TOTAL MARINE LIPID PROFILING BY SHORT COLUMN GAS CHROMATOGRAPHY

JONATHAN JOSEPH KEHOE

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TOTAL MARINE LIPID PROFILING BY SHORT COLUMN GAS CHROMATOGRAPHY

by

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ABSTRACT

Analysis of lipids is a very informative way of determining the physiological state of a marine ecosystem. The information derived from analysis of these hydrophobic, carbon rich compounds is very important to researchers in fields such as aquaculture and biological research.

Thin layer chromatography with flame ionization detection (TLC-FID) has been a common method for identification and quantitation of the lipid classes of samples derived from marine sources such as sediments, plants and animals. However, TLC suffers from some analytical problems such as low sensitivity, non-linear calibration curves, long analysis times and use of copious amounts of hazardous solvent.

Gas chromatography (GC) is an ideal choice for lipid analysis as it allows for automation, high sensitivity, short analysis times and low cost. Marine lipid samples often contain high proportions of polar lipid classes such as the phospholipids (PL) and the acetone mobile polar lipids (AMPL). While neutral lipid classes are readily analyzed by short column GC, AMPL retain on the GC column rendering the column useless after time. Thus GC determination of polar lipids requires enzymatic treatment and derivatization prior to chromatographic analysis.

This project optimized a short column GC method for marine lipid class profiling by incorporating Kuksis' GC profiling strategy (1984) that used the enzyme phospholipase C to hydrolyze PL to diacylglycerols, with the optimized short column GC method for marine neutral lipids developed by Yang (1996). Combination of enzymatic

hydrolysis and short column GC makes a near complete lipid profile of marine lipid samples possible. Hydrogenation of samples allows for compounds to be separated according to their carbon numbers and functional groups.

In this project, the dephosphorylation procedure was optimized for marine samples, which were 50 units of phospholipase C for every milligram of phospholipid present in the sample. Comparison of percent lipid data obtained by short-column GC with Iatroscan TLC-FID data showed that equally accurate and sensitive data could be obtained. Hydrogenation of samples prior to analysis allows for excellent peak resolution and sensitivity to individual compounds within each lipid class. Achieving this information is not possible with TLC-FID when performing total lipid profiles. The pretreatment of samples resulted in 63.7 ± 3.7% recovery of samples, however the overall analytical precision was 1.7% error between replicate samples.

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LIST OF ABBREVIATIONS

ALC	alcohol
AMPL	acetone polar mobile lipids
DAG	diacylglycerols
EE	ethyl ester
FAME	fatty acid methyl esters
FFA	free fatty acids
FID	flame ionization detection
GC	gas chromatography
GC-MS	gas chromatography with mass spectrometric detection
HC	hydrocarbon
KET	ketone
MAG	monoacylglycerols
ME	methyl ester
PL	phospholipid
PUFA	polyunsaturated fatty acids
ST	sterol
SE	steryl ester
TAG	triacylglycerols
TLC-FID	thin layer chromatography - flame ionization detection
TMS	trimethylsilylation
WE	wax ester

LIST OF APPENDICES

1 INTRODUCTION

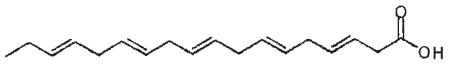
Lipids are classified as carbon rich substances that can be extracted from cells and tissues by non-polar organic solvents. Since lipids have a very high-energy value, they are important fuels in marine ecosystems (Lee *et al,* 1971). Marine lipid samples can be obtained from a variety of sources including sediments, seawater, and plant and animal tissue. Marine sediments often contain the basic skeletal structures and functional groups of the original sources, and therefore serve as a tool to determine the origin of the sediment (Venkatesan *et al,* 1987). Lipid class data from seawater and sediments are useful as indicators for pollution, types of organisms present in the sampling area, and products of anabolic or catabolic processes experienced in these organisms. Marine plants such as microalgae, seaweeds and seagrasses are the primary producers of energy in marine ecosystems. This energy is commonly stored as fatty acids in triacylglycerols (TAG) (Yang *et al,* 1996). Invertebrate animals such as oysters and mussels ingest these plants and therefore serve as the link in the transfer of energy from phytoplankton to the upper trophic levels of the marine food web, such as fish and marine mammals (Fenchel, 1988). For these animals, lipids are a very important source of metabolic energy. In addition, many organisms are able to alter membrane fluidity in response to temperature changes. This is achieved through changes in relative amounts of various unsaturated fatty acids (FA) in the membrane phospholipids (PL). The composition of marine lipids depends greatly on changes in diet and environmental stresses.

Analysis of lipid quantities and compositions is therefore extremely important in monitoring physiological conditions of a variety of marine species. For example,

quantitative evaluation of lipid profiles has specific significance in terms of biomarkers and indices. Lipids and their derivatives have great potential as biomarkers because of their diversity of structures synthesized by plants, bacteria and zooplankton. These compounds can therefore be used to monitor the pathway of carbon as it moves through the ecosystem. In addition, lipids are potential solvents of lipophilic pollutants such as polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB) and p,p′-dichlorodiphenyltrichloroethane (DDT). Therefore they can provide a means of transporting pollutants through marine ecosystems and into marine food webs (Parrish, 1988).

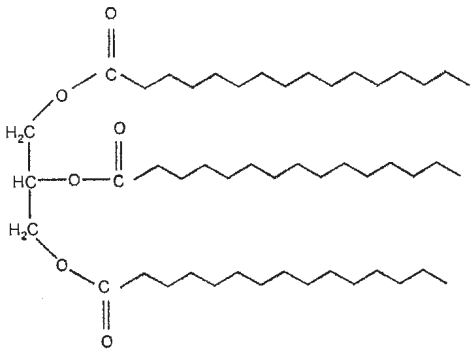
1.1 Lipid classes and structures

Marine lipid samples provide a unique challenge to separation science. Depending on environmental stresses, diet and physiological condition of the organism, there can be as many as 16 different subclasses of lipid (Parrish, 1988). Lipids include many types of compounds containing a wide variety of functional groups. Lipid classes are designated based upon the structure and the biological and chemical nature of the compound. Figure 1.1 shows examples of several of the various marine lipid classes that occur in marine ecosystems. Lipids can be split into two different subclasses, which are the neutral and the polar lipids. In a lipid sample, neutral lipids can include triacylglycerols (TAG), diacylglycerols (DAG), sterols (ST), steryl esters (SE), wax esters (WE), free fatty acids (FFA), free aliphatic alcohols (ALC) and hydrocarbons (HC). The polar lipids include phospholipids (PL) and the glycolipids and other acetone mobile polar lipids (AMPL).

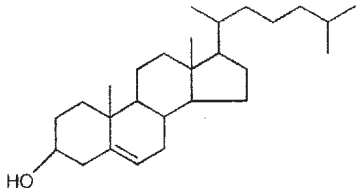


FREE FATTY ACID: Eicosapentaenoic acid (20:5n-3)

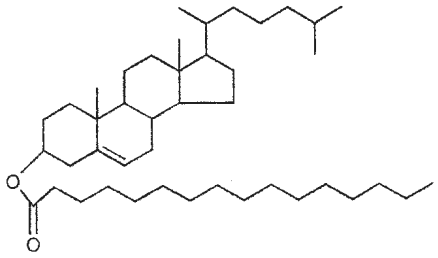
HYDROCARBON: n-Nonadecane



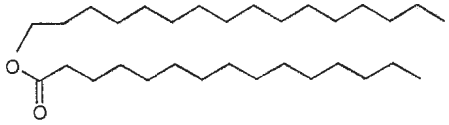
TRIACYLGLYCEROL: Tripalmitin



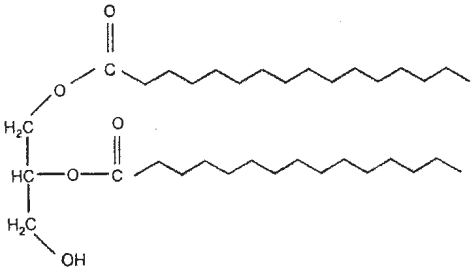
STEROL - Cholesterol



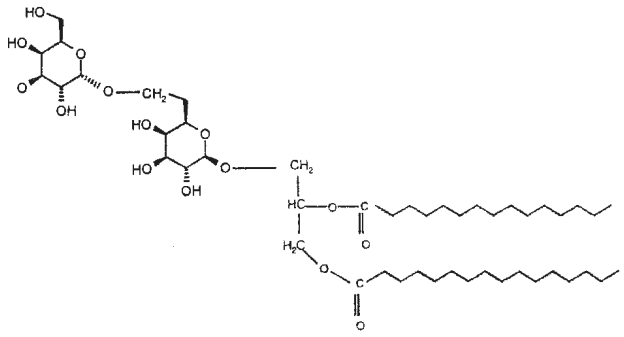
STERYL ESTER: Cholesteryl palmitate



WAX ESTER: Hexadecyl palmitate



DIACYLGLYCEROL: 1,2-Dipalmitin



GLYCOLIPIDS: Digalactosyl diglyceride

Figure 1.1: Various lipid classes and example structures

Each class consists of a set of molecular species that have similar polarities, however there can be several important structural differences between each compound. These differences can include units of unsaturation and carbon chain lengths. Lipid classes can also be grouped in other ways, such as the acyl lipid classes. These classes contain the acyl group (R-C=O) that comes from the presence of a fatty acid within the structure. MAG, DAG, TAG and PL all contain fatty acids chains attached to a glycerol backbone. In routine fatty acid analysis of a lipid extract, these fatty acids are cleaved from the backbone and analyzed as fatty acid methyl esters (Morrison and Smith, 1964) by gas or liquid chromatography.

1.2 Methodology for analyses of marine lipids

Improvement and simplification of the methodology for marine lipid analysis will greatly benefit the field of marine lipid research. Advancements in analytical techniques that lower detection limits and increase sensitivity produce data with higher levels of precision and accuracy. Subsequently, the routine identification and quantitation of key lipid compounds in an aquatic ecosystem can be made to be more time efficient and cost effective.

Marine lipid classes are a group of compounds that are very complex in nature. Lipid classes may consist of many molecular species that are similar in their physico-chemical properties. This makes chromatographic methods the first choice for marine lipid analysis.

1.2.1 Thin layer chromatography – flame ionization detection

Since the early 1980s, thin layer chromatography with flame ionization detection (TLC-FID) has been quite a common method of lipid analysis (Shantha, 1992). Instead of conventional plate TLC followed by scraping, extraction and analysis (e.g. FID detection), Iatroscan TLC-FID has become an important tool in marine lipid research.

The principles behind Iatroscan TLC-FID are generally the same as TLC on silica plates. However, during a routine Iatroscan analysis, samples are individually spotted on a set of silica coated quartz rods. The tips of these rods are immersed in appropriate solvent systems, which rise up the length of the rod. The resulting separations depend on the polarities of the solvent system. Passing the rod along its length through the flame of an ionization detector creates the chromatogram.

Typical lipid class analyses require development in a non-polar solvent mixture, followed by partial FID scan to quantify the resulting eluted compounds. The rods would then be developed in a solvent mixture with increased polarity to elute the more polar lipid classes, followed by FID scan. Finally a strongly polar solvent system is used to elute the polar lipids and scanned again. The compounds within each lipid class are eluted together, resulting in a chromatographic peak for each class. A more detailed description of a typical lipid class analysis is found in Section 2.2.5.1.

The duration of a single lipid class analysis is approximately four hours, regardless of whether one sample or twenty samples simultaneously (a typical maximum in a rack of rods) are analyzed. Iatroscan procedures can be performed to separate a specific class into their individual components, such as the phospholipids (Evans *et al.*, 1996). However, separation of neutral from polar lipids using a silica column is often

required to eliminate interference from the other classes, resulting in increased sample preparation time added to an already lengthy procedure.

Despite having the ability to analyze both polar and non-polar lipid classes, the Iatroscan suffers from several analytical problems (Tvrzicka and Mares, 1990). Insensitivity to low concentrations is a problem since the concentrations of lipid can be quite small in many samples such as algae and juvenile fish. Other problems include non-linear calibration curves, which make quantitation less accurate, as well as variability between each rod. The Iatroscan also suffers in terms of environmental and health concerns, since large amounts of hazardous solvent is discarded after each analysis. Methanol, diethyl ether, acetone and chloroform are common solvents used in Iatroscan analyses. It is these problems that make GC analysis more appealing.

1.2.2 High performance liquid chromatography

High performance liquid chromatography (HPLC) is particularly useful as it eliminates losses of temperature sensitive unsaturated lipids and for the analysis of high molecular weight lipids such as phospholipids and conjugated lipid classes (Shulka, 1988). HPLC utilizes solvents of varying polarities and a solid phase column to separate the lipid samples. The separated solutes are passed through a detector such as an ultraviolet absorbance detector, which provides the chromatogram output (Skoog, 1998).

1.2.3 Gas chromatography

Kuksis and co-workers were the chief developers of lipid class determination by GC. Their first application of the technique involved analysis of human blood plasma,

first using packed columns (Kuksis *et al.*, 1967) and then using fused silica columns (Myher *et al.*, 1984).

Yang and co-workers optimized Kuksis' GC profiling method for use with neutral lipids in samples from cold ocean environments (Yang *et al.*, 1996). However, a method for total lipid analysis (neutral and polar lipids) is desired. Marine samples often contain high proportions of polar lipids, which include phospholipids and glycolipids. These polar lipids are often quite large molecules, which are retained on the column and therefore cannot be analyzed. In addition, due to the retained compounds, the column is rendered useless over time. This problem makes polar lipids very difficult to analyze by gas chromatographic means. Thus GC determination of polar lipids requires enzymatic treatment and derivatization prior to chromatographic analysis.

Of the lipid classes analyzed by this method, TAG has the highest molecular weight, and is very difficult to obtain satisfactory recoveries using longer columns as recoveries of TAG decrease dramatically with long column lengths (Yang, 1996). When using a column of longer lengths, compounds experience increased residence time in the chromatographic system. In conjunction with the required high elution temperatures to elute larger compounds such as TAG, possible polymerization and decomposition of high molecular weight lipids can result. These phenomena can result in lowered recoveries or disappearance of lipid classes from the resulting chromatograms altogether (Mares, 1988). It is desired to have a column length that will retain chromatographic resolution and maximize TAG recoveries. Optimization experiments by Yang determined that a 5.5 m column was most effective in achieving these desired conditions. It for these reasons this method is called a short column GC method. In addition, by using a wide ranging

temperature program, neutral lipid compounds from C16 FFA to C56 TAG are eluted within half and hour and with reasonable separation in terms of carbon numbers, using a high temperature DB-5 column (Myher *et al,* 1984; Kuksis *et al.)*

1.2.4 Comparison of chromatographic methods

TLC-FID is a commonly used method for lipid profiling since it has a high sample capacity and offers analysis of both neutral and polar marine lipids (Volkman *et al.,* 1989*).* However, in comparison to results obtained from a packed column GC the results of from TLC-FID show much greater variability. TLC-FID is not easily automated and has non-linear calibration curves. Slight variations between individual Chromarods contribute to variability in results (Tvrzicka *et al., 1*990). Data obtained by TLC-FID lipid profiling is limited to only ten subclasses after several developments in varying solvent systems, which are often long in duration. GC or HPLC can resolve lipids into their individual compounds (Kuksis *et al.,* 1975). Both GC and HPLC can be readily used in conjunction with a mass spectrometer for even more highly detailed analysis with full automation. The limitations of TLC-FID require that more sensitive, precise and readily automated methods be developed.

In comparison to GC, HPLC offers again the elimination of loss of sensitive unsaturated substances and higher molecular weight homologues. However, GC gives higher sensitivity in detection, shorter analytical times and fewer difficulties in identification (Mares, 1988).

In conclusion, lipid profiling requires improvement over current methods. GC offers the highest quality of analytical data for the lowest cost and highest time efficiency.

It is sensitive and readily automated. However, profiling again is limited to the neutral lipid classes (Kuksis 1994, Yang *et al.*, 1996). This study will help to expand the classes that can be analyzed by GC to include both neutral and polar species.

1.3 Objectives

The objective of this research was to devise a complete automated GC method to measure lipid profiles, including hydrocarbons, free fatty acids, sterols, wax esters, steryl esters, triacylglycerols and phospholipids in marine samples. The method involves extraction, dephosphorylation, hydrogenation, TMS derivatization and analysis with capillary GC at temperatures up to 340 °C on a short, bonded nonpolar ZB-5 liquid phase column.

Essentially, the new GC method incorporates the GC profiling strategy of Kuksis (1984) and the optimized short column GC method for marine neutral lipids developed by Yang (1996). Separations are based on the carbon number of the various molecular species within marine lipid classes, which do not normally possess overlapping molecular weights.

The specific objectives were:

1) To optimize the conditions of high temperature non-polar gas chromatography for a total lipid profile;
2) To analyze data from lipid standards to support total lipid profile data;

3) To optimize the amount of phospholipase C required to digest a known amount of phospholipid in order to digest all the phospholipid present in a sample;

4) To compare the GC results to those from TLC-FID;

5) To verify usefulness by applying the procedure to various marine samples.

2 EXPERIMENTAL

2.1 Materials

2.1.1 Glassware and chemicals

All containers that come into contact with any solvent were made of glass with caps made with Teflon liners. All glassware was made from Pyrex. All glassware was cleaned of trace lipids by rinsing three times with methanol followed by three times with chloroform.

All chemicals and solvents used were either of analytical or chromatographic grade. All lipid standards employed in this research were prepared from chromatographically pure materials (at least 99% purity) supplied by Sigma (St. Louis, MO, USA).

O-rings created specifically for the VELP Solvent Automated Extractor were made using virgin Teflon.

2.1.2 Marine samples

Several of the samples analyzed in this study were obtained in August 2000 from Kelly's Point, in southern Labrador, Canada. From this location, two species of starfish, *Crossaster papposus* and *Ophiura sarsi* as well as a species of scallop, *Chlamys islandica* and copepods of the species *Calanus* were dredged.

Yellowtail flounder were cultured in the Ocean Sciences Centre, Memorial University of Newfoundland. Nearshore capelin samples were collected off William's

Harbour, Southern Labrador, Canada, August 2000. Samples of Pacu, a freshwater fish were collected in São Paulo State, central Brazil.

2.1.3 Total system blanks

Blanks, which involve all procedural steps carried out without a sample, were performed during the analysis of each marine species. Any chromatographic peaks due to background compounds were subtracted from those in the samples.

2.2 Methods

The entire experimental design is outlined in Figure 2.1.

2.2.1 Extraction

Samples analyzed in this research were already extracted prior to receiving them. Aliquots of homogenized marine samples were extracted with a mixture of chloroform and methanol, following the procedure of Folch et al. (1957). The chloroform layer containing the extracted total lipids was transferred to a 15 mL glass vial and was stored under nitrogen at –20 °C prior to sample preparation procedures and analysis.

