EXTRACTION TECHNIQUES AND THE CHROMATOGRAPHIC DETERMINATION OF LIPIDS IN COLD WATER MARINE SPECIES

TARA SUZANNE HOOPER

EXTRACTION TECHNIQUES AND THE CHROMATOGRAPHIC DETERMINATION OF LIPIDS IN COLD WATER MARINE SPECIES

by

Tara Suzanne Hooper

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ABSTRACT

The exhaustive extraction and detection of lipids from marine plant and animal tissues is challenging due to the chemical variability among different lipid classes. An automated procedure based on the Randall method, in which the sample is directly immersed in boiling solvent followed by a solvent wash, has been developed for the exhaustive extraction of lipids from macrophytes and fish. The extraction of wet tissues is obtained by using a solvent system composed of chloroform, methanol and water. The efficiency of this procedure is comparable to traditional extraction methods and it is especially useful for large sample loads.

For the determination of lipids in very small samples such as individual fish larva, short column GC/FID provides an excellent alternative to the more commonly used Iatroscan TLC/FID, due to its lower detection limit as well as its ability to profile lipids based on their carbon number. Results reveal that there were no significant differences in the quantification of triacylglycerols or sterols in individual fish larvae ($p > 0.05$); however, GC/FID is more sensitive, precise, rapid and cost-efficient. Further, with the use of a guard column, the short column GC/FID method has been further expanded to include the qualitative analysis of macrophytes.

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ALC	alcohol
AMPL	acetone mobile polar lipid
DAG	diacylglycerol
FAME	fatty acid methyl ester
FID	flame ionization detector
FFA	free fatty acid
f_w	weight correction factor
GC/FID	gas chromatography with flame ionization detection
HC	hydrocarbon
KET	ketone
MF	modified Folch
MUFA	monounsaturated fatty acid
PL	phospholipid
PUFA	polyunsaturated fatty acid
RM	Randall method
SE	steryl ester
SFA	saturated fatty acid
ST	sterol
TAG	triacylglycerol

TL	total lipid
TLC/FID	thin layer chromatography with flame ionization detection
TMS	trimethylsilylation
WE	wax ester

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Appendix A:

CHAPTER 1

GENERAL INTRODUCTION

Lipids play an important role in the transfer and storage of energy in the marine environment, thus their analysis provides a very informative way of determining the health of a given ecosystem. In addition, detailed knowledge of the specific chemical composition of these complex molecules can provide valuable nutritional information. This is particularly useful due to increased interest in aquaculture; as well, there is ever-increasing public awareness of the health benefits of polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are found in abundance in many marine tissues (Cheung et al., 1998). Further, the hydrophobic nature of lipids make them good solvents for many organic pollutants such as polycyclic aromatic hydrocarbons (PAH's) and polychlorinated biphenyls (PCB's), thus allowing for transport of these pollutants through marine food webs (Parrish, 1988). As well, since some fatty acids (FA) are species-specific and have unique chemical structures, their identification can be useful indicators of biomarkers in marine ecosystems (Parrish et al., 2000). It is estimated that there are over 1000 different FA in nature (Christie et al., 1998).

It is important to extract all lipid classes fully as some classes are retained more tightly within the sample tissue; for example phospholipids are generally more difficult to extract than triacylglycerols due to their strong protein interactions within cell membranes. In most marine tissues, the relative concentration of some FA such as PUFA is generally higher in the polar fraction than in the neutral fraction (Langdon et al., 1981), thus complete extraction of all lipid components is required for accurate analysis of FA profiles. Generally, the lipid yield between different methods can be correlated with extraction efficiency of the polar lipids, which in turn is dependent on the polarity of the solvent used for extraction (Ewald, 1998). Methods currently used for lipid extraction are typically based on procedures developed over 40 years ago and generally involve labour-intensive manual manipulations and require large volumes of harmful organic solvents. Due to the heterogeneous nature of these molecules, their total extraction from marine plant and animal tissues, as well as their detection, clearly pose a complex analytical challenge.

1.1 Lipid Classes and Structures

Marine lipid extracts are complex and can contain up to 16 different subclasses (Parrish, 1988). They are grouped according to their similar chemical and physical properties, relative polarity and functional groups, although there can be much variation within a particular class. Many lipid classes contain 1–3 FA, which are linked via ester bonds to an alcohol backbone (commonly, glycerol), such as triacylglycerols (TAG),

phospholipids (PL) and glycolipids (GL; Christie, 1982; Parrish, 1999). The main storage form of energy is in the form of TAG, while PL, sterols (ST) and GL are important structural components of cellular membranes (Parrish, 1999).

Lipids can be loosely divided into two major classes - the neutral and polar lipids. The neutral fraction includes TAG, diacylglycerols (DAG), hydrocarbons (HC), ketones (KET), ST, steryl esters (SE), wax esters (WE), free fatty acids (FFA), and the aliphatic alcohols (ALC). The polar lipids can be subdivided into the PL and the acetone mobile polar lipids (AMPL); the latter group contains a diverse mixture of molecules including the GL and pigments (such as the chlorophylls and carotenoids; Kates, 1986), as well as the monoacylglycerols (MAG). Examples of some lipids and their chemical structures are presented in Figure 1.1.

