecules. Sterols cannot be synthesized *de novo* from simple precursors, so invertebrates rely on an external sterol supply (Tsitsa-Tzardis *et al*, 1993). Although all three invertebrates were taken from the bottom of Conception Bay, Newfoundland, *Y. hyperborea* may selectively consume fresh phytoplankton, while the two burrowing polychaetes may consume detritus.

In most samples analyzed, the TAG carbon numbers range from 32 to 60. The three marine invertebrates showed similar symmetrical carbon number distributions from 48 to 60 with an apex at 52 or 54. The highest amount of TAG, (12430 ± 9847 μg/g wet weight), was found in *Y. hyperborea*, a three-fold increase over the amount found in *A. proboscidea* (3361 ± 2497 μg/g wet weight) and a ten-fold increase over that in *N. ciliata* (1250 ± 862 μg/g wet weight) (Figure 4.4). The mollusca store TAG as an energy source in many body components rather than in specific organs as in mammals. The small amounts of TAG in *N. ciliata* may be due to fat being mainly deposited in its gut. There

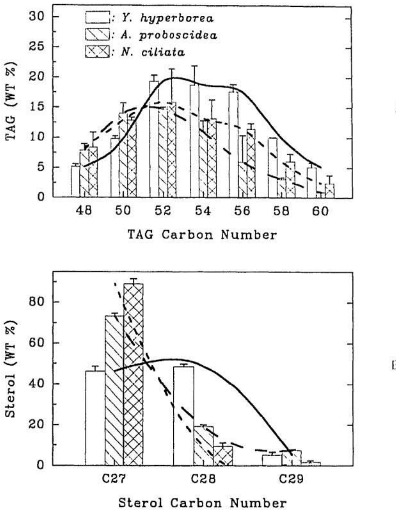


Figure 4.3. Acyl carbon number distributions in neutral lipids in three kinds of marine invertebrates (mean±S.D., n=3, at optimized conditions as listed in Figure 3.3 and with helium as carrier gas)

were much greater standard deviations in the concentrations of TAG compared with sterols in the three invertebrates, which may be the result of biological variation within each species, such as differences between sexes (Lubet *et al*, 1986).

Fatty acids are the fundamental structural components of practically all forms of lipids. The major fatty acid moieties in the neutral lipid fractions from the three invertebrates were 16:0, 18:0, 16:1n-7, 18:1n-7, 20:5n-3 and 22:6n-3, which together accounted for 51-71 % by weight of the total fatty acids (Table 4.2). Compared to fatty acid compositions in *Yoldia limatula* and *Nephthys incisa* from the warmer waters of Narragansett Bay, Rhode Island (Farrington *et al*, 1973), *Y. hyperborea* and *N. ciliata* have a higher percentage of PUFA in their neutral lipids. The predominant fatty acid, 20:5n-3, in neutral lipids of *Y. hyperborea* comprised 32 % of the total acids in its nonpolar fraction, which was slightly higher than those (20-27 %) in total lipids of eggs and adductor muscle from sea scallops (*P. magellanicus*) collected from relatively shallow water in nearby Trinity Bay (Napolitano *et al*, 1992), but 2-4 fold that obtained in Narragansett Bay, a temperature zone (Farrington *et al*, 1973). The PUFA levels in these bivalves from different latitudes appear to be temperature-related. In the species from the same site, 20:5n-3 and total PUFA varied from 32 % and 50 % in fatty acids of neutral lipids in *Y. hyperborea* to 13 % and 39 % in *N. ciliata*. This may reflect different diets and metabolic pathways in individual species.

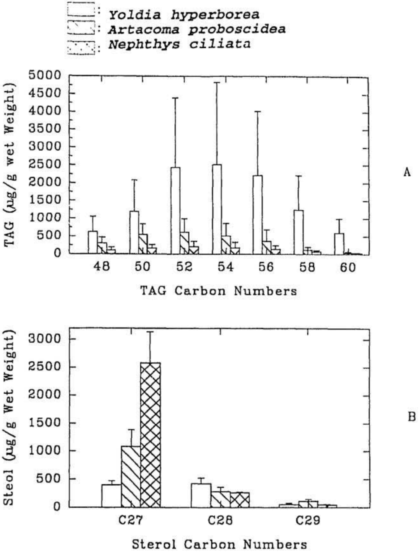


Figure 4.4. Absolute amounts of major neutral lipid compounds in three kinds of marine invertebrates (mean±S.D., n=3, at optimized conditions as listed in Figure 3.3 and with helium as carrier gas)