2.2.2 Phospholipid digestion

Four mL of basic Tris buffer solution (consisting of 1.060g of Tris[hydroxymethyl]aminomethane Tris (basic) and 0.0555g of $CaCl_2$ dissolved in 500mL of distilled H_20, pH adjusted to 7.3 with HCl), 1.3 mL of 1% $CaCl_2$ and 50 units

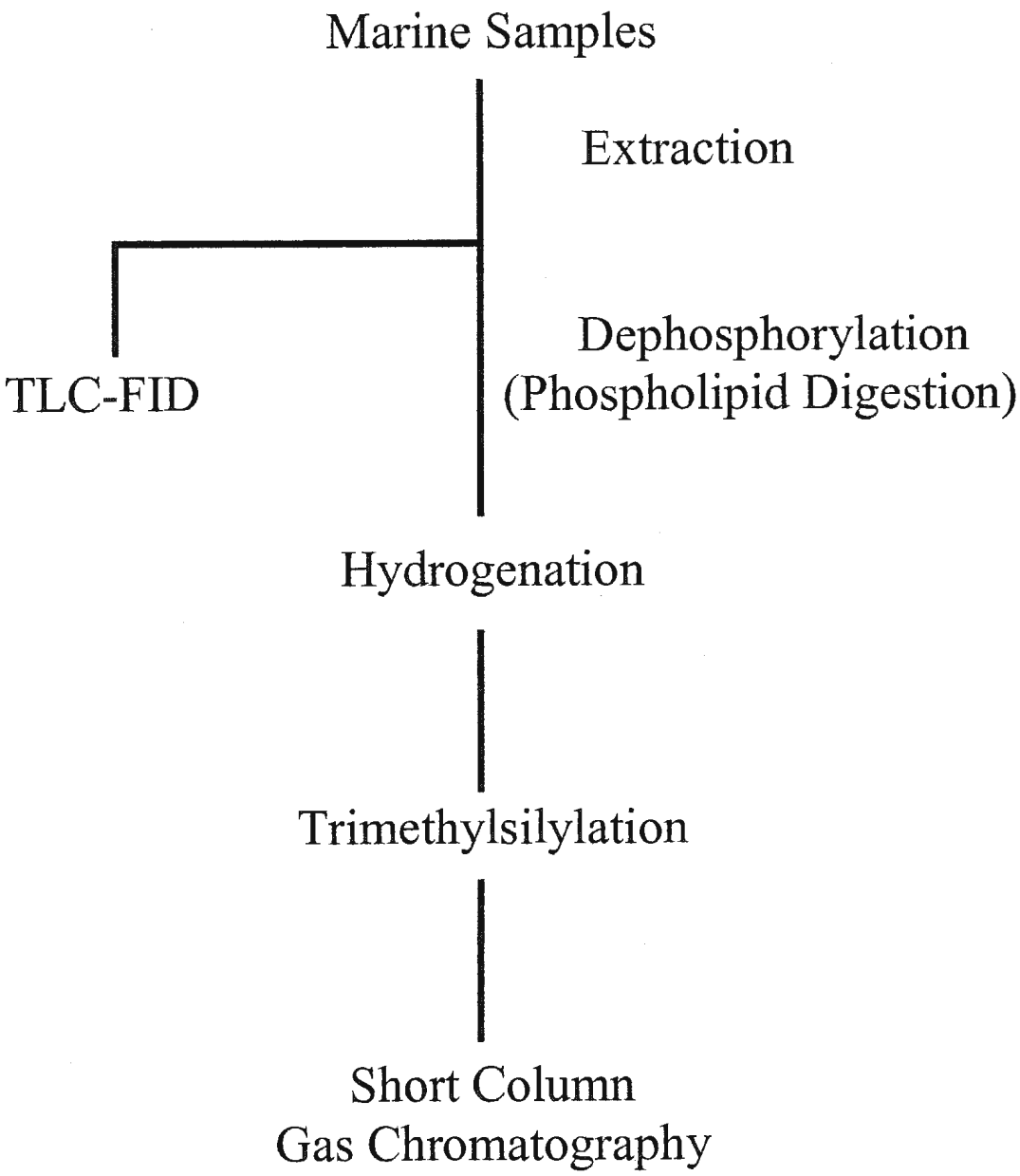


Figure 2.1: Experimental design.

of phospholipase C (Sigma, St. Louis, MO, USA) were mixed in a 25 mL test tube. One unit of phospholipase C is defined by the distributor as the amount of the enzyme that will liberate 1.0 μmol of water-soluble phosphorous from egg yolk L-α-phosphatidylcholine per min at pH 7.3 at 37 °C. One unit of this enzyme (as sold by the distributor in 3.2 M $(NH_4)_2S0_4$, pH 6) is equal to 0.996 μL of solution. To the buffer/enzyme mixture, 2 mL of diethyl ether was added to create an organic layer, followed by a 0.5 mL aliquot of total lipid chloroform extract (see Section 2.2.1). For the optimization of the phospholipid digestion by phospholipase C, a 0.5 mL aliquot of ~2 mg/mL dipalmitoyl α-phosphatidylcholine (Sigma, St. Louis, MO, USA) was added in place of the lipid extract.

The entire mixture was then sonicated at ~37 °C for two hours, while tilted at 45° to increase the interface between the aqueous and organic layers. Prior work by the Parrish lab showed that increased surface area facilitated the hydrolysis reaction. After two hours, five drops of 0.1N HCl were added to the test tube and the tube vortexed. The tube was centrifuged at 1000 rpm for two minutes and the lower organic layer was removed and placed in a 10 mL vial.

2.2.3 Hydrogenation

An aliquot of phospholipase C digested sample in ~ 5 mL chloroform and 5 mg fresh platinum oxide (Sigma, St. Louis, MO, USA) was added to a 10 mL vial. The solution was bubbled with a gentle flow of hydrogen gas for 20 minutes without stirring. The hydrogen-filled vial was capped and sealed, then sonicated for 1.5 – 2 hours. The

hydrogenated sample was then filtered with a lipid cleaned GFC glass fiber filter paper to remove the catalyst.

2.2.4 Trimethylsilylation

An aliquot of lipid sample was placed in a 2 mL vial and evaporated to dryness by nitrogen. This dried lipid sample was mixed with two drops of N,O-bis(trimethylsilyl)-acetamide and two drops of N,O-bis(trimethylsilyl)-trifluroacetamide (Sigma, St. Louis, MO, USA), then sealed under nitrogen and heated at 70 °C in an oven for 15 minutes. The excess trimethylsilylation (TMS) reagents were evaporated off under nitrogen and the TMS derivatives were dissolved in 0.5 mL of hexane prior to GC profiling.

2.2.5 Chromatographic methods

2.2.5.1 Thin layer chromatography – flame ionization detection

Aliquots of the samples were directly separated into lipid classes on silica-gel coated Chromarods-SIII using four different solvent systems and measured in an Iatroscan MK V (Iatron Laboratories, Tokyo, Japan) after development with each solvent system (Parrish, 1987) as follows.

Each sample was spotted with an appropriate volume of extract on an individual rod, with a 20 μL Hamilton syringe. The sample spots were then focused to a narrow band using 100% acetone. After 5 minutes in a constant humidity chamber, the rods were developed using a 99:1:0.05 hexane/diethyl ether/formic acid mixture (60 mL) for 25 minutes. The rods were dried in the constant humidity chamber for five minutes before

developing the rods again in the same solution for a further 20 minutes. Repeating the development helps to sharpen the eluted peaks. The first partial FID scan (78% of the rod from the top) was then performed, which detects the HC, SE/WE and KET lipid classes that have eluted up the rod from the origin.

Upon completion of the first development and detection, the rods were developed in a tank containing an 80:20:1 hexane/diethyl ether/formic acid mixture (60 mL) for 40 minutes and then scanned by the FID (89% of the rod from the top) for the TAG, FFA, ALC and ST lipid classes.

The final development stage involved developing the rods twice in 100% acetone (60 mL) for 15 minutes to elute the AMPL (dried in between developments). After drying in the constant humidity chamber for 5 minutes and then developing in a final solvent of a 5:4:1 methanol/chloroform/water mixture (60 mL) for 10 minutes twice (dried in between developments) the rods were then scanned for a final time for their entire lengths for the AMPL and PL lipid classes.

The three resulting chromatograms from the three FID scans were then combined to form one complete chromatogram of the lipid classes and the resulting data were analyzed using the T Data Scan Chromatography Analysis program (RSS, Bemis, TN, USA).

2.2.5.2 Gas chromatography

Total lipid profiles were investigated using a Hewlett Packard 6890 Plus GC system equipped with a Hewlett Packard 7863 automated injector and a FID. The analytical short column was a 5.5 m ZB-5 fused silica column (0.32 mm ID and 0.25 μm

film thickness) coated with a cross-linked 5% phenylmethyl silicone (Supelco, Bellefonte, USA). The carrier gas (helium) 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 the fuel gas (hydrogen) was adjusted to 30 mL/min and the air flow was set at 300 mL/min. The heater temperature of the FID was set at 345°C. The column oven temperature was programmed to rise from the initial temperature of 60°C 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, where it was held for 13.58 minutes. Data acquisition, baseline subtraction, and chromatogram re-plotting were performed with the Hewlett Packard Chemstation software.

The parameters for autoinjection, the cool on-column injection temperature program, initial column temperature, type of carrier gas, injection technique, length of column and septum types in the GC were based on previous work by Yang (1996).

In order to obtain good quantitative data from several different lipid classes, a wide carbon number range of standard lipids including hydrocarbons, free fatty acids, ketones, wax esters, sterols, steryl esters, DAG and TAG were used. Retention times of peaks that appear between these standards were calculated by mathematical interpolation.

3 Results and Discussion

3.1 Optimization of dephosphorylation procedure

Ensuring the complete dephosphorylation of phospholipid compounds is essential for this type of GC analysis. Phospholipids are typically quite large and complex polar molecules that are retained on a GC column, rendering the column useless after time. This procedure follows that of Kuksis' blood plasma profiling procedure (Kuksis, 1975), which makes use of the enzyme phospholipase C to cleave phospholipids of their phosphate group to yield their diacylglycerol (DAG) moieties. Figure 3.1 shows the PL compound dipalmitoyl phosphatidylcholine and the bond that is cleaved to produce the DAG compound dipalmitin. DAG compounds can be easily analyzed by gas chromatography after TMS derivatization. The information gathered from the identification and quantitation of the DAG will give direct information about the PL originally present in the sample. It is possible that free DAG can be present in a sample, however they are generally in low quantities if present. In this project, samples identified by Iatroscan as having no DAG present were selected for analysis.

Iatroscan TLC-FID was used to determine the amount of phospholipase C required to effectively cleave a PL to DAG in a sample by monitoring the decreasing peak areas of PL. As the amount of enzyme added increases, the remaining amount of undigested PL decreases to ~0.03 mg (from 1 mg) after 45 units of enzyme. A graph illustrating the decrease in PL with increased enzyme is shown in Figure 3.2. However, by reacting with a slight excess of enzyme, the maximum amount of PL that is converted to DAG can be ensured. Therefore, 50 units of enzyme would be the optimal amount.

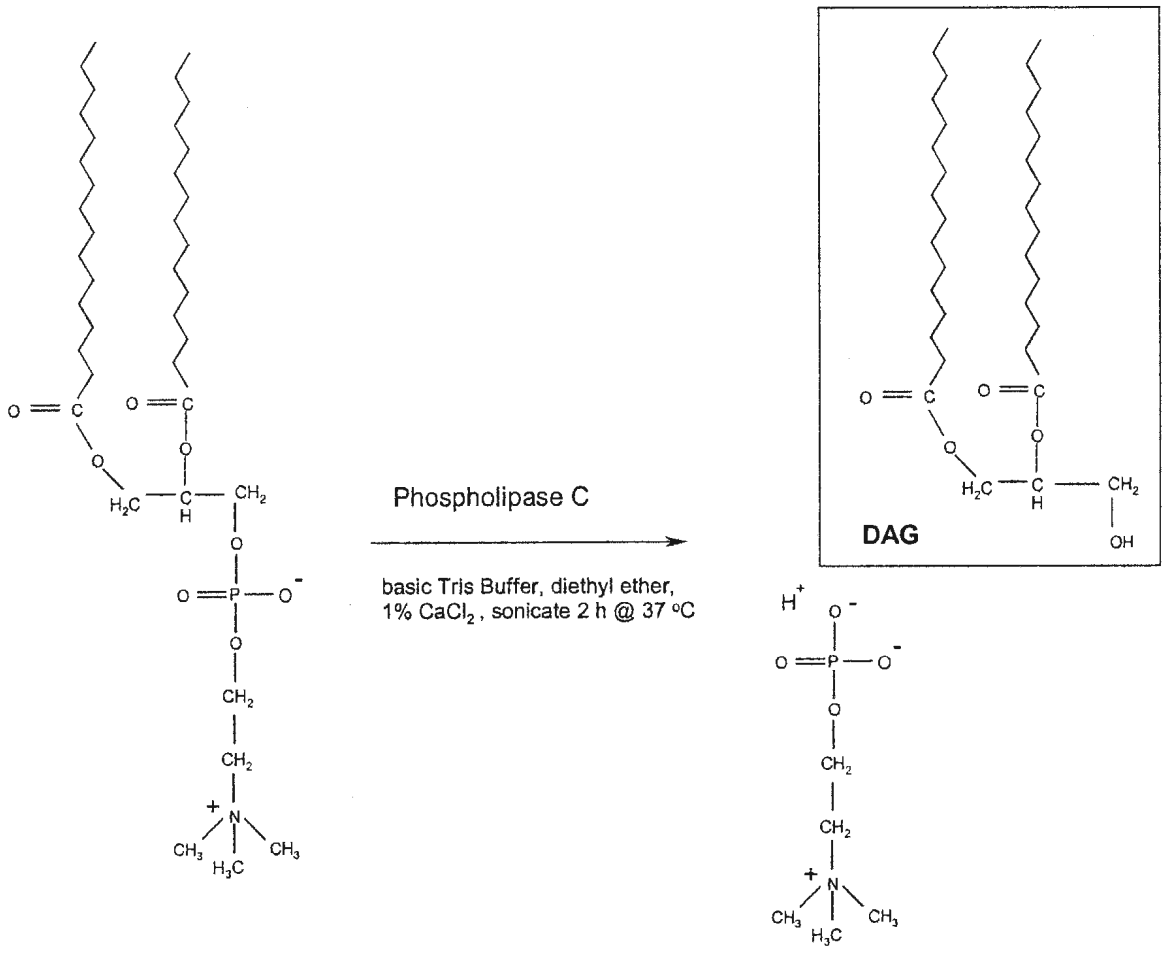


Figure 3.1: PL molecule dipalmitoyl phosphatidylcholine cleaved to DAG through enzymatic hydrolysis.

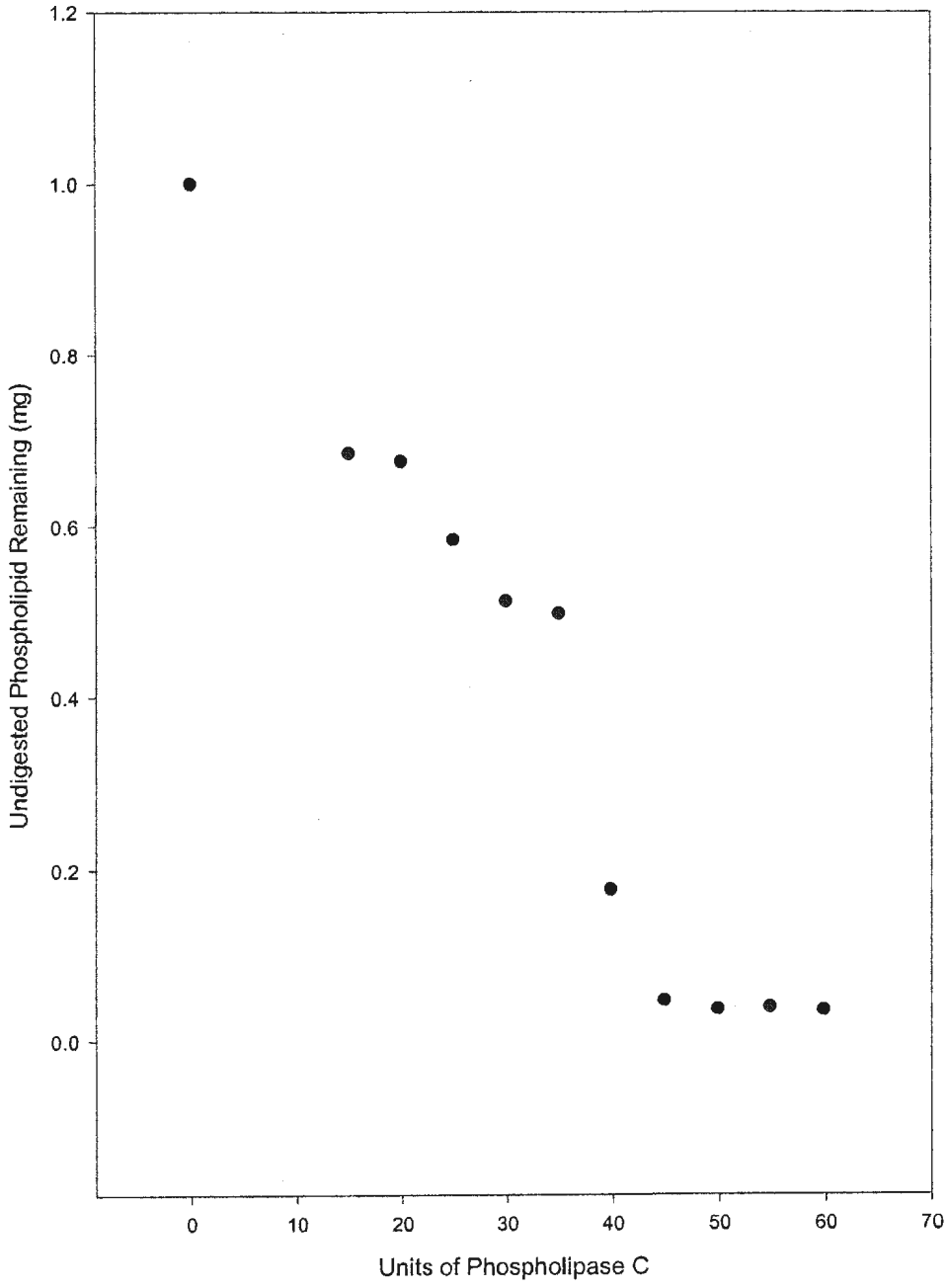


Figure 3.2: Optimization of dephosphorylation procedure. Portions (0.5 mL) of a 1.964 mg/mL standard solution of α-phosphatidylcholine dipalmitoyl were reacted with increasing amounts of phospholipase C as per the procedure outlined in Section 2.2.2.

In order to hydrolyze all the PL present in a sample, some prior knowledge of how much PL was present in the sample is necessary. An estimate of the PL per sample can be obtained from previous literature and TLC-FID results or experience in order for a suitable amount of enzyme to be added. If a percentage of PL in the wet or dry weight of a sample is known or estimated, then successful dephosphorylation can be performed. GC analysis of the TMS-DAG can then be used to identify and obtain quantitative information about each individual PL compound (by carbon number) along with the other lipids present in the sample.