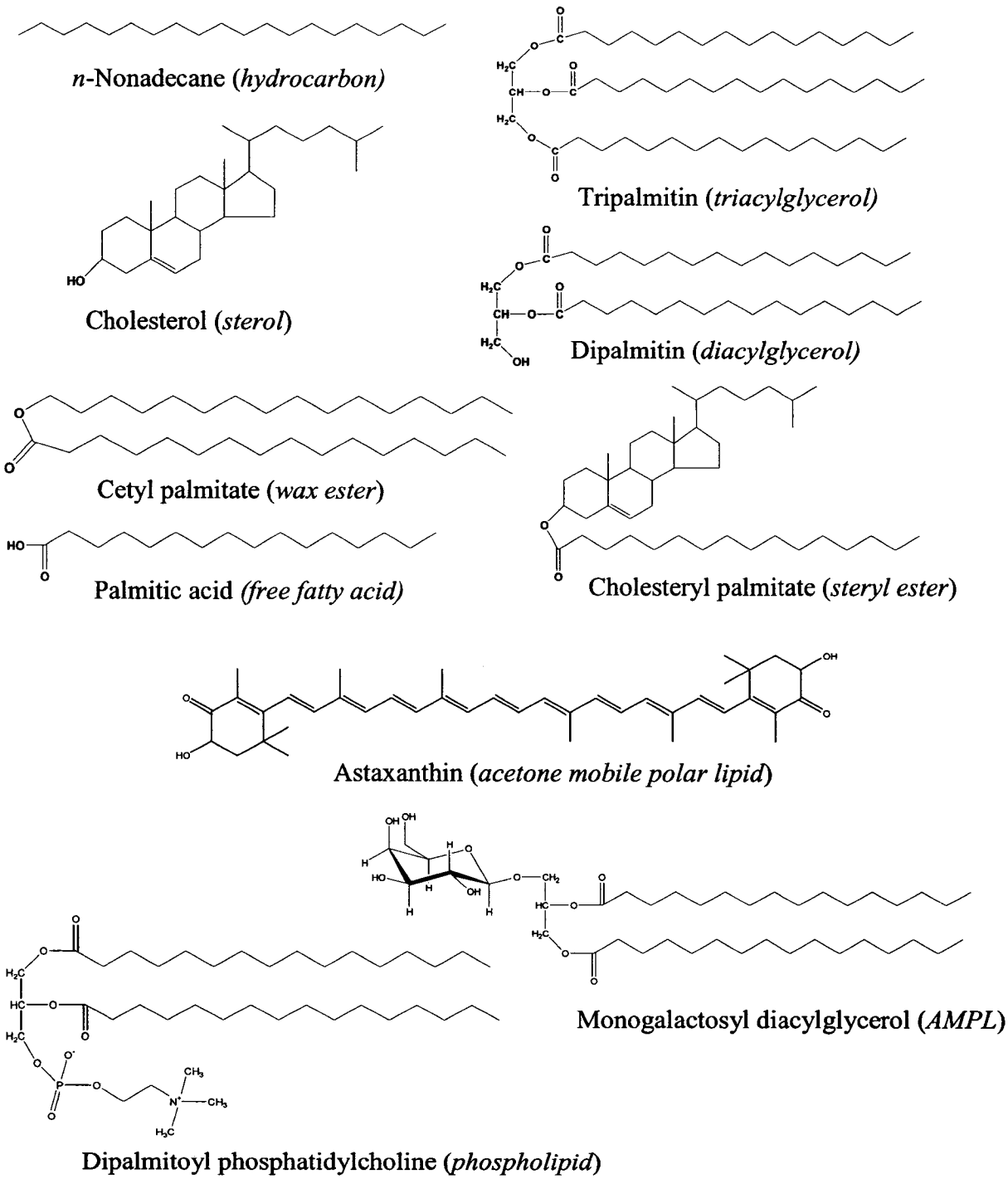


Figure 1.1. Representative chemical structures of major lipid classes

1.2 Objectives

The first and primary objective of this research project was to develop a protocol for the extraction of wet marine plant and animal tissues using an automated solvent extractor, based on the Randall method (RM). To validate this procedure, it was compared to traditional liquid extraction techniques commonly used for marine lipids, including the "Bligh and Dyer" procedure (Bligh & Dyer, 1959), the "Folch" method (Folch et al., 1957), Soxhlet and a modified form of the Folch et al. procedure commonly used in our laboratory. The principal aim was to develop an automated procedure that would quantitatively extract all lipid classes, while reducing the many manual manipulations commonly encountered with traditional methods. This would result in a more standardized method, requiring less hands-on time, thus simplifying lipid extraction procedures for large (> 1g) marine samples.

The second objective was to develop a micromethod for the quantitative determination of neutral lipids in individual fish larvae using gas chromatography with flame ionization detection (GC/FID), and to compare these results to thin layer chromatography with flame ionization detection (TLC/FID), which is currently the method of choice for the quantitative determination of lipids in marine samples. Since individual lipid classes share common similarities regarding their chemical properties (such as functional groups and relative polarities), gas chromatographic methods provide a useful tool for lipid profiling. GC also offers the advantage over TLC due to lower detection limits as well as the ability to resolve individual lipid components.