Table 4.2. Fatty acid composition (wt %) of neutral lipids in three marine invertebrates

Fatty Acids	*Yoldia hyperborea* Mean (n=3)[1]	± S.D.	*Nephthys ciliata* Mean (n=6)	± S.D.	*Artacoma proboscidea* Mean (n=5)	± S.D.
14:0	2.10	0.87	2.77	1.36	2.47	0.63
15:0	0.62	0.39	1.47	0.48	1.74	0.16
16:0	12.37	2.33	14.02	3.24	22.46	5.12
17:0	0.70	0.35	0.04	0.09	0.65	0.90
18:0	3.70	1.45	7.41	5.21	4.12	3.11
20:0	1.49	0.97	1.79	1.00	1.45	2.82
22:0	0.18	0.18	0.92	1.56	0.30	0.45
Total Saturated FA[2]	21.24	3.53	30.99	7.47	35.01	3.84
14:1n-9 ?	1.62	1.61	0.61	0.80	3.78	0.60
16:1n-9 ?	–[3]	–	1.36	1.96	0.48	0.25
16:1n-7	13.09	3.92	4.92	4.91	14.89	6.00
18:1n-9	3.19	0.52	3.69	2.72	2.88	0.47
18:1n-7	7.04	1.32	5.71	3.70	10.33	3.02
18:1n-5 ?	–	–	0.96	0.96	1.84	0.64
20:1n-9	–	–	0.05	0.13	0.24	0.53
20:1n-7 ?	1.93	0.46	1.16	0.96	0.03	0.07
22:1n-9	0.87	0.80	0.35	0.42	0.01	0.01
24:1	0.52	0.93	0.83	1.30	0.12	0.15
Total Monoenic FA[2]	28.20	4.38	20.34	8.27	32.18	9.06
16:2n-6 ?	1.11	0.51	1.70	2.49	0.92	0.65
16:2n-4 ?	1.05	0.97	1.79	1.83	2.06	1.09
18:2n-6	1.25	0.36	1.75	1.50	1.30	0.37
18:2n-4 ?	0.87	1.08	1.01	2.17	0.85	1.12
20:2n-6	0.01	0.02	0.82	2.00	0.57	0.78
Total Dienoic FA	4.28	1.81	7.06	4.90	5.70	2.47
18:3n-6	–	–	0.10	0.26	–	–
18:3n-3	0.69	0.70	1.05	1.29	0.34	0.20
20:3n-6	0.34	0.77	0.97	1.70	0.54	0.80
Total Trienoic FA	1.04	1.41	2.12	2.52	0.89	0.63
16:4n-3 ?	2.24	1.32	0.67	0.69	0.15	0.16
16:4n-1 ?	0.43	0.95	1.81	2.40	0.16	0.16
18:4n-3	1.21	0.42	0.85	1.20	0.15	0.13
18:4n-1 ?	2.63	1.89	1.95	1.72	0.27	0.25
20:4n-6	1.58	0.31	1.16	1.06	1.01	0.26
20:4n-3 ?	0.26	0.17	0.57	0.91	0.59	0.20
22:4n-6	0.06	0.19	1.98	1.45	1.89	0.07
22:4n-3 ?	0.20	0.23	0.83	0.96	0.25	0.26
Total Tetraenoic FA	8.64	1.81	9.83	2.83	4.48	0.68
20:5n-3	31.62	5.63	16.01	5.21	14.75	1.72
21:5n-3	0.91	0.14	0.79	1.21	0.69	0.42
22:5n-3	0.63	0.48	3.32	1.63	3.39	2.00
Total Pentaenoic FA	33.16	5.40	20.12	5.13	18.83	3.38
c22:6n-3	3.44	1.37	9.55	6.49	2.91	0.64
P/S	2.38	0.54	1.70	0.75	0.94	0.18
Sum PUFA	50.56	5.15	48.67	8.92	32.81	6.79
Sum(n-3)	41.21	5.57	33.64	7.29	23.23	4.18
22:6/20:5	0.11	0.06	0.63	0.54	0.20	0.03
Mean FA C.L.[4]	18.05	0.23	18.16	0.19	17.47	0.45
Mean D.B./FA[5]	2.48	0.19	2.23	0.29	1.62	0.19

1: each sample duplicated
2: including some minor fatty acids not listed
3: not detected
4: mean fatty acid chain length
5: mean double bond per fatty acid

4.3 Eggs and Larvae from Laboratory Held Fish

Halibut and cod are abundant in the relatively cold waters of the North Atlantic, and they are considered to be potentially important species for the North Atlantic aquaculture industry.