3.2 Hydrogenation

The accumulative effects of various functional groups, unsaturation and carbon number contribute to the chromatographic behavior of the compounds in a lipid sample. Unsaturation, or the presence of double bonds, plays a major factor in terms of identification and separation of compounds by GC. Compounds such as fatty acids, which are similar in structure are separated according to their carbon chain lengths and their unsaturation. Short hand nomenclature for fatty acids is generally of the form a:bωc where a is the number of carbons in the chain, b is the number of double bonds and ω-c is the position of the double bond closest to the terminal methyl group. In terms of short column GC elution order, on a 30m non-polar column, an 18:1ω7 fatty acid would elute before an 18:2ω4 fatty acid. Both of these fatty acids elute before a 20:5ω3 fatty acid.

In this GC project, a wide variety of compounds are being analyzed which differ greatly in structure and complexity. Problems arise where compounds of differing carbon

numbers and differing degrees of unsaturation co-elute. By hydrogenating the sample, unsaturation as a variable for chromatographic separation is eliminated and separation is now dependent only upon carbon number and functional groups. Figure 3.3 shows two GC chromatograms of a copepod sample (A) before and (B) after hydrogenation as per the procedure outlined in section 2.2.3. Peak resolution in Figure 3.3 B increases dramatically, especially in the region of the WE (approximately between 8 and 16 minutes), which is shown, and quantitation of compounds based on carbon number becomes easier and more accurate. These chromatograms and results will be discussed further in Section 3.7.

3.3 Investigation of procedures by Iatroscan TLC-FID

Lipid extracts of capelin obtained from Williams Harbour, Labrador were subjected to the phospholipid digestion and hydrogenation procedures, separately and then consecutively. The samples were then analyzed by Iatroscan to monitor the effects of each procedure. The original Iatroscan chromatogram of the capelin sample is shown in Figure 3.4 A. The capelin extract contains HC, TAG, ST, two types of AMPL (indicated by two peaks) and PL. Ideally, only the PL should be affected by the dephosphorylation procedure.

The dephosphorylation had no effect on the relative amounts of the overall lipid classes with the exception of DAG and PL. By comparison of peak areas, 95% of the PL was converted to DAG. Figure 3.4 shows the capelin sample (A) unmodified and (B) after dephosphorylation and hydrogenation. In Figure 3.5 (B), the PL peak is visibly decreased

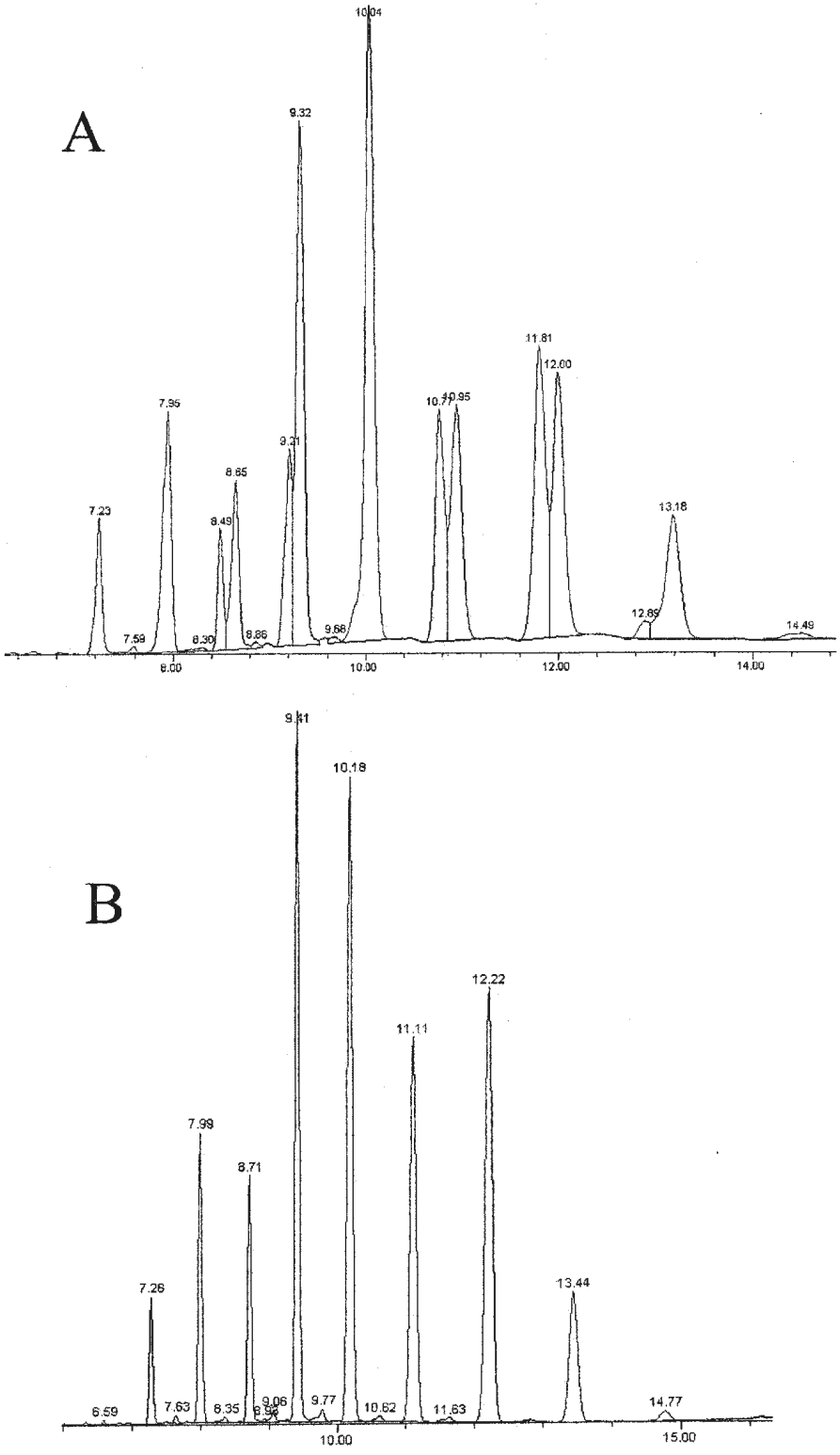


Figure 3.3: Partial chromatograms of (A) non-hydrogenated and (B) hydrogenated copepod sample, showing the WE peaks.

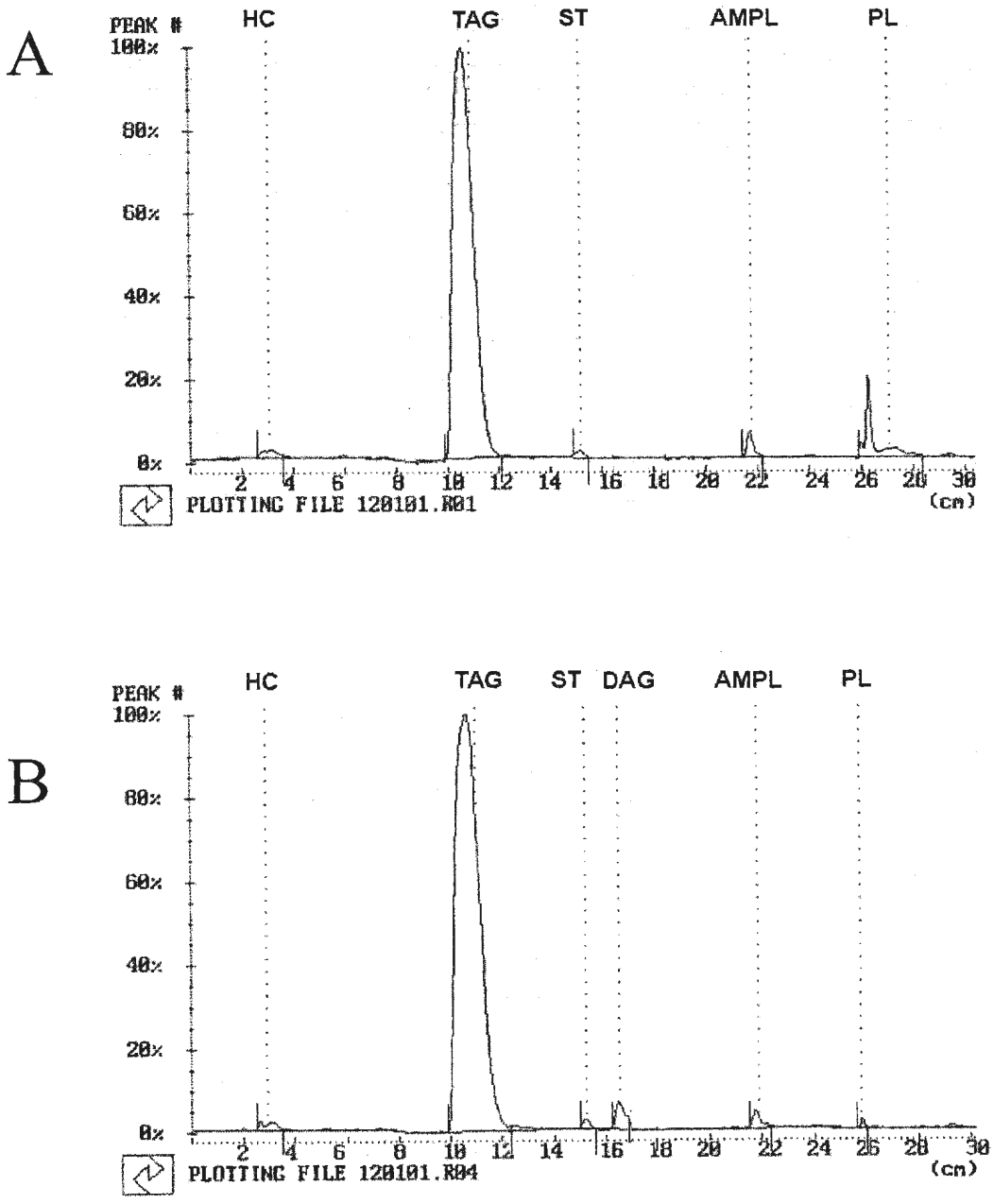


Figure 3.4: Comparison of (A) an unmodified capelin extract with (B) a capelin extract after dephosphorylation and hydrogenation.

and the expected DAG peak is now present. However a loss of ~20% overall lipid occurred during the dephosphorylation and hydrogenation procedures. This was monitored by comparison of TAG concentrations before and after. Improvement in this area would be beneficial to the overall recovery of samples. A possible solution to this problem would be use of an internal standard to monitor how much sample loss occurs between the beginning and the end of a sample preparation for GC. A TAG with a low carbon number such as tricaprin (C30:0) would be an appropriate choice (Kuksis, 1975), since it elutes by short column GC analysis much earlier (approximately 9 minutes) than other typical TAG peaks (longer than 18 minutes) found in marine samples. A problem using tricaprin would be risk of overlapping peaks with another compound with 30 carbons, or similar polarity. Recovery studies using standards could be performed in order to optimize the procedures to achieve the conditions for maximum recovery. General losses are to be expected; however they can be minimized.

Hydrogenation did not affect the TLC retention distances of any of the lipid classes in the capelin extract and relative peak areas were also not affected, except for the PL and DAG as mentioned above. In other words, the amount of TAG relative to HC and ST were not affected by the hydrogenation step. Short column GC was then used to analyze a capelin sample that was dephosphorylated, hydrogenated and TMS derivatized in order to compare the amounts of lipid from both GC and Iatroscan data. The results obtained are found in Section 3.5.1.

3.4 Lipid class identification and calibration by short column GC

Separations of lipids on a short GC column are the result of structural complexity, carbon number as well as the functional groups of each compound. As a result, compounds are eluted in order of: hydrocarbons, ketones, alcohols, fatty acids (hydrogenated species of the same carbon number will always elute in this order), wax esters, diacylglycerols, steryl esters, and triacylglycerols. Table 3.1 shows the retention times of the common carbon numbers of each lipid class. Retention times of compounds marked with an asterisk denote those compounds determined using single standards, while other retention times were determined using mathematical interpolation between standards as well as through comparison with peaks from marine samples. In the case of using marine samples to determine retention times, Iatroscan data of the extracts made it possible to select samples whose lipid classes would be sure to separate very well by the short column GC method. For example, a winter flounder sample contained FFA, ST, PL and TAG, all of which elute at very different retention times. In the case of determining TAG retention times, using previous knowledge that the major compounds in the TAG lipid class are odd in carbon number (three even carbon-numbered fatty acid chains and the three-carbon glycerol backbone) as well as knowledge that C51, C55 and C57 elute at 18.18, 20.94 and 22.36 minutes respectively, the retention times of the rest of the TAG compounds could be predicted. Groupings of lipid classes in terms of carbon number become simple to predict with experience, with the TAG profile resembling a Gaussian distribution at times when TAG is of high proportion in the extract as in the capelin extract and flounder extract in Figures 3.5 and 3.6, respectively.

Table 3.1: Retention times (in minutes (! 0.01) of lipid classes by carbon number, as determined by standard calibration and mathematical interpolation.

Carbon number	HC	KET	ALC**	FFA**	ME	EE	ST**
14	1.69	1.86	2.07				
16	2.14	2.64*	2.84*	2.91*	3.00*		
18	2.67*	3.44	3.49	3.63	3.74*		
20	3.54	4.27	4.21	4.32*	4.43*	5.11*	
22			4.87	4.97*	5.07*		
24				5.58	5.67*	5.74	
26							6.63
27						6.37	7.12*
28							7.49
29							7.79*
30							8.06*

Carbon Number	WE	DAG**	SE	TAG
32	7.99			
34	8.71			
35		9.08		
36	9.41*			
37		9.95*		
38	10.18			
39		10.82		
40	11.11			
41		11.85*		
42	12.22			
43		13.02	14.81*	
44	13.44*			
45		14.28	16.28*	
46	14.66			
47		15.60	17.76*	
48	15.88			
49		16.81	19.25*	16.91
51				18.18*
53				19.56
55				20.94*
57				22.36*
59				24.18
61				26.21
63				30.09
65				33.97

* - retention times determined by single standards (Sigma).
** - classes that were TMS derivatized prior to calibration.

Using similar information about the other lipid classes can also aid in identification of peaks. For example, SE, DAG and the previously mentioned TAG are typically odd carbon-numbered compounds. Even carbon-numbered compounds of these classes are possible and do exist, but generally are of much smaller abundance. Odd-carbon numbered fatty acid chains are generally less common in marine samples than those containing even carbon numbered chains, especially the 16, 18, 20, 22 and 24 carbon FFA chains. This is also true of KET and WE. In the cases of DAG and TAG, the three-carbon glycerol backbone in addition to the even-carbon numbered fatty acid chains make these classes typically odd in carbon number. Sterols in marine samples are typically highest in C27 with some samples having minor amounts of C26 and C28. Terrestrial markers such as C28 and C29 ST as well as C30, a dinoflagellate marker can also be observed in some marine samples (Hudson et al, 2001). Since the C27 ST peak is generally the largest ST peak, it becomes simple to locate it (7.1 minutes) and then locate the C26, C28, C29 and C30 peaks (if present) eluting 0.3 minutes from each other using the short column. In cases where WE, SE and DAG are all present in the sample, good separation can be difficult at times, resulting in some shouldering of GC peaks. However, WE are usually even carbon-numbered, and DAD and SE are odd carbon-numbered, so overlap is generally not a problem.

FID responses of several lipid standards were calibrated using the short column GC method. These standards consisted of the following selection of neutral lipids: hydrocarbons, ketones, free fatty acids, wax esters, steryl esters, an alcohol, diacylglycerols and triacylglycerols. Classes marked with a double asterisk in Table 3.1 denote lipid classes that were TMS derivatized prior to calibration. These lipids were

calibrated in order to be able to determine the amounts of each lipid class quantitatively. In a situation where many lipids of the same class appear in a sample, it is not a viable option to have a standard for each individual lipid. Therefore, experiments were performed to determine whether two standards differing in carbon length from each class would have the same response factor. If the resulting calibration equation between each of the standards was similar, then it could be used to determine the amounts of all types of lipids. In other words, one standard could be used to calibrate a whole class of lipids. Calibration curves for each standard were calculated and compiled using increments of 10, 20, 40, 60, 80 and 100 ng of standard injected. These calibration curves were then used in the quantitation of each sample analyzed. A representative selection of the calibration curves can be found in Appendix 1.

3.5 General comparison of short column GC and TLC-FID results

Each sample extract analyzed using the short column GC method was dephosphorylated, hydrogenated and TMS derivatized prior to chromatography. Without TMS derivatization (see Section 2.2.4), chromatograms of compounds containing a free OH functional group, such as FFA, ALC, ST, MAG and DAG suffer from peak tailing due to strong interaction with the liquid phase of the column. By performing a TMS derivatization, these compounds become more volatile (and less polar, which results in weaker retention time compared to an underivatized compound) and improved peak resolution is observed. Iatroscan lipid class analysis does not require TMS derivatization, however derivatized standards were used in the GC calibration.

The quantitation of PL by the short column GC method involves quantifying the DAG in the sample after dephosphorylation. It must be noted that the structure and/or identity of the phosphate headgroup that is removed by the procedure cannot be obtained. However, useful information can be obtained about the carbon-rich (non-polar) DAG backbone of the PL. There is generally one DAG produced by the dephosphorylation for each PL molecule present in the extract. By subtracting the mass of the TMS group from the DAG and then adding a phosphate headgroup to the compound, one can calculate the amount of PL. The phosphate head group phosphatidyl choline was selected since it is the most abundant animal diacylglycerophospholipid (Gurr *et al.*, 1971). Therefore, the percentage of PL in the sample calculated by short column GC should be the equivalent to the percentage of PL determined by the Iatroscan. Hence, percent lipid data is a useful way to determine whether the information obtained by the short column GC method is comparable to that obtained by the Iatroscan.

Prior to GC analysis, an Iatroscan was used to analyze the samples for lipid classes. Some of these Iatroscan analyses were performed close to a year prior to the GC analysis, and this storage time may contribute to elevated levels of FFA present in the samples, due to breakdown of the acyl lipid classes.

3.5.1 Short column analysis of capelin extract

This capelin extract was the same extract used to investigate the dephosphorylation and hydrogenation procedures in Section 3.3. Capelin are of interest to researchers since they are the prey of many marine animals, including larger fish, mammals and birds. Table 3.2 shows the percent lipid class data obtained by the short

Table 3.2: Percent lipid data for capelin extract determined by short column GC and Iatroscan.