Another aim of this project was to further expand on GC procedures previously developed in our lab (Yang et al., 1996; Kehoe, 2003), based on the pioneering efforts of Kuksis et al. (1967), to include the analysis of marine plant tissues. Until now, the analysis of plant tissues was not explored in our lab due to presence of the relatively polar AMPL fraction, which is found in abundance in plants.

A flow chart of the experimental design is presented in Figure 1.2. Due to the contrast between the primary objectives, this thesis has been divided into two separate chapters, each with its own introduction, materials and methods section and results and discussion. This thesis closes with some overall conclusions.

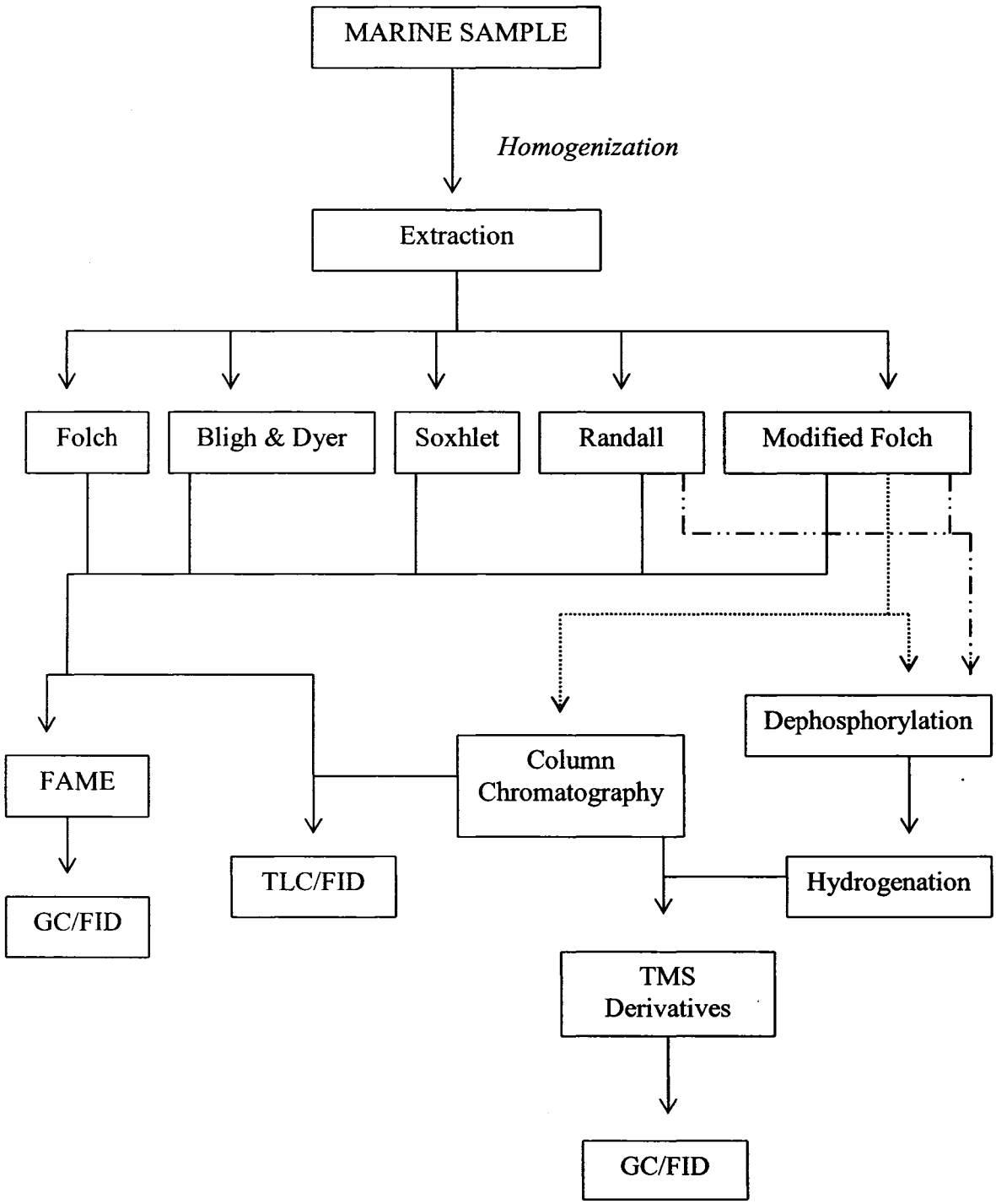


Figure 1.2. Overview of experimental design

CHAPTER 2

LIPID EXTRACTIONS

2.1 Introduction to Lipid Extractions

The chemical intricacy among different lipid classes, as well as the wide range of marine sample matrices, can pose specific analytical challenges for exhaustive lipid extraction. Successful solvent extraction requires that all lipid components be soluble, thus the solvents used must be polar enough to remove lipids from their protein interactions and to remove them from membranes (Smedes & Askland, 1999). The Folch et al. (1957) and the Bligh & Dyer (1959) protocols, which use methanol (MeOH) and chloroform ($CHCl_3$) to extract lipids, with the endogenous water in the sample as the ternary component, are two classical lipid extraction procedures on which many current marine lipid extraction methods are based. The main differences between these two methods are the initial solvent ratios and the proportion of solvent to sample. $CHCl_3$ and MeOH based solvent systems have been shown to be very effective for the removal of polar lipids, which generally occur in a high proportion, in relation to TAG, in marine tissues such as Atlantic cod and marine macrophytes (Dodds et al., 2004).