4.3.1 Eggs of Atlantic Halibut (*Hippoglossus hippoglossus*)

Quality of eggs and larvae are the most important determinant for cultivation of Atlantic halibut, the largest flatfish in the North Atlantic. The quality of different egg batches in individual fish over the spawning seasons and the maturity of the adult fish influence egg and larval viability (Daniel *et al*, 1993).

The neutral lipid profiles in eggs spawned by two captive halibut over the spawning season show different TAG carbon number patterns (Figure 4.5: the solid line representing the results from a repeat spawner and the dashed line a first time spawner). The repeat spawner produced a greater percentage of higher carbon number TAG which indicates storage of a higher proportion of long chain PUFA. By comparison with the first time spawner with 16 % of TAG in the higher acyl carbon numbers from 57 to 60, there was 22 % in the same range in the repeat spawner. A time-course plot through the spawning season shows the same trends in C_{52} and C_{54} TAG spawned by both halibut. Both of these TAG declined in later batches (Figure 4.6). C_{52} TAG could have a fatty acid composition of 16:18:18, while C_{54} TAG could have a composition of 16:18:20. The physiological meaning behind these changes in certain TAG carbon numbers is still not

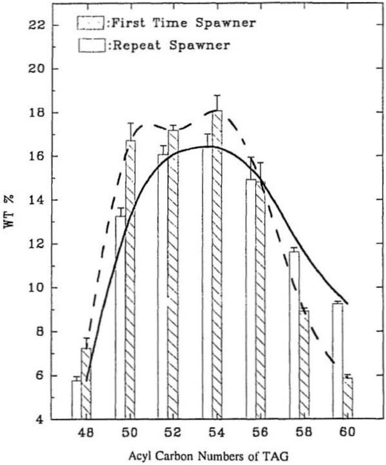


Figure 4.5. Acyl carbon number distributions in neutral lipids in the eggs of two Atlantic halibut (*Hippoglossus hippoglossus*) (mean±S.D., n=14, at optimized conditions as in Figure 3.3 and with helium as carrier gas)

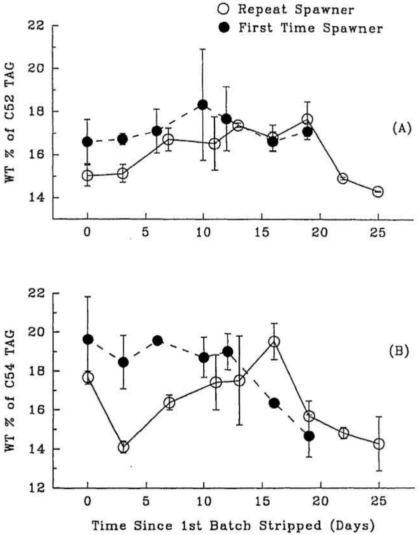


Figure 4.6. Time course of C_{52} and C_{54} TAG in the eggs through the spawning season of two Atlantic halibut (*Hippoglossus hippoglossus*) (mean±S.D., n=2, at optimized conditions as listed in Figure 3.3 and with helium as carrier gas)

as clear as that for the changes in their fatty acid moieties.

The trends in the neutral lipid carbon numbers coincide with decreased 20:5n-3 and increased 18:0 in their fatty acid moieties in later batches (Figure 4.7). One small component, 22:5n-3, also decreases significantly over the spawning season ($r = -0.836$, $p < 0.001$, $n = 16$).

These suggest that the earlier to middle batches are better for halibut larvae prior to first feeding due to larger proportions of long chain PUFA, especially the essential fatty acids. These could be utilized for a prolonged period after hatching. Also, the eggs from the repeat spawner seem to be superior to those of the first time spawner in terms of neutral lipid quality. This difference in egg quality was reflected in the fertilization success of two fish. The eggs from the repeat spawner had a higher average fertilization success (61 %) by comparison with those from the first-time spawner (44 %).

Unlike the time-course of 22:6n-3 in the total lipids from another pair of halibut (Parrish *et al*, 1993b), this essential fatty acid in this pair of halibut seemed to fluctuate irregularly in the neutral lipids during their spawning season. Accumulation of data on egg neutral and polar lipids from a reasonable number of halibut in different years will probably help make the biological significance clearer.