Lipid Class	Carbon Number	% Lipid GC	% Lipid Iatroscan
HC	14	0.13	
	16	0.12	
	18	0.13	
	∑HC	**0.39**	**0.65**
FA	18	0.23	
	20	0.19	
	22	0.31	
	24	0.24	
	∑FFA	**0.97**	**0.76**
ST	27	1.16	
	28	0.07	
	∑ST	**1.23**	**0.93**
DAG (PL)	39	1.65	
	41	2.12	
	43	2.55	
	45	1.86	
	47	2.20	
	∑DAG (PL)	**10.37**	**7.58**
TAG	49	0.82	
	51	3.61	
	52	0.44	
	53	7.20	
	54	1.45	
	55	15.85	
	56	2.31	
	57	18.50	
	58	1.21	
	59	20.95	
	60	0.97	
	61	7.25	
	62	0.61	
	63	6.46	
	64	0.51	
	65	3.41	
	∑TAG	**91.55**	**90.08**

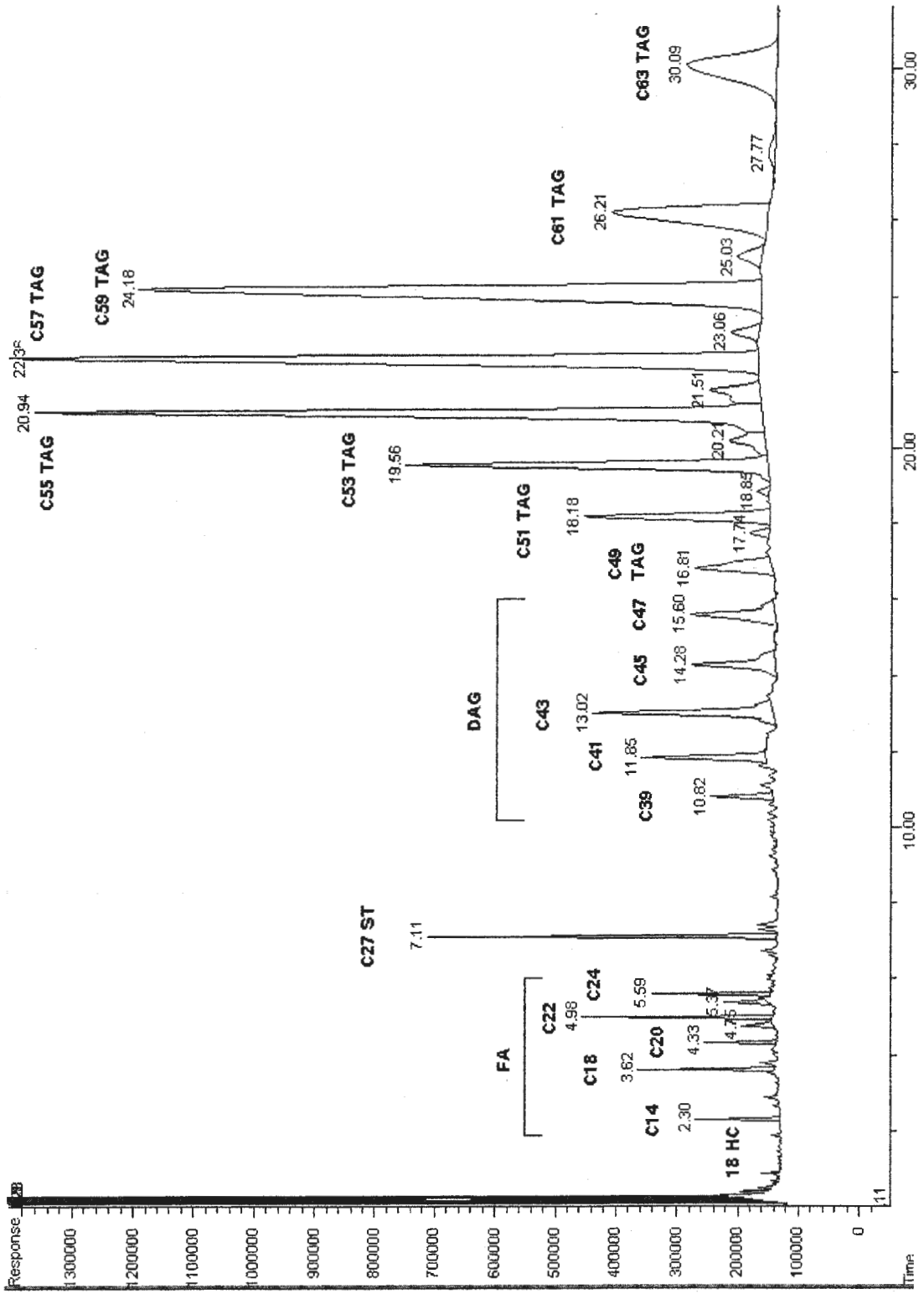


Figure 3.5: Capelin extract short column GC chromatogram after dephosphorylation, hydrogenation and TMS derivatization.

column method, which was very close to that of the Iatroscan. Figure 3.5 shows the chromatogram of the capelin lipid extract after the procedures. The lipid classes are very distinguishable, and resolution is quite good. This capelin extract shows that the GC method is applicable to the analysis of lipid classes. Further analysis of other types of marine samples will help confirm the applicability of the short column GC method.

3.5.2 Yellowtail flounder

Marine aquaculture is a rapidly expanding field in which research is being done to help improve the culture of many marine finfish and shellfish. Recently, the development of yellowtail flounder aquaculture methods has been under investigation at the Ocean Sciences Centre, Logy Bay, Newfoundland (e.g. Copeman, 2001). Several factors that make yellowtail flounder a good candidate for cold-water aquaculture include its profitable foreign market, high filet-to-body ratio, low commercial supply and relatively high growth rates at low temperatures (Brown et al., 1995; Brown, 2000). One of the main aspects of research into the aquaculture of these fish involves devising conditions that will satisfy the goals of minimizing the mortality rate and maximizing the number of marketable individuals. Possibly the most important of these conditions is selecting an appropriate feedstock to achieve the desired goals of successful aquaculture. Monitoring of the lipid classes and fatty acid levels of the flounder with time is an excellent way to determine the level of nutrition required so that the flounder receive optimal nutritional value. Using the short column GC method, a detailed analysis of the lipid classes of the fish is possible.

Data obtained from the short column GC method were compared to data from the Iatroscan to show the efficiency of the method, and to determine whether the same information could be obtained. Figure 3.6 is the chromatogram obtained from a 500 μL aliquot of the original flounder extract after dephosphorylation, hydrogenation and TMS derivatization. The chromatogram shows the expected peaks, including FFA, ST, DAG (from PL) and TAG. Table 3.3 compares the total amounts of lipid classes obtained from the GC to those from the Iatroscan. In comparison to the Iatroscan method, the levels of FFA in the extract are much higher in the GC sample, mostly due to a large amount of C24 FFA. This increase in the expected amount was due to a C24 FFA internal standard having been added to the extract prior to analysis. Therefore this amount was subtracted before calculating the final amount of C24 FFA. As for the rest of the lipid classes, an overall average of 63.7 ± 3.7% recovery was determined after the procedures, which is consistent with the other samples analyzed with this method.

In comparison to the Iatroscan, the data acquired through the GC method is equally effective in determining the lipid class data of the sample; however, more information is generated about how the compounds within those lipid classes are divided by carbon number. The yellowtail flounder extract was used to determine the overall analytical precision. Three 0.5 mL aliquots of the extract were dephosphorylated, hydrogenated and TMS derivatized. The amount of PL was calculated, and the three samples were found to be 567 ± 10 μg, i.e. 1.7% error between replicate samples.

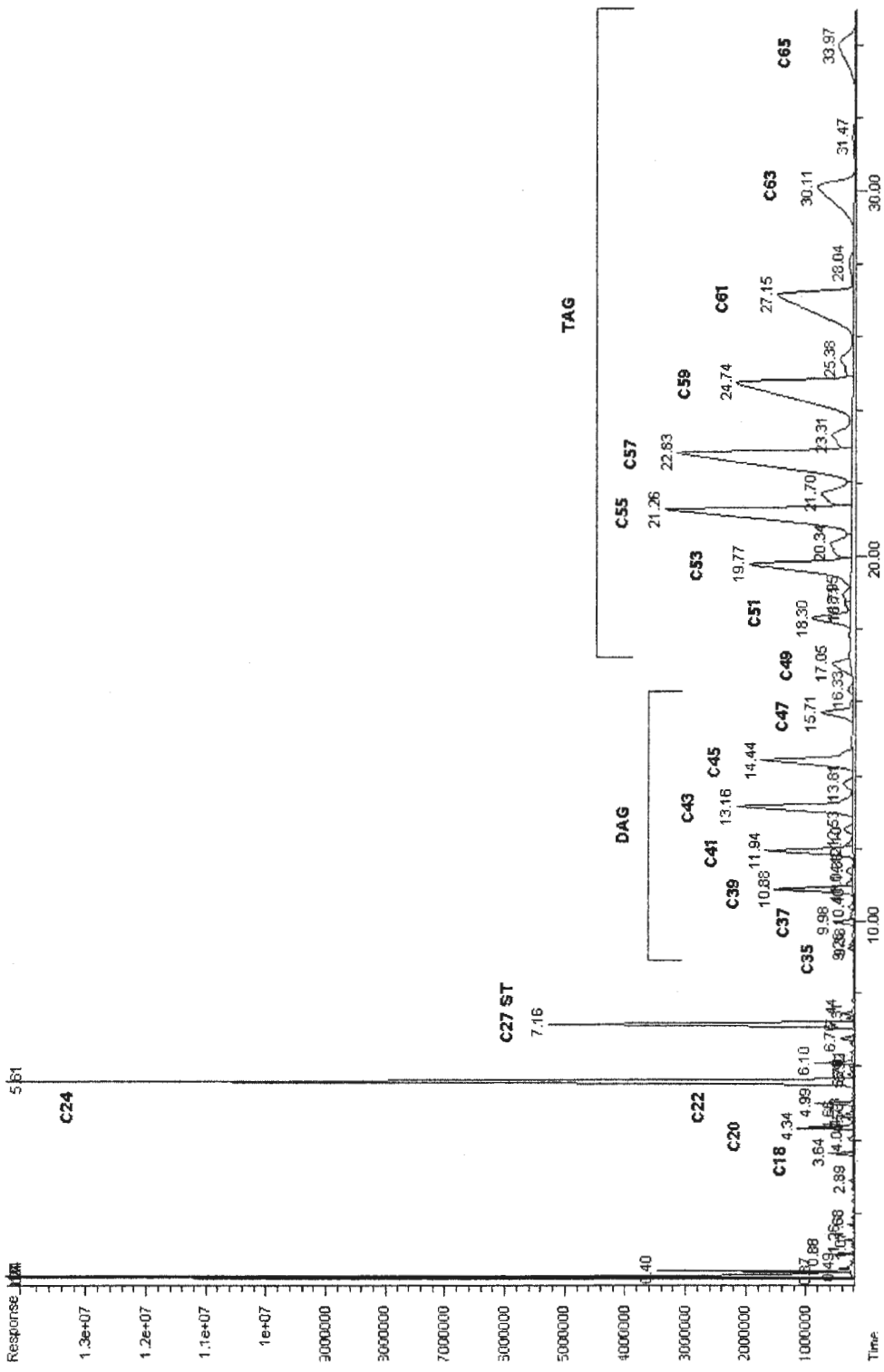


Figure 3.6: Yellowtail flounder short column GC chromatogram after dephosphorylation, hydrogenation and TMS derivatization.

Table 3.3: Percent lipid data for yellowtail flounder extract determined by short column GC and Iatroscan.

Lipid Class	Carbon Number	% Lipid GC	% Lipid Iatroscan
FFA	16	0.05	
	18	0.16	
	20	0.32	
	22	0.22	
	24	0.39	
	∑FFA	**1.08***	**1.92**
ST	27	3.56	
	28	0.10	
	29	0.14	
	∑ST	**3.79**	**4.78**
DAG (PL)	35	0.38	
	36	0.38	
	37	0.73	
	38	0.43	
	39	2.64	
	40	0.57	
	41	3.47	
	42	0.64	
	43	5.50	
	44	0.67	
	45	4.51	
	46	0.73	
	47	1.99	
	∑DAG (PL)	**22.63**	**16.46**
TAG	51	2.77	
	52	0.68	
	53	7.75	
	54	1.66	
	55	14.01	
	56	2.27	
	57	16.60	
	58	1.61	
	59	13.06	
	60	1.34	
	61	9.47	
	62	0.58	
	63	4.88	
	64	0.27	
	65	2.52	
	∑TAG	**71.78**	**76.79**

* - calculated after subtraction of C24 FFA internal standard.

3.5.3 Sea scallop female gonad

Aquaculture of seas scallops is another scenario in which the monitoring of lipid classes is a useful tool in determining the optimal breeding rates of these animals. The lipid levels of female scallop gonads are of importance in particular since scallop eggs derive their nutritional reserves from lipids provided by the gonads (Pazos et al., 1997). In other words, hatchery success is directly related to the lipid status of the eggs when spawned, therefore high lipid levels in the gonads are desirable. Lipids of interest include TAG for their energy storage properties and PL for their importance in cell growth (as part of cell membranes). Therefore, analysis of scallop gonad lipids by short column GC would be an excellent use of this method.

The results obtained by the short column GC compared to those obtained by the Iatroscan (Table 3.4) were very close, with higher levels of FFA due to degradation of the DAG and TAG. Figure 3.7 shows a chromatogram of the scallop gonad extract, which shows that the amount of even carbon-numbered DAG in the sample was high, as they were in the scallop muscle (Section 3.5.4) and spiny sunstar (Section 3.5.6). However, the literature did not suggest that high levels of odd-carbon numbered fatty acid chains are present in these scallops, however it could be accounted for in the spiny sunstar (see Section 3.5.6).

3.5.4 Sea scallop muscle

Lipid class analysis of the muscle tissue of sea scallops is also important in aquaculture of these animals. Nutritional information is of importance to scallops farmers, as well as to monitor the physiological well being of the animals. High levels of polyunsaturated fatty

Table 3.4: Percent lipid data for sea scallop female gonad extract determined by short column GC and Iatroscan.

Lipid Class	Carbon Number	% Lipid GC	% Lipid Iatroscan
HC	14	0.08	
	16	0.10	
	18	0.07	
	20	0.11	
	∑HC	**0.36**	**0.44**
MKET	16	0.35	
	18	0.12	
	20	0.05	
	22	0.10	
	∑MKET	**0.62**	**0.90**
FFA	16	0.13	
	18	0.55	
	20	0.35	
	22	0.60	
	24	0.27	
	∑FFA	**1.90**	**1.25**
ST	26	0.78	
	27	0.78	
	28	1.04	
	29	0.41	
	30	0.10	
	∑ST	**3.11**	**5.50**
DAG (PL)	35	0.59	
	36	0.21	
	37	1.09	
	38	0.21	
	39	2.86	
	40	1.34	
	41	5.83	
	42	3.27	
	43	6.06	
	44	0.21	
	45	2.70	
	46	0.21	
	47	2.09	
	48	0.21	
	49	1.99	
	∑DAG (PL)	**28.87**	**22.31**

SE	41	0.25	
	43	0.27	
	45	0.22	
	47	0.64	
	∑SE	**1.37**	**1.30**
TAG	51	2.77	
	52	0.68	
	53	7.75	
	54	1.66	
	55	14.01	
	56	2.27	
	57	16.60	
	58	1.61	
	59	13.06	
	60	1.34	
	61	9.47	
	62	0.58	
	63	4.88	
	64	0.27	
	65	2.52	
	∑TAG	**71.78**	**76.79**

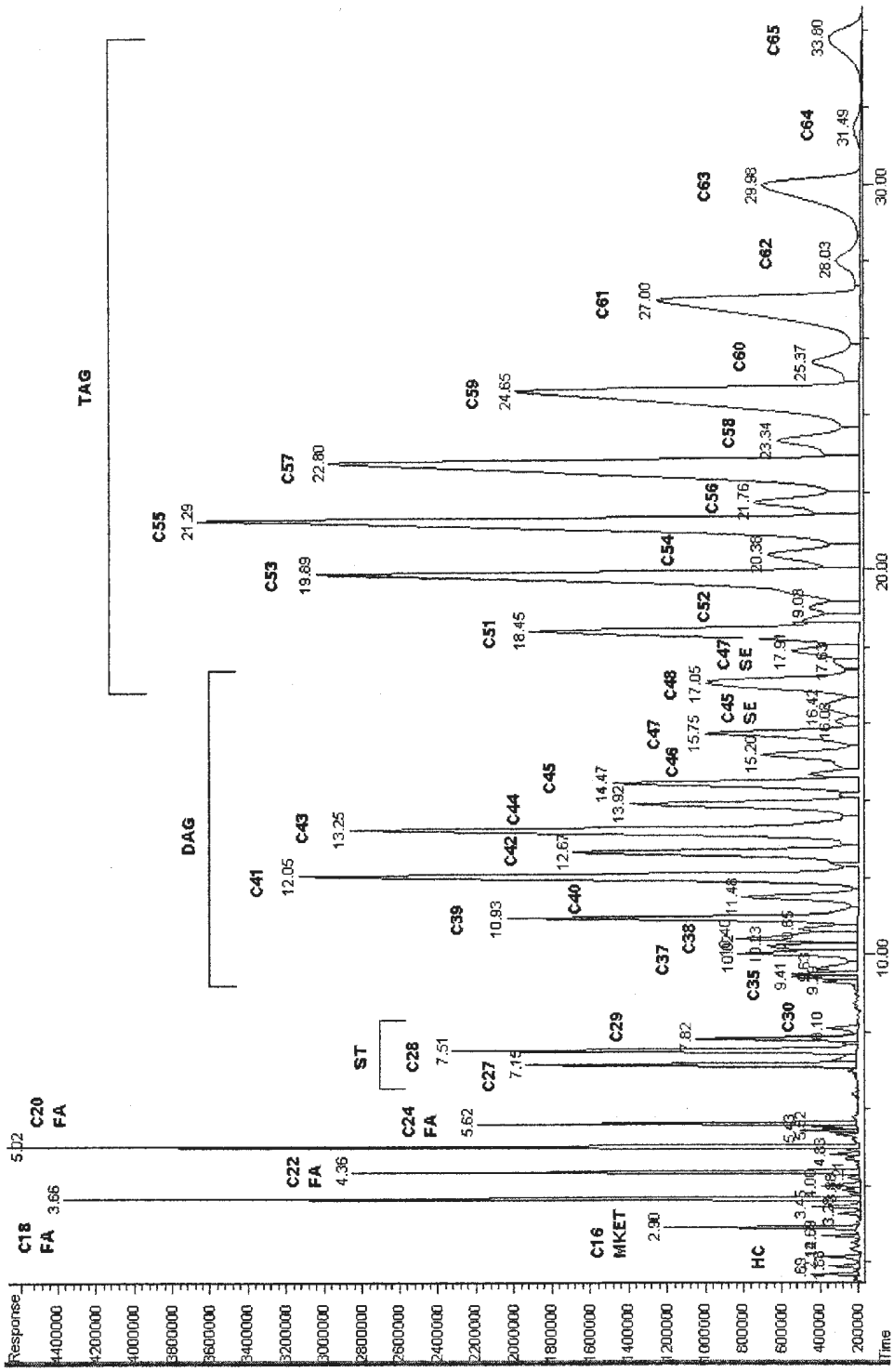


Figure 3.7: Sea scallop female gonad short column GC chromatogram after dephosphorylation, hydrogenation and TMS derivatization.

acids (PUFA) including docosahanoic acid (DHA, 22:6ω3) and eicosapentaenoic acid (EPA 20:5ω3) are commonly found in scallops, bound in the PL and TAG. Sterols are also an abundant lipid in sea scallops, as well as other mollusks such as mussels. Using the short column GC method, these lipid levels can be obtained very quickly. The percent lipid data was compared between both the Iatroscan and GC results to determine the effectiveness of the GC method.