The original protocols for these methods use very large samples and are time consuming, thus modifications are often made to simplify the procedures. Unfortunately,

the nature of these modifications is rarely mentioned, let alone detailed, although variations in extraction efficiency can easily occur if improper solvent ratios and solvent-to-sample ratios are used.

These procedures, if used properly, can exhaustively extract all major lipid classes from the sample matrix, resulting in minimal damage to the chemical structures of the native components (Carpenter et al., 1993). Maintaining the original integrity of the lipid components is particularly important in marine lipid research since a great deal of emphasis is placed on the identification of polyunsaturated fatty acids (PUFA), found in abundance in cold-water marine samples. Long-chain PUFA, many of which are highly unsaturated, are particularly prone to chemical oxidation.

Due to the variability among sample matrices, which include sediments, animals, plants and seawater, an extraction method developed for one sample type may not necessarily be as useful for another. For instance, the extraction of plant materials can pose specific analytical challenges due to the presence of cell walls, which are not found in animal tissues (Wiltshire et al., 2000). Plant tissues generally contain a relatively high proportion of acetone mobile polar lipids (AMPL), including glycolipids and pigments such as chlorophyll–*a*. The Folch et al. and Bligh & Dyer protocols, which were originally developed for the extraction of very large animal samples, are frequently referenced for the extraction of plant lipids (e.g. Vaskovsky et al., 1996; Moreau et al. 1998). It is important to validate the effectiveness of these classical methods for the quantitative extraction of lipid species not generally found in high abundance in animals.

Some of the main drawbacks of these extraction techniques include long extraction times as well as the large volumes of organic solvents that must be used for the procedures. In addition, the many manual operations involved can lead to variability in results depending on the skill of the analyst (Manirakiza et al., 2001). Further, the complex manual manipulations involved in the traditional procedures, resulting in prolonged exposure of the analyst to the solvents, can potentially cause adverse health consequences.

The presence of excess water in the sample can greatly affect the nature of the organic solvent mixture, and too much water in the sample can possibly lead to decreased yields of total lipid extracted (Nelson, 1991; Manirakiza et al., 2001). An increase in the water content can result in an increase in the extraction of non-lipid material (Nelson, 1991). As well, care must be taken to ensure that the proper solvent ratios are correct, or there could be loss of some of the more polar lipids in the sample such as the phospholipids (PL) and glycolipids (GL; Christie, 1982).

Plant tissues are reportedly particularly difficult to extract due to the presence of very active lipases; these lipases rapidly hydrolyze PL and GL, increasing the amount of free fatty acids (FFA) in the extract. It has been recommended to inactivate these enzymes by replacing MeOH with isopropanol during preliminary sample homogenisation, prior to extraction (Nichols, 1963). Treatment of the samples with boiling water prior to extraction has also been reported to be an effective method for the deactivation of these enzymes (Christie, 1973; Couture et al., 1988; Budge & Parrish, 1999).

Gravimetry is commonly used for quantitative lipid determination (Folch et al., 1957; Bligh & Dyer, 1959; Phillips et al., 1997; Jensen et al., 2003), however it provides no information on the individual components present in the sample, thus no information on the extraction efficiency of particular lipid classes can be obtained. Problems can be encountered, such as overestimation of lipid content, if any non-lipid materials are extracted and the samples are not properly dried prior to weighing (Phillips et al., 1997; Dodds et al., 2004). As well, increased solvent polarity can lead to erroneously high results for total lipid determination (Manirakiza et al., 2001).

Proper quantitative analysis requires that oxygen-exposure of the lipid extracts be kept to a minimum; PUFA, which are found in high abundance in marine tissues, are prone to oxidation (Budge & Parrish, 2003), thus care must be taken to reduce any changes in the native chemical composition.

2.2 Lipid Extraction Procedures

2.2.1 Folch et al.

This classic procedure, originally published in 1957 by J. Folch, M. Lees and G.H. Sloane-Stanley, was originally designed for the isolation of brain lipids. This method uses $CHCl_3$ and MeOH in an excess of 20:1 (or greater) solvent to sample ratio to quantitatively extract lipids, which were determined gravimetrically in the original study. The extraction of the wet tissue is followed by a wash with either water or a weak salt solution, and the critical final solvent ratio for this method is 8:4:3, $CHCl_3$:MeOH:H_2O.

The Folch procedure has been well established to quantitatively remove lipids from marine tissues, however its main drawback is the excess solvent required for extraction.

2.2.2 Bligh & Dyer

The Bligh & Dyer procedure, developed in 1959, was originally designed for the extraction of lipids from cod flesh, which contains a very high proportion of phospholipids. This method is generally the benchmark for marine lipid analysis, however modifications are frequently made in order to simplify the generally cumbersome procedure. The Bligh & Dyer method also makes use of critical solvent ratios of MeOH, $CHCl_3$ and H_2O. A recent study has shown that the success of the Bligh & Dyer method is highly dependent on the amount of organic phase recovered (Smedes & Askland, 1999). As well, it has recently been demonstrated that this procedure can lead to a reduction in extraction efficiency at high (>2% w/w) lipid concentrations (Iverson et al., 2001).