4.3.2 Cod Larvae

Quantitative measurement of the rates of ingestion and assimilation of nutrients from different prey taxa should advance our understanding of the feeding dynamics of

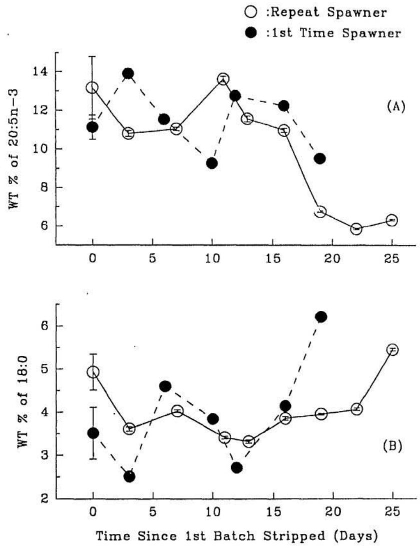


Figure 4.7. Time course of 20:5n-3 and 18:0 fatty acids in the neutral lipids of eggs through the spawning season of two Atlantic halibut (*Hippoglossus hippoglossus*) (mean±S.D., n=2, FAME analysis conditions as listed in the Section 2.2.5.1)

early stage cod larvae. The carbon number distribution of sterols in cod larvae (*G. morhua*), (Figure 4.8), was composed of approximately 99% C_{27} sterols, with C_{28} sterols being almost absent regardless of the variations in the three feeding experiments. In the larvae fed the high prey diet there were slightly lower levels of C_{27} (98 %), and almost twice the amount of C_{28} sterols. There was also much greater variability in the sterol compounds from these larvae. This suggests that diet does influence sterol composition in cod larvae, but only to a small degree.

This result is in accord with the literature (Frasier, 1989): Sterols in fish larvae are usually impervious to diets, while TAG may readily be affected by feeding. Therefore, sterols may be useful to normalize the TAG content to a size-specific basis. Unfortunately, our sample size was too small (15 or 30 larvae/sample) to determine TAG content. Not only was the TAG amount in each sample below the TLC-FID limit but it also caused TAG peaks to be so small in the automated GC that they were almost merged with the baseline. Further study may require manual injection with a significantly larger sample size.

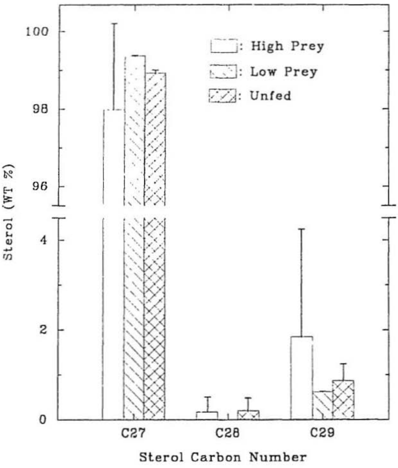


Figure 4.8. Carbon number distributions of sterols in neutral lipids of cod larvae (*G. morhua*) in feeding trials (mean±S.D., n=3, at optimized conditions as listed in Figure 3.3 and with helium as carrier gas)

5 CONCLUSIONS

From this study, it would appear that large solvent plug sizes, fast injection rates, use of hydrogen as a carrier gas with high flow rates, and use of short capillary columns enhance recoveries of high molecular weight neutral lipids. Sample hydrogenation allows for the correct evaluation of carbon number distributions in sterols and avoids discrimination in samples with high proportions of long chain PUFA. With incorporation of these procedures, neutral lipid carbon number profiles may be useful in defining physiological characteristics of diverse marine species.

Up to now the profiles of neutral lipid components from marine samples, particularly from cold oceans, are few and scattered over a wide range of species. Absence of powerful analytical methods may be one of the major shortcomings. Automated high temperature GC on hydrogenated samples may offer a precise tool for the measurement of highly unsaturated neutral lipid samples with acyl carbon numbers up to 62 or slightly greater.

The applications indicate that neutral lipid profiles by GC not only characterize carbon number patterns according to marine biological origins, but also differentiate the distributions within the same species which differ only in diet, and physiological or environmental conditions. This makes lipid determination by high temperature GC versatile as compared to its counterparts. TLC-FID only reaches subclass separation and does not allow insights into the variations in individual neutral lipid compounds. HPLC with its relatively low resolution does not uniformly resolve compounds based on their molecular weights.

By combining with dephosphorylation, this optimized GC method can be extended to analyze phospholipid moieties along with neutral lipids as there is enough room in the chromatograms to insert di- and monoacylglycerol peaks (Kuksis *et al*, 1989). Further study should verify the usefulness of the proposed method in samples rich in other lipid compounds, such as wax esters and hydrocarbons.

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