Iatroscan results in Table 3.5 show very high levels of PL, upwards of 73.8%, followed by ST at 20.9%, TAG at 2.1%, SE at 1.3% and also HC at less than 0.1%. Figure 3.8 shows the GC chromatogram obtained from the scallop muscle extract. The short column GC gave similar percent lipid data: 78.62% PL, 15.64% ST, 0.36% SE and 1.02 % TAG, however the levels of FFA and HC were slightly elevated at 0.74% and 0.6% respectfully. The elevated levels of HC may be possibly due to use of filter paper that was not completely lipid clean. The FFA increase may have been due to the drop in overall SE, TAG and PL levels in the extract over the storage time of approximately one year.

An interesting feature of the GC chromatogram is the appearance of high amounts of DAG with even carbon numbers. It is unlikely that there would be such high levels of C15, C17, C19 and C21 FFA bound in the DAG compounds. However, the percent lipid GC results are supported by the amount of PL that was determined in the extract, according to the Iatroscan results. Further detailed fatty acid analysis of this sample would be appropriate to check the levels of odd carbon numbered fatty acids.

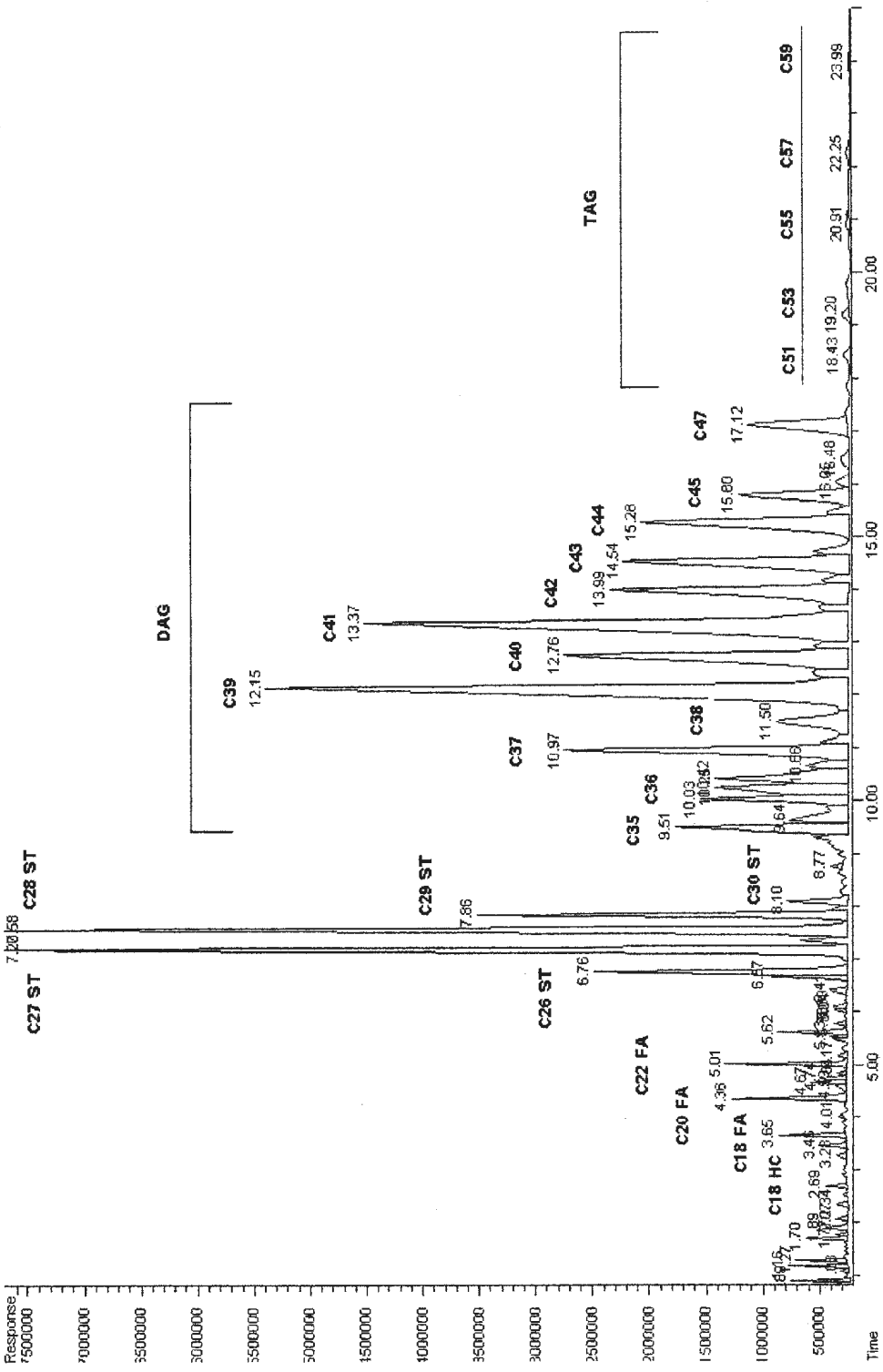


Figure 3.8: Short column GC chromatogram of scallop muscle tissue extract after dephosphorylation, hydrogenation and TMS derivatization.

Table 3.5: Percent lipid data for scallop muscle extract determined by short column GC and Iatroscan.

Lipid Class	Carbon Number	% Lipid GC	% Lipid Iatroscan
HC	14	0.17	
	16	0.15	
	18	0.11	
	20	0.15	
	∑HC	**0.58**	**0.07**
FFA	18	0.14	
	20	0.24	
	22	0.22	
	24	0.15	
	∑FFA	**0.74**	**0.09**
ST	26	1.52	
	27	5.91	
	28	5.75	
	29	2.08	
	30	0.38	
	∑ST	**15.64**	**20.94**
DAG (PL)	35	3.25	
	36	1.46	
	37	2.52	
	38	3.06	
	39	5.40	
	40	2.31	
	41	15.92	
	42	7.54	
	43	14.70	
	44	6.02	
	45	5.63	
	46	5.41	
	47	2.68	
	48	2.72	
	∑DAG (PL)	**78.62**	**73.88**
SE	45	0.26	
	47	0.10	
	∑SE	**0.36**	**1.30**
TAG	51	0.18	
	52	0.12	
	53	0.05	

54	0.10	
55	0.05	
56	0.12	
57	0.06	
58	0.09	
59	0.05	
60	0.08	
61	0.05	
62	0.07	
63	0.00	
∑TAG	**1.02**	**2.10**

3.5.6 Spiny sun star *Crossaster papposus*

The common sun star, *Crossaster papposus*, relies on lipids as an important reserve of energy in the form of TAG and alkyldiacylglycerols. In addition, cyclic changes in the lipid content of these animals have been correlated with their reproductive cycles (Sargent *et al.*, 1983). Therefore, lipid determination and analyses is of importance to researchers who are interested in the biochemistry of this animal.

The percent lipid data determined by Iatroscan and short column GC is shown in Table 3.6 as well as the GC chromatogram obtained by the short column GC method (Figure 3.9). The short column GC data agrees well with the Iatroscan percent lipid results. Of particular interest in the short column GC data is the presence of a large percentage of even carbon-numbered DAG compounds in the sample, which is indicative of large amounts of odd chained fatty acid groups in the PL of these animals. This phenomenon of odd chain fatty acids in asteroids such as these is discussed by Sargent *et al.* (1983) and the references therein.

3.6 Other Applications

Once the short column GC method had been validated for use in routine analyses, the method was used to solve real analytical problems. In some situations where Iatroscan could not provide adequate information about a sample, the short column GC method was used to authenticate questionable Iatroscan data. In addition, in situations where the limits of Iatroscan begin to appear, the short column GC method was used to expand the chemical information about the extracts.

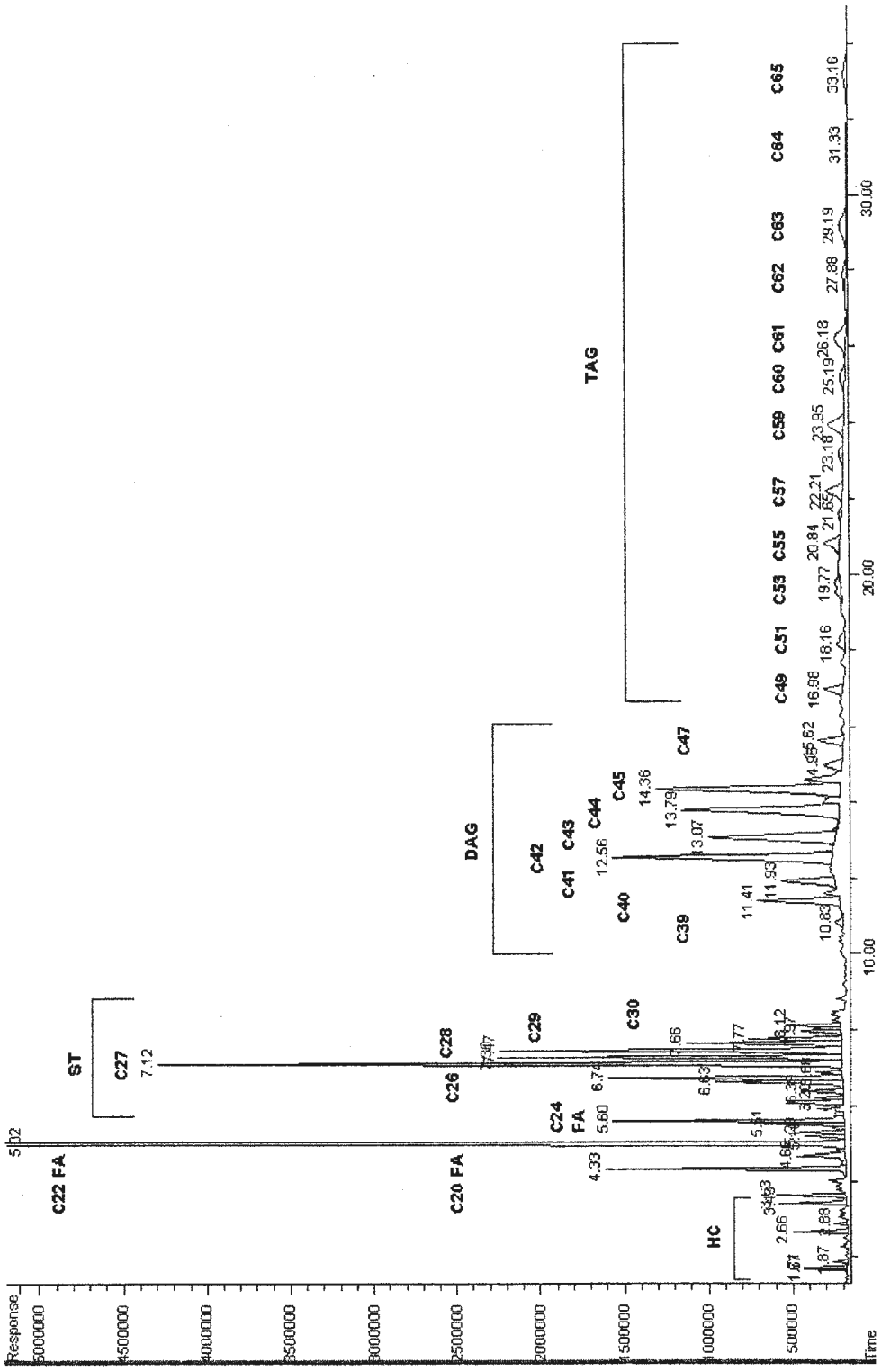


Figure 3.9: Short column GC chromatogram of *Crossaster papposus* extract after dephosphorylation, hydrogenation and TMS derivatization.

Table 3.6: Percent lipid data for ***Crossaster papposus*** extract determined by short column GC and Iatroscan.

Lipid Class	Carbon Number	% Lipid GC	% Lipid Iatroscan
HC	14	0.59	
	16	0.51	
	18	0.39	
	20	0.52	
	∑HC	**2.01**	**1.60**
FFA	18	0.31	
	20	0.98	
	22	8.14	
	24	1.02	
	∑FFA	**10.45**	**9.90**
ST	26	4.18	
	27	8.13	
	28	4.19	
	29	1.14	
	30	0.53	
	∑ST	**12.32**	**16.37**
DAG (PL)	39	1.46	
	40	3.94	
	41	3.53	
	42	11.42	
	43	8.85	
	44	10.53	
	45	12.04	
	46	2.06	
	47	2.19	
	48	2.05	
	∑DAG (PL)	**58.08**	**45.75**
TAG	49	1.10	
	50	0.42	
	51	0.78	
	52	0.28	
	53	0.73	
	54	0.18	
	55	1.96	
	56	0.40	
	57	1.31	
	58	0.58	
	59	1.72	
	60	0.79	
	61	1.48	

	62	0.69	
	63	1.33	
	64	0.51	
	65	0.90	
	∑TAG	**14.04**	**16.89**

3.6.1 Determination of ethyl esters in rotifers

Long chain PUFA such as DHA and EPA are essential fatty acids in the early growth, survival and lipid composition of many larval fish (Copeman et al, 2002). In the aquaculture of fish, live-foods that are commonly used as feed for larval fish include rotifers and *Artemia* sp. However, both of these live-feeds are naturally low in long chain PUFA, which again are important in larval fish nutrition. Therefore, rotifers and *Artemia* grown as larval fish food ingest feed that is enriched with PUFA-rich commercial products such as Algamac in order to increase their nutritional value for consumption by the cultured animal. Other feed enrichments include *Isochrysis galbana* (T-Iso), though not rich in PUFA, does improve the nutritional benefits of the live feed for the larval fish. The lipid levels of live feed can be analyzed by Iatroscan to help determine their nutritional value. However, Iatroscan analysis of rotifers by the Parrish lab has shown difficulty in confirming the identity of a peak that elutes at the position of an ethyl ester of PUFA. All three samples contain the same lipid classes (HC, TAG, FFA, ST, some AMPL and PL), as well the unidentified peak which appears at 7 cm. Further attempts at identification of the peak in Parrish lab included co-spotting with standards of fatty acid methyl ester (ME), ketone (KET) and fatty acid ethyl ester (EE) to determine if the peak will co-elute with either of these standards. The unidentified peak elutes before both KET and ME however the peak only co-elutes with the EE in some samples but not in others. The short column GC method was used to confirm the identity of the peak.

The three samples all contained C20 FFA, C22 FFA in high amounts and also C24 FFA in smaller amounts. These chromatograms are shown in Figure 3.10 A to C shows the short column GC chromatograms of rotifers that are (A) unenriched, (B) enriched with Algamac and (C) enriched with I. galbana. Ethyl esters were the peaks that were

tentatively identified as C22 EE, and C24 EE. Only qualitative data was obtained, since the EE peaks were identified by mathematical interpolation. Standards of C18 and C20 EE were used to predict the retention times of the C22 and C24 EE. The presence of these ethyl esters may be due to the rotifer feed, which may have some of its rich PUFA in ethyl ester form. Further lipid analysis of the rotifer feed may confirm this.

The ST region of the chromatograms (C26 to C29 ST) is interesting as well since there seems to be an extra peak between C28 and C29 sterol in the enriched rotifers. It is possible that this compound may be AMPL, specifically a C28 or C29 monoacylglycerol. In addition the peak found just before the C27 ST peak may be underivatized C27 ST. However, if this were the case then it would be expected that the other ST peaks would also show signs of underivatized sterol. Coupling the short column GC method with mass spectrometric detection would be useful to determine the true identity of these compounds.

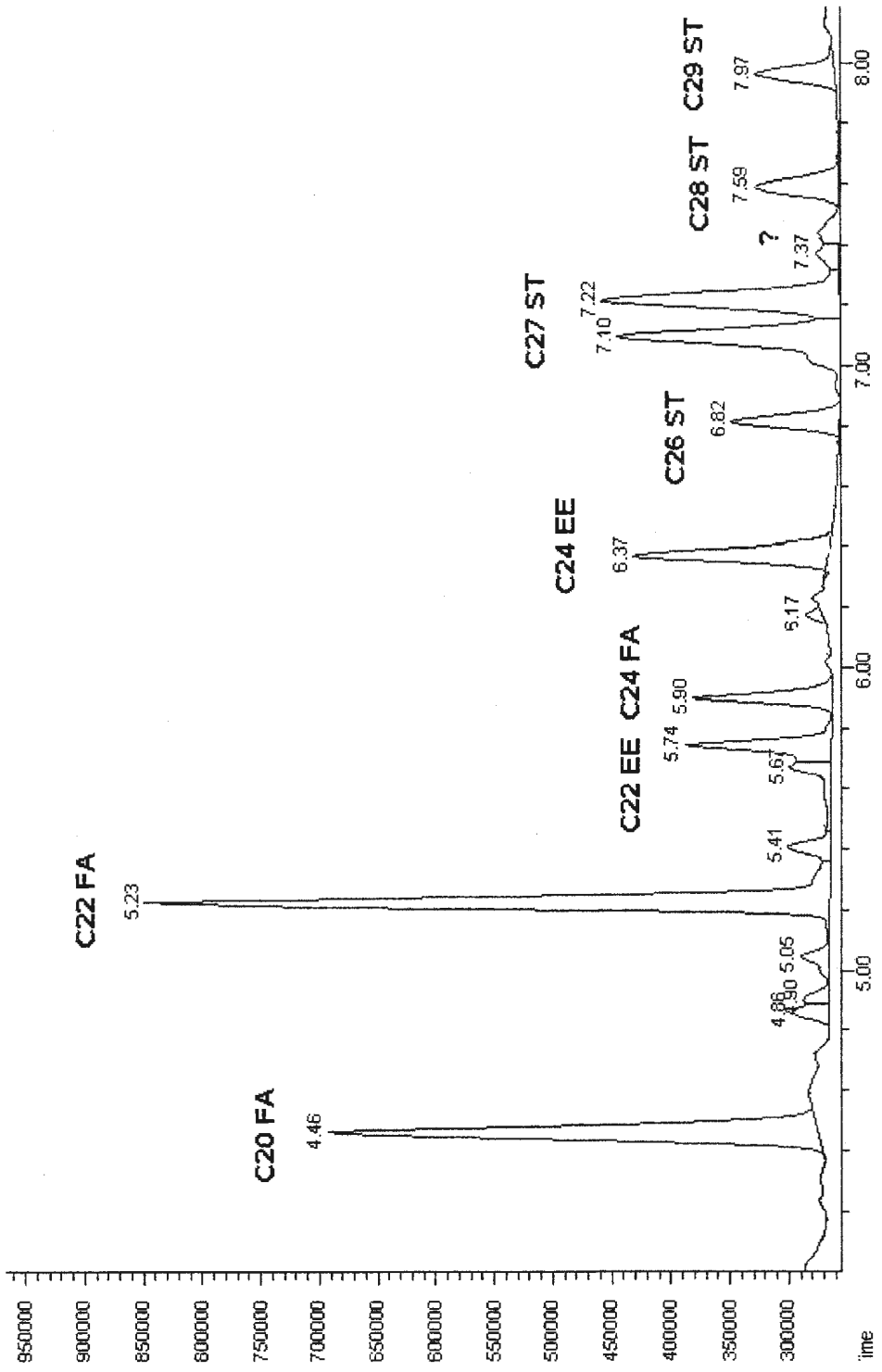


Figure 3.10 A: Partial chromatogram of unenriched rotifer extract after dephosphorylation, hydrogenation and TMS derivatization.