Both the original Folch et al. and the Bligh & Dyer methods involve homogenization of the sample in an excess amount of solvent, followed by a vacuum filtration step and a water wash, after which the biphasic mixture, without any interfacial fluff (particulate matter), is allowed to stand until complete phase separation occurs. The top, aqueous layer is removed, and in the case of the Folch et al. procedure, the lower organic layer is washed three times with a "synthetic upper phase". These latter two steps are frequently omitted in favour of centrifugation and direct removal of the organic phase

with a Pasteur pipet, however this current study followed the protocols as stated in the original articles with the exception that individual lipid classes were quantified via Iatroscan TLC/FID instead of total lipid determination by gravimetry.

2.2.3 Soxhlet

Conventional Soxhlet extraction is a semi-continuous reflux method in which the homogenized sample, held in a glass fibre or cellulose thimble, is rinsed with cold distilled solvent. The sample is soaked in solvent that is periodically siphoned off, re-distilled and returned to the sample until all the lipid components are extracted. The lipids are collected in a round bottom flask, which contains the boiling solvent. Classical Soxhlet is well accepted for the extraction of lipids from a variety of foodstuffs, however these procedures often used dried samples and solvents such as diethyl ether or hexane to extract the lipid components. These solvents, due to their non-polar nature, are not miscible with wet samples, resulting in a water barrier between the solvent and the sample, thus samples are usually oven dried prior to extraction. It has been reported however that drying samples can lead to a decrease in lipid yield, if samples are not rehydrated prior to extraction (Dunstan et al. 1993). As well, these solvents generally cannot sufficiently extract the polar lipids without harsh chemical pre-treatments, such as acid hydrolysis, prior to extraction.

2.2.4 Randall

The Randall method (RM), otherwise know as Soxtec or the submersion method, is a variation of traditional Soxhlet, differing in that the sample is actually immersed in boiling solvent followed by a rinse step. E.L. Randall designed the original extraction apparatus in 1974, which allowed for the simultaneous extraction of up to six samples at once. Traditionally, as with Soxhlet, the extracting solvent is hexane or diethyl ether (Randall, 1974; Thiex et al., 2003), however, as mentioned previously, these solvents are not suitable for wet samples and are generally not polar enough for the quantitative removal of polar lipid classes from cold-water marine samples.

This method has been successfully applied for the extraction of lipids from a wide variety of foodstuffs (Thiex et al., 2003), and it is used as the AOCS official method for the extraction of meats and meat products (AOCS Official Methods of Analysis, 1998). To our knowledge, there have been no previously published reports using this method with marine samples for lipid class and fatty acid analysis.

2.2.5 Supercritical Fluid Extraction

This technology has the advantage of being relatively rapid compared to conventional liquid extraction techniques. It also generates minimal solvent waste and has the advantage of extracting lipids at low temperatures, making it useful for temperature-sensitive compounds such as many long-chain PUFA (Cheung et al., 1998). The common extracting fluid is supercritical carbon dioxide (SC-CO_2), which is often

amended with a modifier such as ethanol or 90% isopropanol (Johnson et al., 2003) in order to increase the polarity of the very non-polar solvent.

Several aspects must be optimised for proper extraction efficiency, in particular, temperature, pressure of the extractant, as well as particle size and moisture content of the sample (Cheung et al., 1998). Samples with high water content often result in inefficient extractions since water acts as a barrier between the sample and the SC-CO_2 (Dunford et al., 1997). As a result, samples with high moisture content are often freeze-dried before analysis (Yamaguchi et al., 1986; King et al. 1989; Temelli et al., 1995; Dunford et al., 1997).

This method is advantageous due to its compatibility with fatty acid profiling methods (King et al., 1989; Snyder et al., 1996; Taylor et al., 1997). Recent advances in this field have led to the pairing of extraction and *in situ* synthesis of fatty acid methyl esters (FAMEs), which allows for a reduction in the time required for FA profiling compared to current conventional methods (Carrapiso, 2000). Some shortcomings of this technology include cost and incomplete extraction of samples under certain conditions, as well as the simultaneous extraction of non-lipid components (Snyder et al., 1984; Hardardottir et al., 1988; Dunford et al., 1997).

2.2.6 Microwave

This relatively recent technology has the advantage of being inexpensive, safe and rapid, and it can be used in water-rich matrices (Pare et al., 1997). A satisfactory

procedure for the extraction of lipids from seeds, foods, feeds and soils was developed which involves irradiation of the sample with short 30 s bursts of energy with an appropriate solvent (Ganzler et al., 1986). The use of microwave energy can cause lipid oxidation, thus gravimetric determinations of lipids may be over-estimated (Yoshida et al., 1990).

2.2.7 Ultrasound

This technique involves the use of sonication resulting in acoustic cavitation, a high-energy reaction that produces high contact between solvent and solute (Suslick, 1990). One major advantage of this relatively new technique is the short analysis time (Mecozzi et al., 2002). However, the heat created in addition to the radicals produced during the sonication can lead to oxidation and modification of the sample (Hoffman et al., 1996; Taylor et al., 1997).

2.3 Methods for Lipid Analysis and Detection

A more detailed overview of methods used for lipid analysis and detection will be presented in Chapter 3, however a brief summary of some common lipid detection methods used in cold-water marine lipid research, in particular those that were used during this research, is presented here.