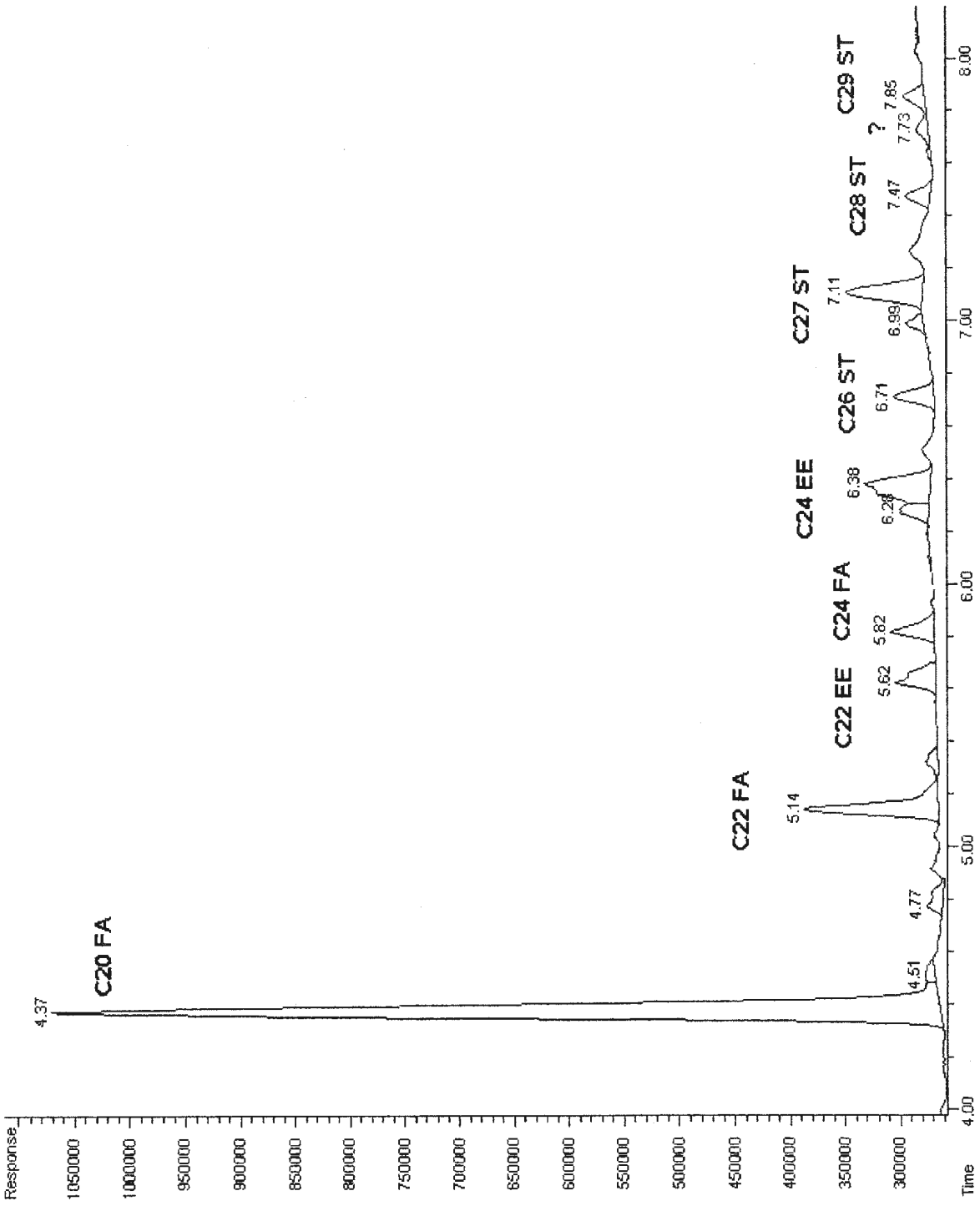


Figure 3.10 B: Partial chromatogram of Algamac-enriched rotifer extract after dephosphorylation, hydrogenation and TMS derivatization.

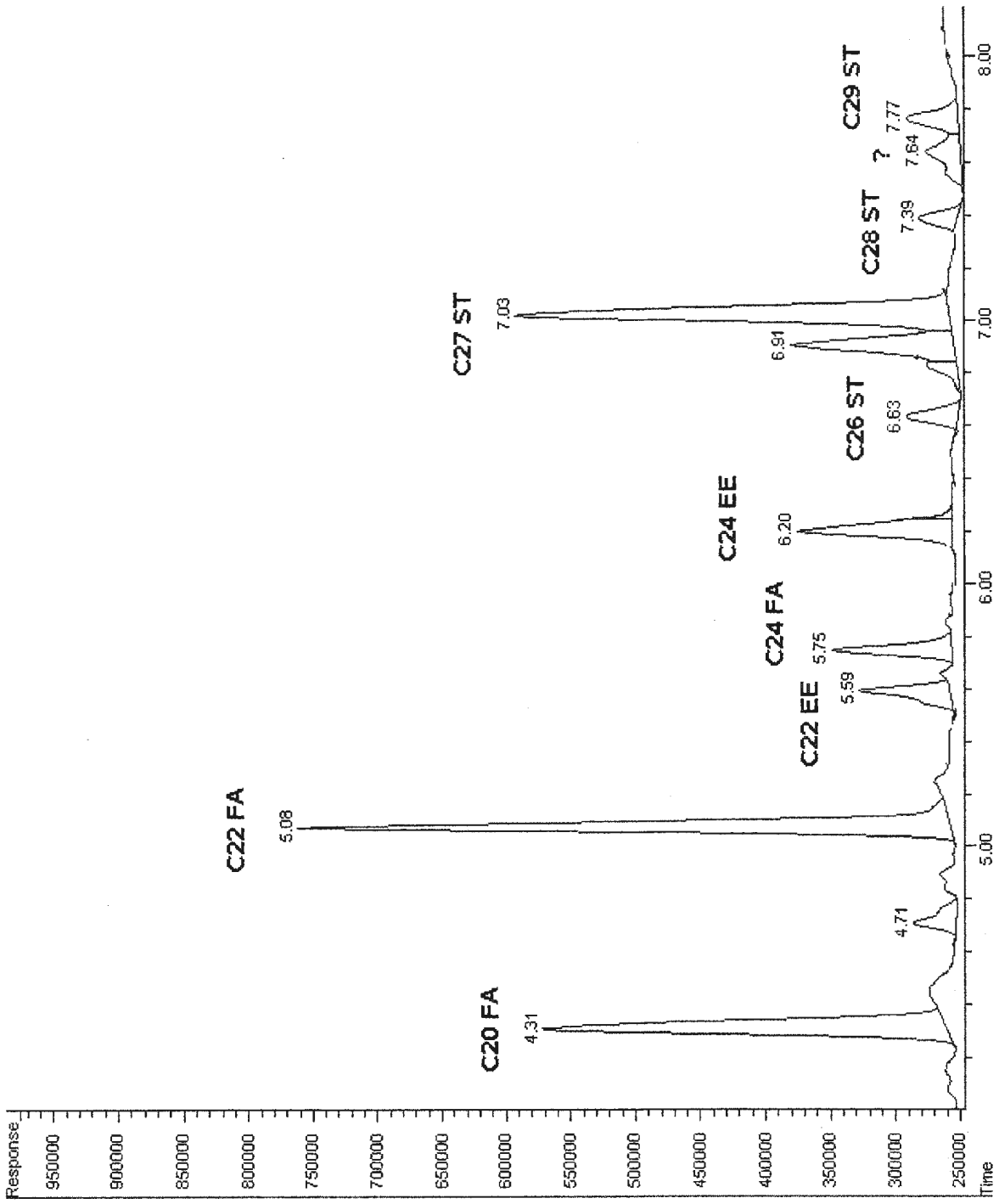


Figure 3.9 C: Partial GC chromatogram of *I. galbana* enriched rotifer extract after dephosphorylation, hydrogenation and TMS derivatization.

3.6.2 Determination of wax esters in the copepod *Calanus hyperboreus*

Wax esters can be found in many types of marine organisms such as copepods, decapods, euphausiids, mysids, chaetonaths, squid and several fish (Lee, Hirota, 1973). Wax esters are an important energy reserve for copepods, which are the dominant prey for many species of marine larvae in the wild. In higher latitudes, copepods have been found to contain greater than 80% of their lipids as WE (Kattner & Krause, 1987). Using Chromarods (in Iatroscan TLC-FID) for analysis of total lipid profiles, it is difficult to resolve SE peaks and WE peaks, yet there are sometimes two peaks in the WE/SE region. An example comes from the work of Stevens (personal communication) where two peaks where determined by Iatroscan, tentatively identified as wax ester 1 and wax ester 2. Further confirmation of the identities of these peaks was necessary to determine the true amount of wax ester in the extract. The Iatroscan chromatogram in Figure 3.11 shows the two peaks, labeled WE1 and WE2, the identities of which had several possibilities. The two peaks could have been SE, WE or a combination of the two lipid classes. This short column GC method was used to identify the lipid class composition and carbon number distribution of the copepod lipid extract, as well as illustrate the importance and influence of hydrogenation. The sample provided by Stevens consisted of extract from 48 copepods; the WE component isolated using the method of Ohman (1997). Assuming the two peaks were WE, an Iatroscan used to determine that the concentration of the WE lipid class found 1.16 g/L in the extract.

Figure 3.12 shows a GC chromatogram of the hydrogenated copepod extract. The retention times of interest fall between 7 and 13.5 minutes, which is the range for wax ester compounds. Peaks at 7.28, 7.99, 8.71, 9.41, 10.18, 11.11, 12.22 and 13.44 min are

the even carbon numbered WE from C30 through C44 (see Table 3.1). These even carbon-numbered wax esters are the major peaks, and there are trace amounts of odd carbon-numbered wax esters in between each major peak. One small SE peak eluted at 14.77 minutes, which was C43 SE or quite possibly the same compound used in the standard, cholesteryl palmitate. These chromatographic results show that the peaks WE1 and WE2 observed in the Iatroscan chromatogram were 97.9% WE by peak area. Using WE standards, quantitation of the peaks of interest gave a concentration of 999 mg/L of WE and 21.9 mg/L of SE. Based on Iatroscan results, 88.3% recovery of SE and WE compounds after the hydrogenation procedure was indicated. As mentioned in Section 3.2 and demonstrated here, hydrogenation permits a straightforward determination of the lipid compounds in the sample with this method. There is no confusion of compound identity due to unsaturation, as compounds elute by carbon number within their class.

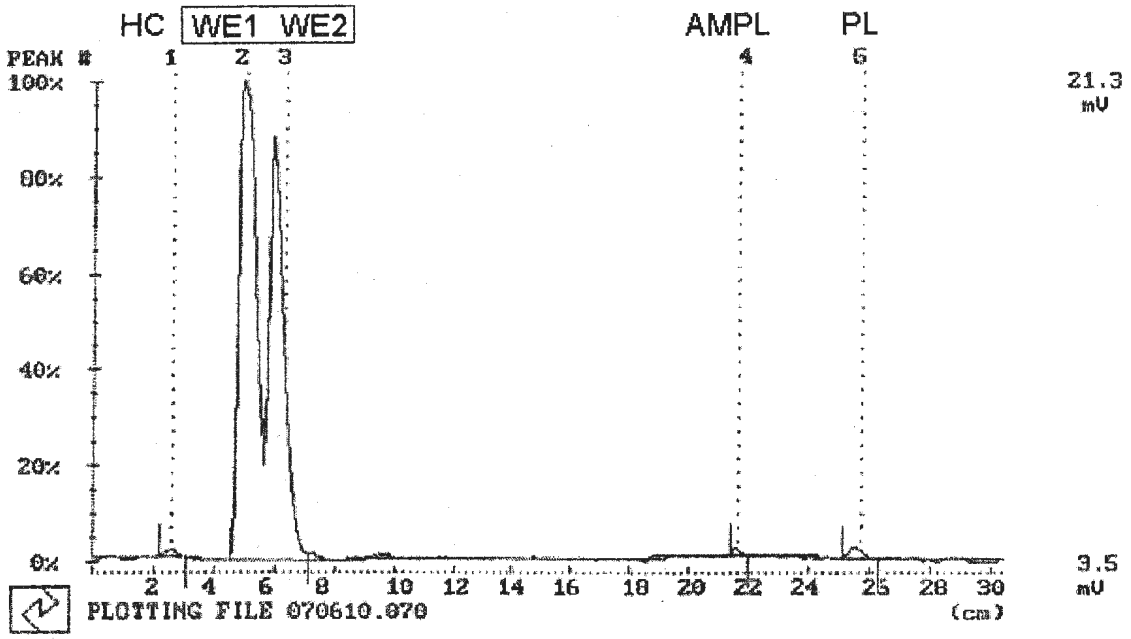


Figure 3.11: Iatroscan chromatogram of *Calanus hyperboreus*, indicating the two unknown WE peaks, WE1 and WE2.

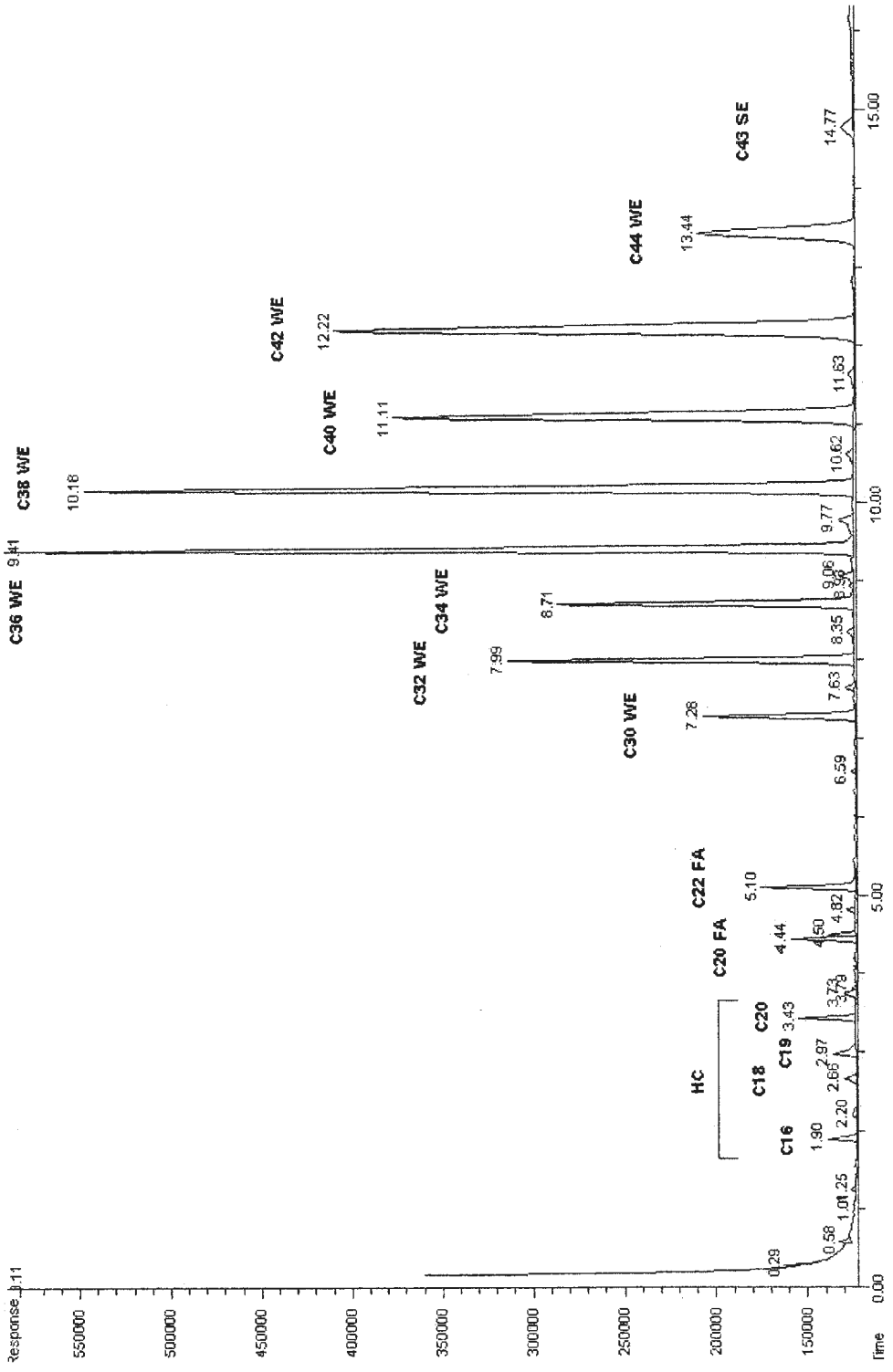


Figure 3.12: Partial GC chromatogram of hydrogenated *Calanus hyperboreus* after hydrogenation, and TMS derivatization.

4 CONCLUSIONS

4.1 Conclusions

Analysis of lipids is a very informative way of determining the physiological state of a marine ecosystem. Lipids can give insight into the nutritional value of various feeds in aquaculture, as well as the prey that an animal may ingest in the wild. The lipids of an animal give important details about its own health and nutritional state. The information derived from lipid analysis is very important to researchers in fields such as aquaculture and biological research. Monitoring of lipid data can ensure that the animals they are studying are as healthy as possible.

The short column GC method is an effective method for analyzing lipid classes. Like the Iatroscan, the GC method collects data about what lipid classes are present and their quantities, however additional information about the compounds within those classes can be obtained. Combined with hydrogenation of lipids, the resulting chromatographic separation is based primarily on functional groups, carbon number. The short column and a wide-ranging GC temperature program allows for such a mixture of different compounds including high boiling point compounds to be separated effectively. However, polar lipid classes such as the AMPL and PL are retained on the GC column and therefore require chemical modification before GC analysis. This project has investigated an enzymatic hydrolysis (dephosphorylation) of the PL compounds, cleaving them to their non-polar DAG moieties that can be readily analyzed by the short column GC method after TMS derivatization. Improvement in overall recovery of lipid after the dephosphorylation and hydrogenation procedures is desired in order to optimize the

overall recovery of the method. Total lipid class data obtained by the short column GC method was on the same level as that of the Iatroscan, as shown by the comparison of percent marine lipid class data obtained by both methods in Section 3.5. However, the short column GC method is also able to provide information outside the limits of the Iatroscan. In Section 3.6, peaks that were difficult to identify and characterize by Iatroscan were easily identified by short column GC.