2.3.1 Thin Layer Chromatography with Flame Ionization Detection

Thin layer chromatography with flame ionization detection (TLC/FID) is an analytical method commonly used for the separation and quantification of lipid classes. Currently, Iatroscan TLC/FID, using silica-coated rods instead of plates, is the most widely used method for separating both neutral and polar lipids classes from marine samples (Shantha, 1992). Briefly, samples are spotted onto silica-coated quartz rods (Chromarods), and then placed in an appropriate solvent system. The rods can then be either partially or fully scanned by being passed through the flame of the detector. The rods can be immersed in solvents of variable polarity and re-scanned to an appropriate length of the rod, thus making this method suitable for separating a large proportion of lipid classes from the non-polar hydrocarbons (HC) to the relatively polar PL and AMPL. However, this method suffers some drawbacks such as the large amounts of solvents used, as well as the increased susceptibility to lipid oxidation during the lengthy manual procedure (about 4 hr for up to 20 samples; Ruiz-Gutierrez et al., 2000). As well, problems with analysis can arise due to variability between rods, non-linear calibration curves and low sensitivity at low lipid concentration (Tvrzicka & Mares, 1990). Further, acyl lipids containing highly unsaturated fatty acids will give a lower detector response, thus acyl lipid classes such as TAG and PL can be underestimated (Shantha, 1992). Hydrogenation of the lipid extracts prior to analysis can lead to increased detector response as well as improved peak resolution (Shantha & Ackman, 1990).

2.3.2 Gas Chromatography

Gas chromatography is frequently used in lipid analysis, particularly for FA identification. Accurate FA analysis in cold-water marine species, which contain a high proportion of PUFA, can be challenging (Budge & Parrish, 2003). Fatty acids are freed from lipid moieties such as TAG and PL via an acid or base catalysed trans-esterification reaction, in which fatty acid methyl esters (FAMEs) are produced, which are stable and volatile, and can be readily analysed with GC/FID.

Free fatty acids (FFA) are generally not found in nature, and elevated levels of FFA in total lipid extracts are believed to be the sign of enzymatic degradation (Gurr & Harwood, 1991; Budge & Parrish, 1999).

2.3.3 Short-Column Gas Chromatography

This profiling method, using a short (5.5 m) capillary column, groups compounds in each class according to their carbon number (Parrish et al., 2000). This method is advantageous because it can be applied to a wide range of marine samples, is readily automated and has high sensitivity (Yang et al., 1996). Further, GC is relatively cheap to run and has rapid analysis times. A GC procedure has been developed for the profiling of neutral lipids in animals (Yang et al., 1996), which has recently been further expanded to include the indirect detection of PL via enzymatic dephosphorylation of PL to produce diacylglycerol (DAG; Kehoe, 2003). More details of this procedure and its applications are presented in Chapter 3.

2.4 Experimental

2.4.1 Glassware and Chemicals

All materials that came into contact with lipids were made of either glass or were Teflon-coated. All materials were lipid cleaned by rinsing 3 times with MeOH followed by 3 times with $CHCl_3$. Alternatively, glassware was heated in a muffle furnace at 450°C for at least 4 hr. All solvents used were of analytical or chromatographic grade. Standards used for calibration and verification, prepared from chromatographically purified materials, were supplied by Sigma (St. Louis, MO, U.S.A.).

Water that was used for experimental analysis was first washed three times with $CHCl_3$ in a separatory funnel and stored in a glass bottle for future use. Formic acid used for column chromatography was also washed with chloroform prior to neutral lipid separation.

2.4.2 Marine Samples and Sample Storage

Cold-water marine samples were of both plant and animal origin. Two macrophytes, *Chondrus crispus* (Irish moss) and *Agarum cribrosum* (sea colander), were collected from the beach at Middle Cove, Newfoundland in September 2003 (*A. cribrosum*), May 2004 and November 2004 (*C. crispus*). Only wet samples, fresh in appearance, were chosen. The algae were frozen at –20°C until preparation. Fish samples of *Gadus morhua (*Atlantic cod) and *Mallotus villosus* (capelin) were caught from the coastal waters at Bonne Bay, Newfoundland in July 2003, under the multidisciplinary

Coasts Under Stress research initiative, and frozen whole. Samples of muscle tissue, free of skin, were taken from the thawed fish, wrapped in aluminium foil and stored at –80°C until preparation and extraction.

2.4.3 Sample Preparation

Algal samples, after thawing, were left untreated or were rinsed with either distilled room temperature or boiling water for *ca* 60 sec. prior to mincing. Plant and fish samples were thawed and minced into *ca* 0.5 cm pieces with scissors (algae) or a surgical knife (fish), and then portions were weighed to the nearest 0.01 mg on a Mettler Toledo analytical balance. The weighed samples were covered with either $CHCl_3$ or an azeotrope solution (without the addition of the water component, see section 2.4.6.5). The solvent was covered with N_2 and the sample was stored at –20°C until extraction.

It likely would have been useful to homogenize the samples and then sub-sample from the homogenate, thus reducing sample variability. Attempts were made to homogenize the algal samples with a mortar and pestle prior to adding solvent, however due to the tough nature of the algal fibres, this proved to be unsuccessful. It was not possible to homogenize the samples in solvent prior to sub-sampling due to the differences in solvent proportions among the different extraction procedures. The best alternative seemed to be to mince the samples into small pieces and sample from this mixture prior to adding solvent and freezing the sample, until extraction could be carried out.

2.4.4 Total System Blanks

Procedural blanks, which involved all the same manipulations used for experimental samples, were carried out and then subtracted where appropriate.

2.4.5 Sample Homogenization

Fish and algae samples were homogenized on ice with a Brinkman Polytron blender in the solvent specific to the extraction method. The homogenization times varied but routinely took up to 10 min. for large algal samples.

2.4.6 Extraction Procedures

2.4.6.1 Traditional Folch et al.

Fish and algal samples weighing 1 g were homogenized in 2:1 $CHCl_3$:MeOH (v/v) to a final dilution of 20:1 solvent to sample ratio. According to the protocol, it was assumed that the sample had the specific gravity of water. After homogenization, samples were vacuum filtered through a 90 mm Buchner funnel, intermittently being flushed with N_2, and the funnel was covered with a watch glass in order to increase the vacuum. The resulting extract was transferred to a 50 mL graduated cylinder, its volume recorded, and 0.2-fold its volume of water was added. The resulting biphasic mixture was flushed with N_2 and shaken well. The cylinder was sealed with Teflon tape and placed at $-20^{\circ}C$ overnight to allow phase separation. The resulting final phase ratio was 8:4:3 $CHCl_3$:MeOH:H_2O. The upper aqueous phase was then removed with a pipet and

the lower phase was rinsed three times with a synthetic upper phase previously prepared from pure solvent (see below). The rinsed lower phase was transferred to a round bottom flask. The graduated cylinder was rinsed three times with the synthetic lower phase to ensure quantitative removal of lipids.

Synthetic upper and lower phases: $CHCl_3$, MeOH and H_2O in the proportions of 8:4:3 (v/v/v) were mixed in a separatory funnel. The resulting upper aqueous phase and the lower organic phase were separated and stored in clean glass bottles for future use.

2.4.6.2 Modified Folch

This procedure was adapted from the original Folch et al. procedure (Parrish, 1999), using the same solvent ratios as the traditional Folch method (8:4:3; $CHCl_3$:MeOH:H_2O). According to this procedure, samples weighing up to between 10-150 mg dry weight were homogenized, on ice, in 3 mL of 2:1 $CHCl_3$:MeOH. After homogenization, 1 mL of 2:1 $CHCl_3$:MeOH and 0.5 mL of water were added to the suspension and the mixture was flushed with N_2 and capped. The mixture was vortexed, sonicated in an ice bath for 4 min. and then centrifuged for 3 min. to separate the organic and aqueous phases. The lower, organic layer was removed by a double-pipeting technique and transferred to a 15 mL vial. The pipet was rinsed with 3 mL of $CHCl_3$ and the sonication/centrifugation procedure was repeated at least three times. All $CHCl_3$ extracts were pooled in a 15 mL vial, evaporated to near dryness under N_2, transferred to a 2 mL vial and stored under a blanket of N_2 at $-20^{\circ}C$ until analysis.

In the current study, sample sizes averaged from 0.5 – 4 g wet weight (0.1 – 0.8 g dry weight). Solvent volumes were increased for larger samples (>150 mg dry weight) in order to achieve a final solvent ratio of 8:4:3 $CHCl_3$:MeOH:H_2O. In addition, the final solvent-to-sample ratio was at least 4:1 (wet weight samples).

2.4.6.3 Bligh & Dyer

Fish samples weighing 4 g and 1 g were homogenized for 2 min. in 15 mL of 2:1 MeOH:$CHCl_3$. The appropriate amount of H_2O, determined from dry weight measurements, was added to provide a final solvent ratio of 1:2:0.8 $CHCl_3$:MeOH:H_2O (before dilution), which included the water present in the sample. Five mL of $CHCl_3$ were added to the homogenate and the sample was blended for another 30 sec., then 5 mL of water was added and the mixture was homogenized for a further 30 sec. The final solvent ratio was 2:2:1.8 $CHCl_3$:MeOH:H_2O. The mixture was then filtered under vacuum through a 90 mm Buchner funnel, intermittently being flushed with N_2 and the funnel was covered with a watch glass. The filter cake was rinsed with 5 mL $CHCl_3$ and filtered. The filter and the filter cake was re-homogenized with *ca* 10 mL of $CHCl_3$ and filtered again. The filtrate was transferred to a 50 mL graduated cylinder and the layers were allowed to separate overnight under N_2. The top, aqueous layer of the final biphasic mixture was almost completely removed and the bottom layer was rinsed three times with a small amount of MeOH. The bottom, organic layer was transferred to a round bottom flask and rotary-evaporated to near dryness. The resulting extract was transferred to a 15 mL vial, the flask rinsed well with $CHCl_3$, and all rinses were added to the original

extract. The pooled extracts were evaporated under N_2 to their desired concentration. For both the 1 g and 4 g samples, the solvent-to-sample ratio was always greater than 4:1.

2.4.6.4 Soxhlet

Homogenized samples, weighing 1 g and 4 g, were added to a lipid cleaned Soxhlet apparatus and the sample tube was rinsed well with azeotrope solution to ensure complete transfer of the entire sample. The total volume of solvent used for extractions was 125 mL. The temperature of the heating mantle was controlled with a Variac autotransformer, which was adjusted to 40% of maximum output. This corresponded to a drip rate of 60-65 drips $min.^{-1}$ and 4 cycles hr^{-1}.