Previously, this short column GC method for marine lipid samples was restricted to the neutral lipids. Now one of the polar lipid classes, PL, can be readily analyzed. Phospholipids can now be added as a new lipid class to Yang's neutral lipid profiling method (1996). More information can now be obtained about both the carbon number patterns in marine derived samples, and the physiological and environmental condition of various marine species.

4.2 Future work

This short column GC method allows for profiling of all lipid classes with the exception of AMPL. These compounds would be retained on the GC column, rendering the column useless over time with a sufficient amount of these polar compounds injected. AMPL includes a number of different compounds, some of which include MAG and glycolipids. Quantitative MAG data can be determined by this method, upon TMS derivatization of the two free OH groups of the compound. However, glycolipids are more similar to PL in terms of their polar structure. They contain a head group, which may be removed with an appropriate enzyme such as β-galactosidase (Sigma), in similar fashion to the dephosphorylation procedure. Development of a such method for achieving

this result would allow for an almost full lipid profile of samples, regardless of AMPL concentration. Another class of compounds that need to be addressed is the pigments, such as plant chlorophyll. Pigments also pose a similar problem as the AMPL, in that they tend to retain on the short column. They elute along with AMPL on the Iatroscan, and are usually quantitated as such. A method of dealing with the pigments will be required to develop a "universal" short column GC method for marine lipid profiling. A guard column may be a temporary solution to these problems, as the harmful AMPL and pigment fraction would remain on the guard column. Once the guard column was rendered useless, it could be replaced and the analytical column could continue to be used for an extended period of time. Interference and the risk of significantly shortening the lifetime of the short column could be eliminated.

Glyceryl ethers are also a lipid class that should be addressed in future work. While not a lipid class that is common in all samples, the Iatroscan can sometimes detect these eluting close to TAG. Similar in structure to TAG, with an ether group instead of an acyl group, these glyceryl ethers would be expected to elute close to TAG on the GC as well. To accommodate the separation of these compounds, varying the temperature program in the TAG region may aid in separating these compounds more efficiently.

Combining this method with mass spectrometric detection would be an interesting step in furthering the applicability of this method. Confirmation of peaks would thus be performed, especially for peaks that elute closely. This would make the short column GC method a very powerful tool in marine lipid research.

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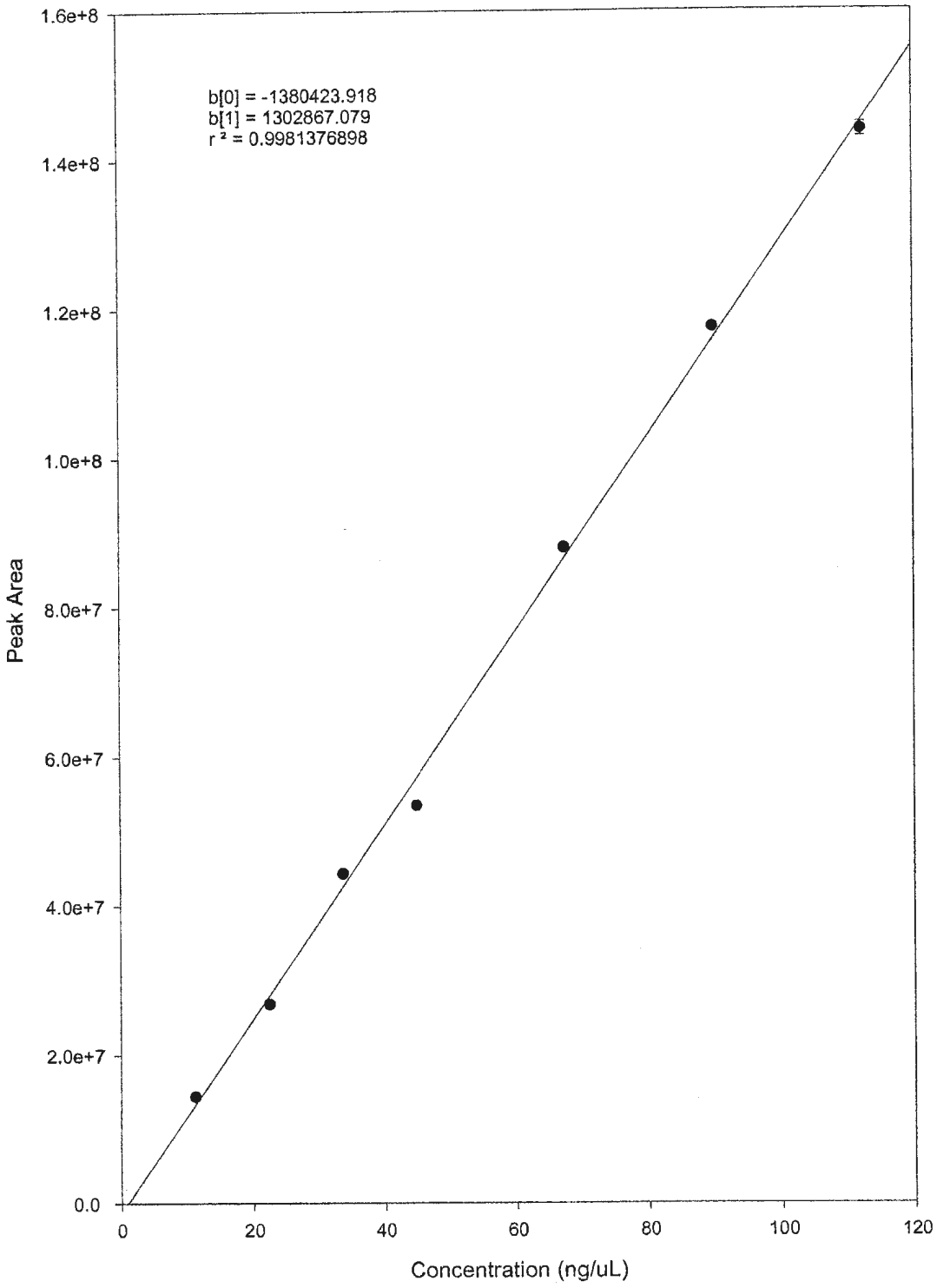
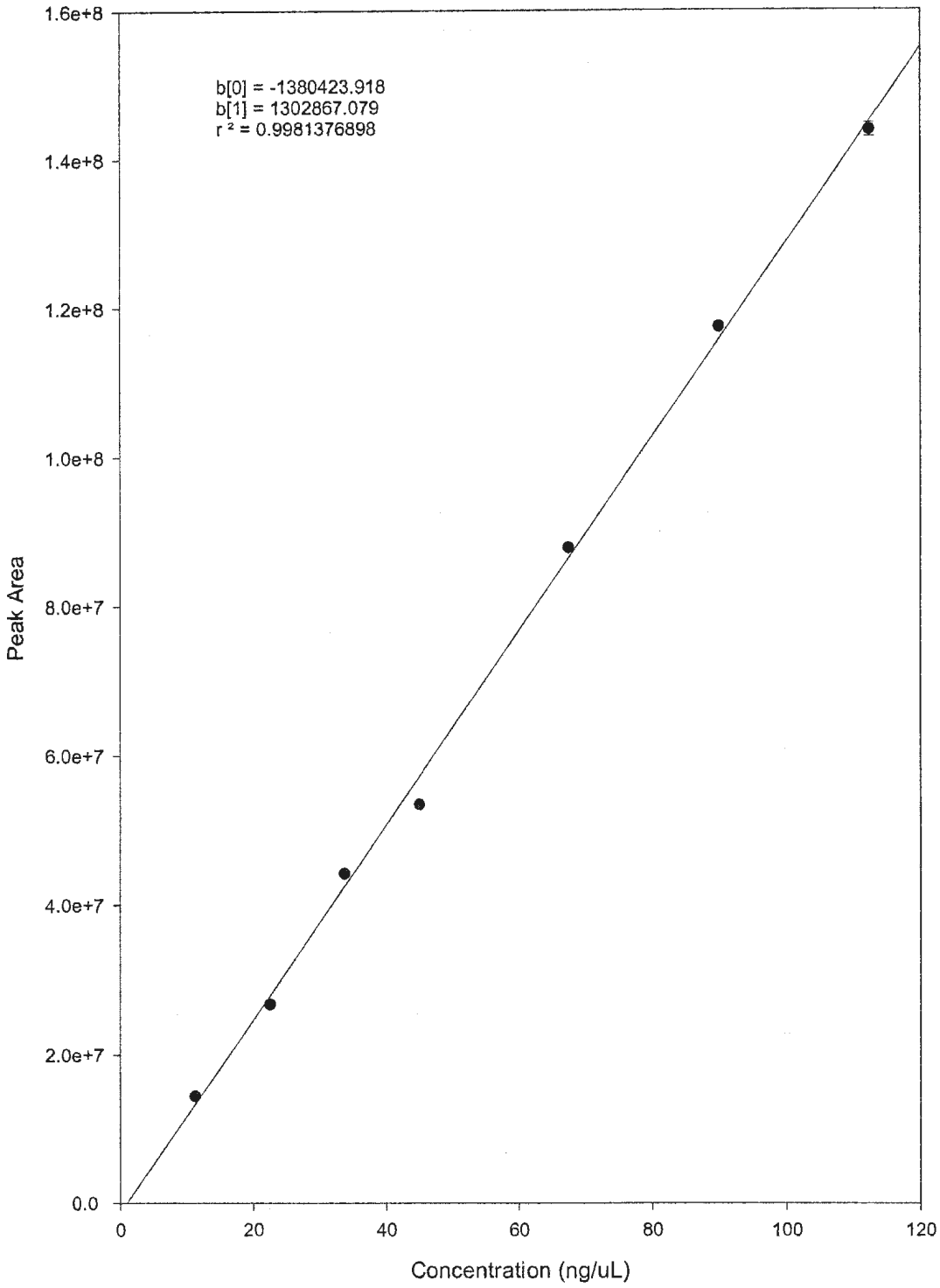
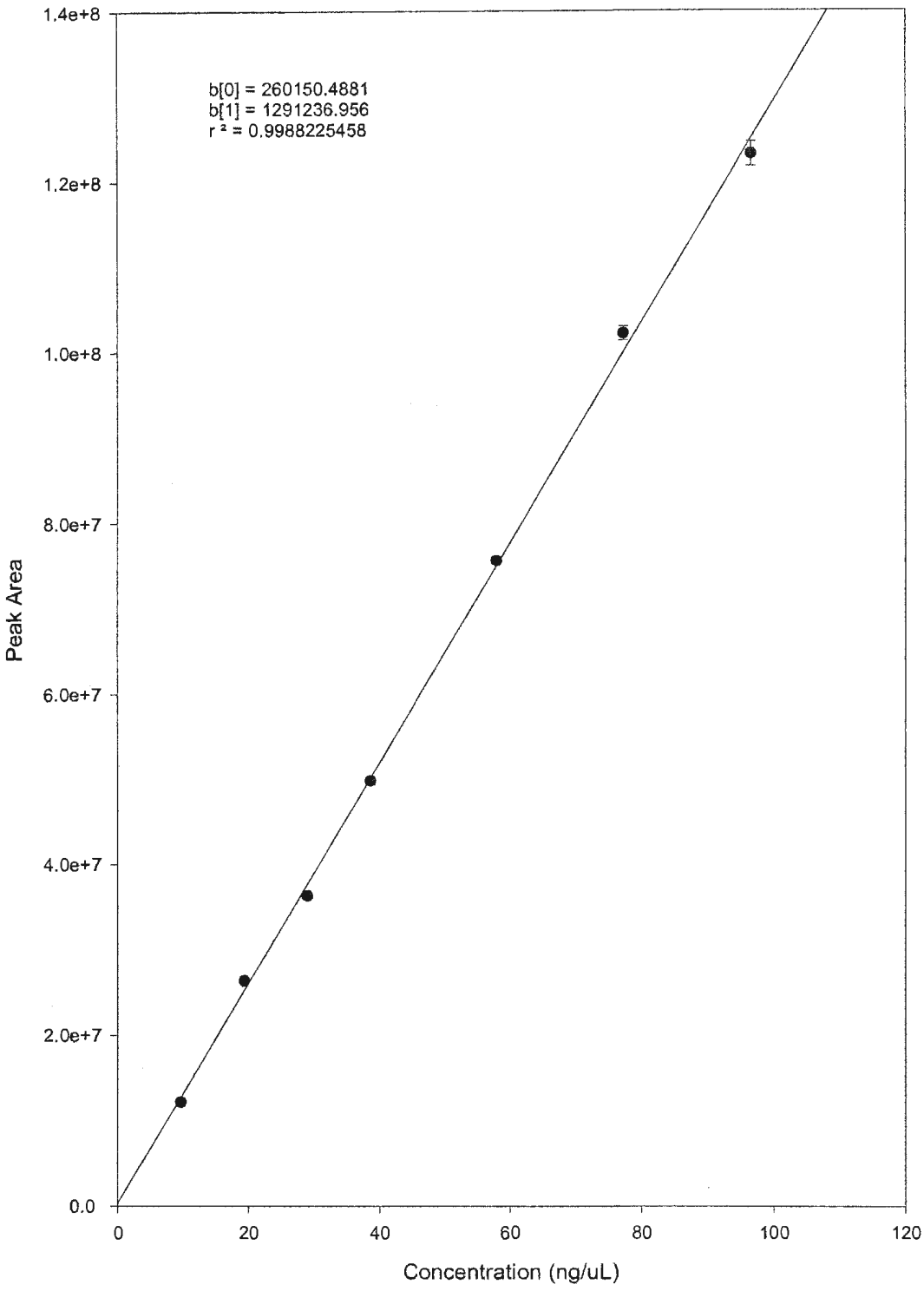
APPENDIX A



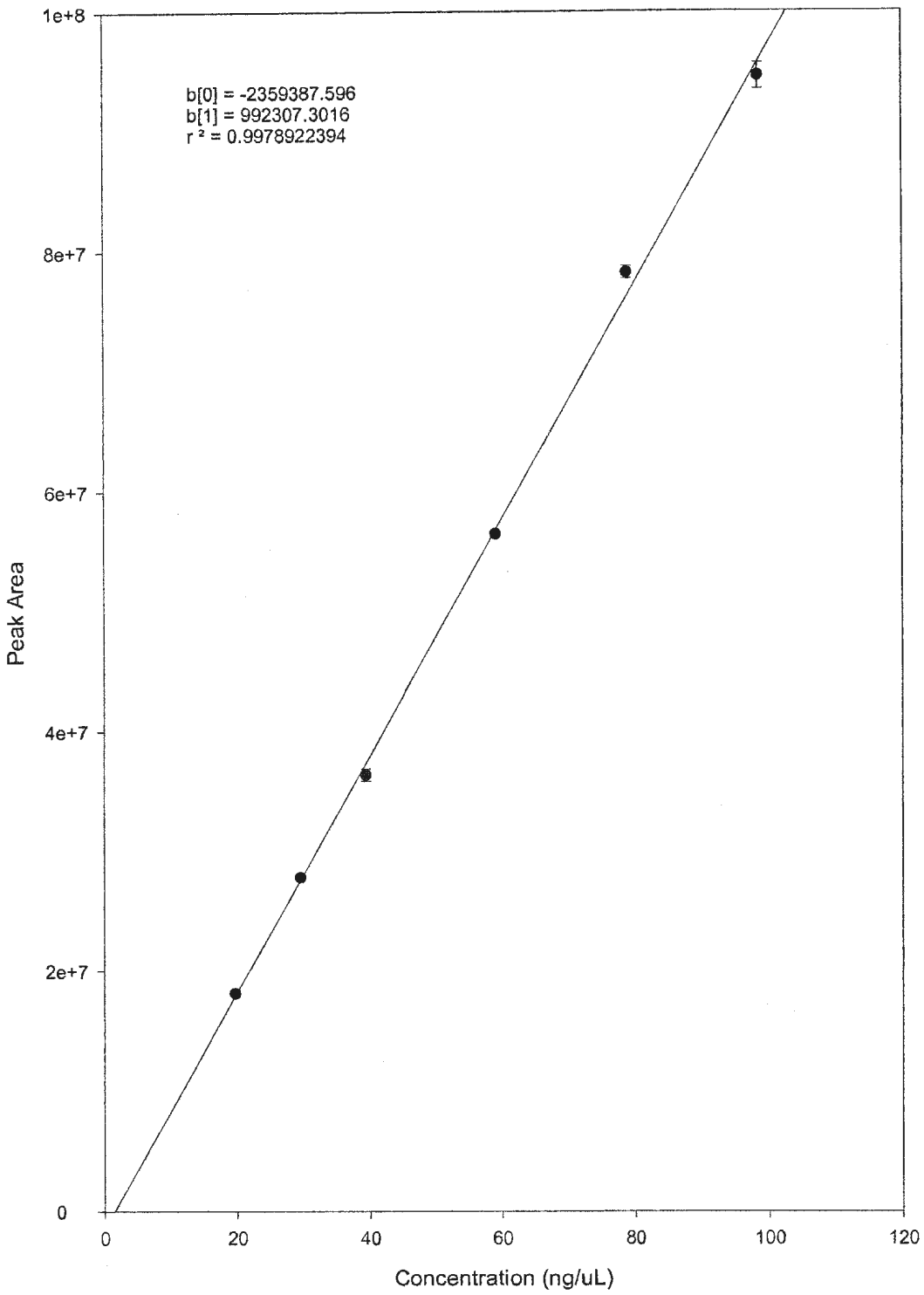
Calibration curve for C19 HC (n-nonadecane)



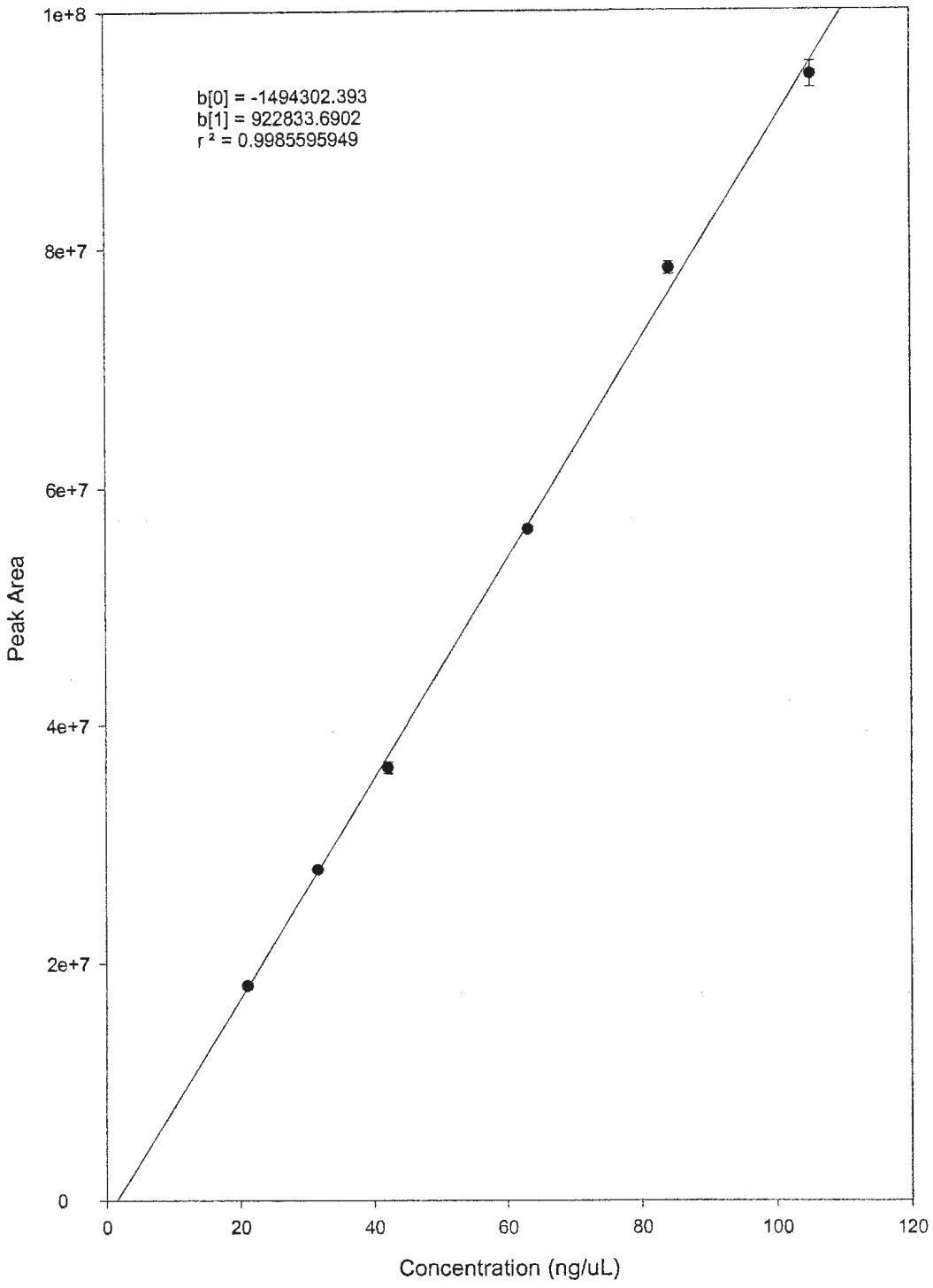
Calibration curve for C16 KET (3-hexadecanone)



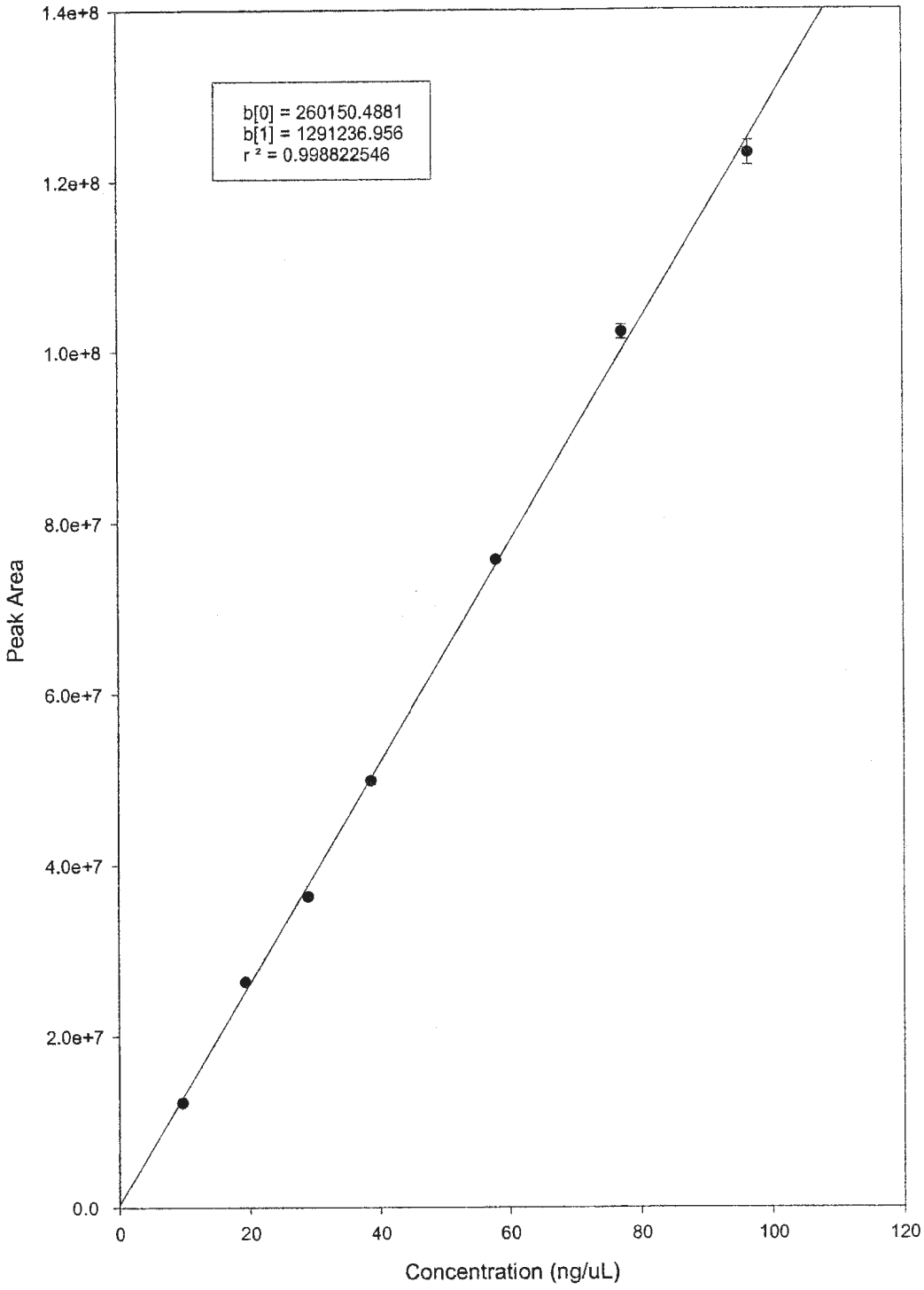
Calibration curve for C44 WE (behenic acid behenyl ester)



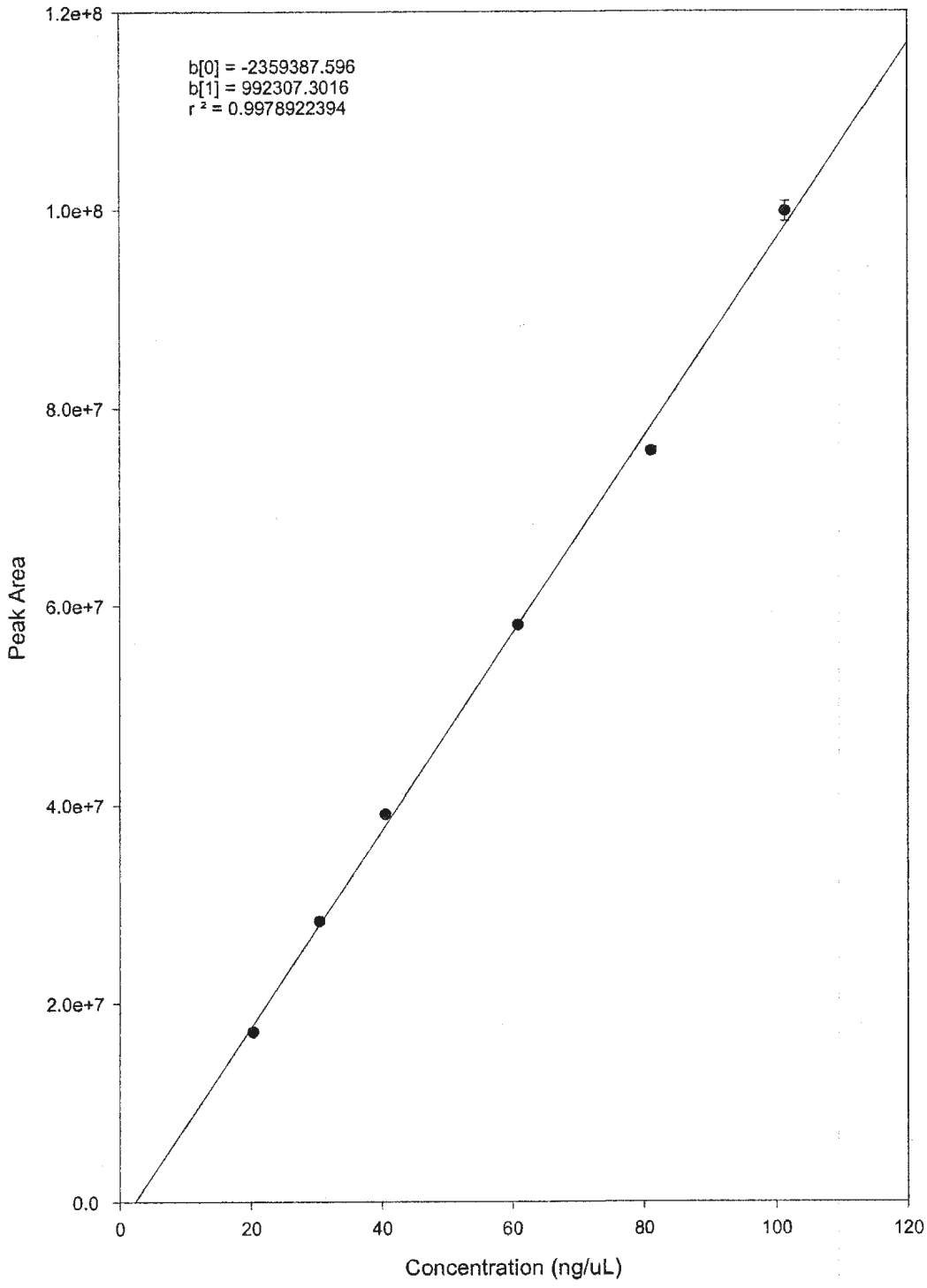
Calibration curve for C43 SE (cholesteryl palmitate)



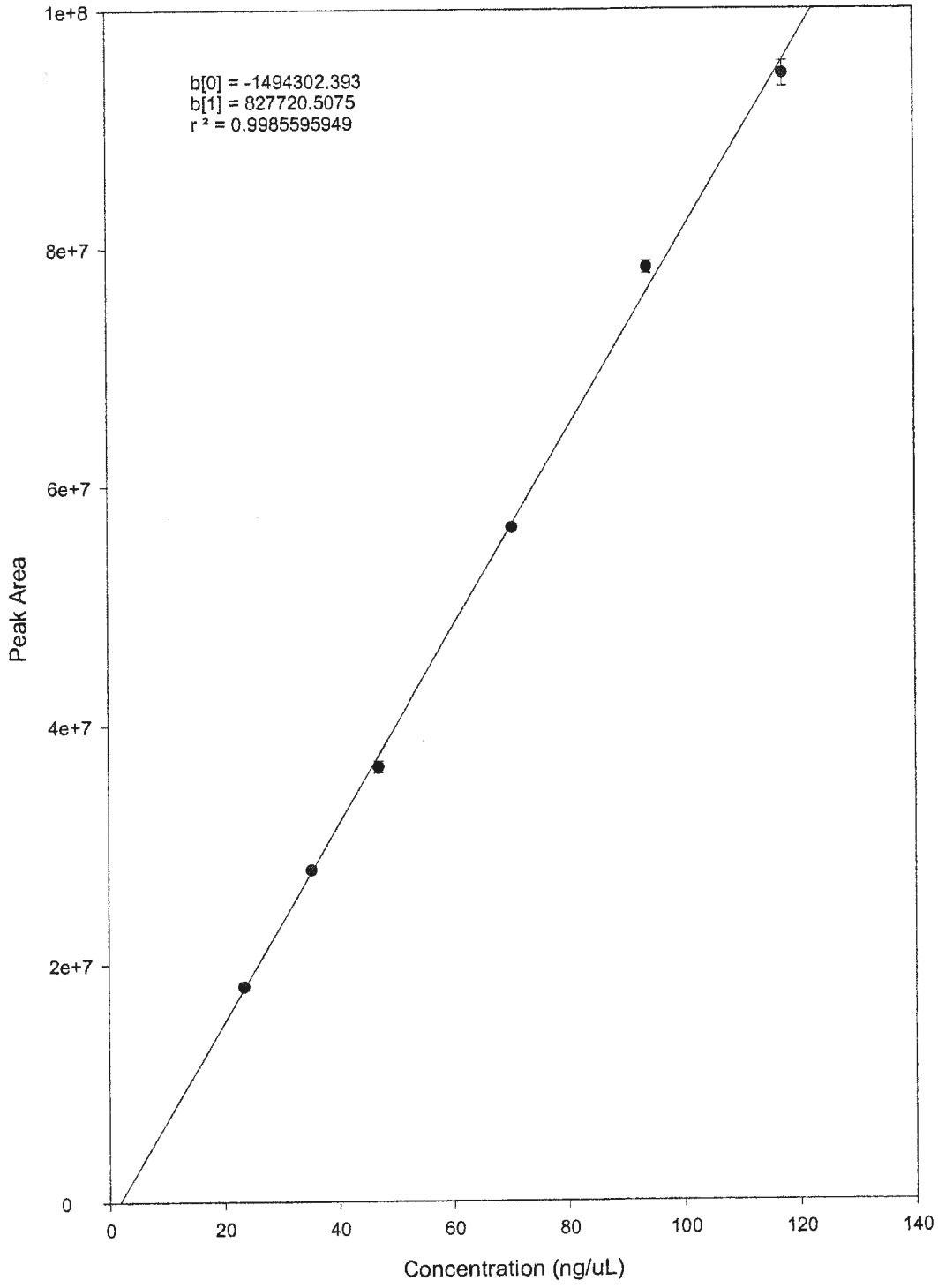
Calibration curve of C47 SE (cholesteryl archidate)



Calibration curve for C27 ST-TMS (cholesterol)



Calibration curve of C48 TAG (tripalmitin)



Calibration curve of C54 TAG (tristearin)

APPENDIX B

A commissioning of a VELP Scientifica SER148/6 Extractor Unit for marine lipid extraction was attempted. The VELP autoextractor performs extractions of organic material in a similar fashion to a Soxhlet extractor. An appropriate solvent is chosen and placed in a glass cup and heated on a heating block. A glass condenser apparatus is lowered onto the cup to form a tight seal. A sample within a cellulose-paper cone is lowered into the boiling solvent. Solvent vapour rises up the glass tube and is sent back down over the sample by the water-cooled condenser, thus maintaining the level of solvent in the solvent cup. After a sufficient amount of time, the sample is raised out of the boiling solvent and the condensed solvent pours over the sample to wash any lipid residue into the glass cup. The condensed solvent is then collected, thus concentrating the final extracted material to a volume suitable for transfer to a sample vial.

Several problems were observed with the VELP autoextractor. Ideally, six samples should be able to be extracted simultaneously, all of which are heated by a single heating block. However, uniform heating could not be maintained across the entire block. Uneven heating of six replicate samples would result in variations in the amounts of extract obtained from each of the six samples. The problem could not be repaired therefore the manufacturer was contacted to replace the block. However, the most significant problem in using the VELP autoextractor involved the O-rings used to make a tight seal between the condensing apparatus and the glass cup containing the solvent. In lipid extraction, a common solvent used is chloroform or chloroform mixtures with other

solvents (Folch *et al,* 1957). The O-rings provided with the autoextractor were composed of either butyl rubber or Viton. The instrument manual recommended that Viton rings be selected for seals when using chlorinated solvents. However, the Viton O-rings showed significant breakdown upon contact with chloroform liquid and vapour. Reaction with the chloroform produces unidentified contaminants that could interfere with lipid data. These contaminants would be seen visually as the solvent would become yellow after these O-rings came in contact with chloroform.

Several approaches to resolving this problem were attempted. A Teflon O-ring identical in size and shape to the Viton O-ring was constructed, however the material was too rigid to properly fit into the O-ring holders in the same fashion as the more flexible Viton O-ring. A very thin Teflon ring was flexible enough to be fitted into the O-ring holder however the seal was completely lost. Teflon rings of the correct thickness that were smaller in diameter would move around inside the O-ring holder or fall out onto the heater. The next approach to the problem was to wrap a Viton O-ring tightly in Teflon tape, however chloroform vapour could still attack the Viton material.

A new Teflon O-ring was constructed (by the Ocean Sciences Centre engineering team) that fits into the O-ring holder, remains stationary during extraction and does not fall if the cup was removed. The O-ring was made to sit on top of the glass, with a thin sleeve that would fit snugly inside the cup. The O-ring was the correct thickness for a good seal and the sleeve would ensure stability. Preparation for an extraction would simply require putting the O-ring on top of the rim of the glass cup, and then the apparatus could be lowered onto cup to create the seal. Since the O-ring does not move,

simply placing the cup in the correct position on the heating block ensures proper placement. A diagram of the new O-ring is shown in Figure B1.

Once the heating and seal problems were solved, choosing of an appropriate solvent or solvent mixture as well as optimization of the extraction procedure can now commence. Time limitations did not allow for full optimization, which could be performed by my successor on this project.

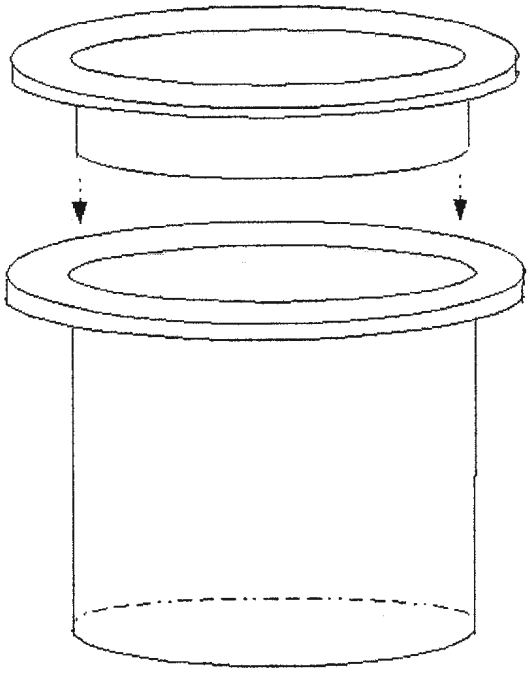


Figure B1: New Teflon O-ring design