The open system was continuously flushed with a gentle stream of N_2 during the entire extraction procedure. The system was insulated with tinfoil to allow more efficient heat transfer, resulting in an increased reflux rate. The solvent used for extraction was an azeotrope composed of $CHCl_3$:MeOH:H_2O in the proportions of 84:14.2:1.8 (v/v/v; bp 52.3°C). Generally, a solvent mixture composed of $CHCl_3$/MeOH in the proportion of 85.5:14.5 (v/v) was prepared, and the appropriate amount of water (and/or wet sample with known moisture content) was added to produce the proper final ratio required for the azeotrope. Thus, the water present in the sample was included in the final ratio of the solvents. Extractions were carried out for 6 hr, after which time the sample was removed, flash evaporated to near dryness and the extract was quantitatively transferred to a 15 mL vial.

2.4.6.5 Randall Method

Extractions were carried out using a Velp Scientifica Extractor, model SER 148, purchased from Fisher Scientific. This unit has the capacity to extract up to 6 samples simultaneously and allows for temperature programming between 100°C and 260°C. Three programs can be selected: immersion, wash and recovery. Each can be programmed up to 16.6 hr. This apparatus extracts lipids based on the Randall technique (Randall, 1974) in which the homogenized sample, held in a cellulose thimble, is initially submerged directly in boiling solvent, followed by a wash step with a continuous flow of distilled solvent. After extraction, the solvent can be evaporated and recovered. An outline of the procedure is presented in Figure 2.1. A picture of the apparatus is presented in Figure 2.2.

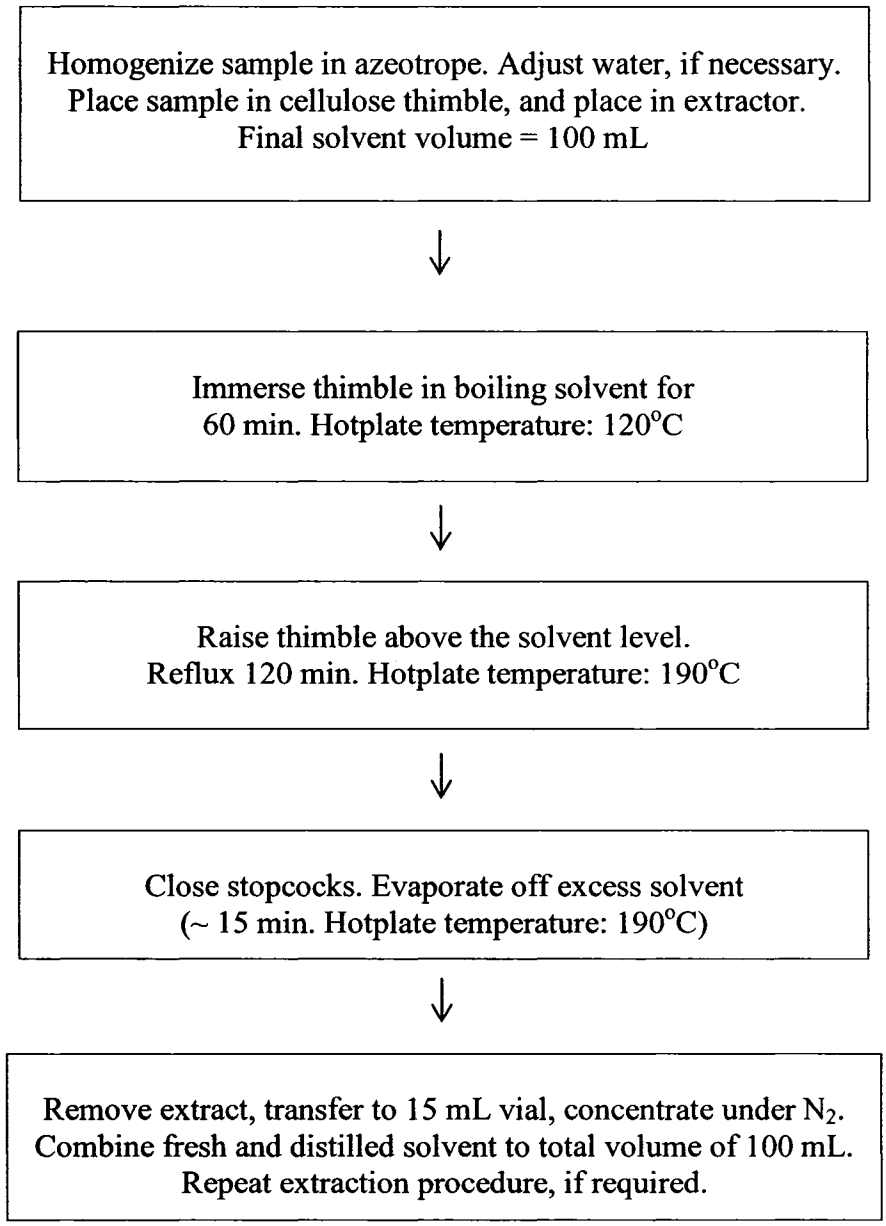


Figure 2.1. Randall procedure for the extraction of marine samples using a Velp Scientifica automated solvent extractor

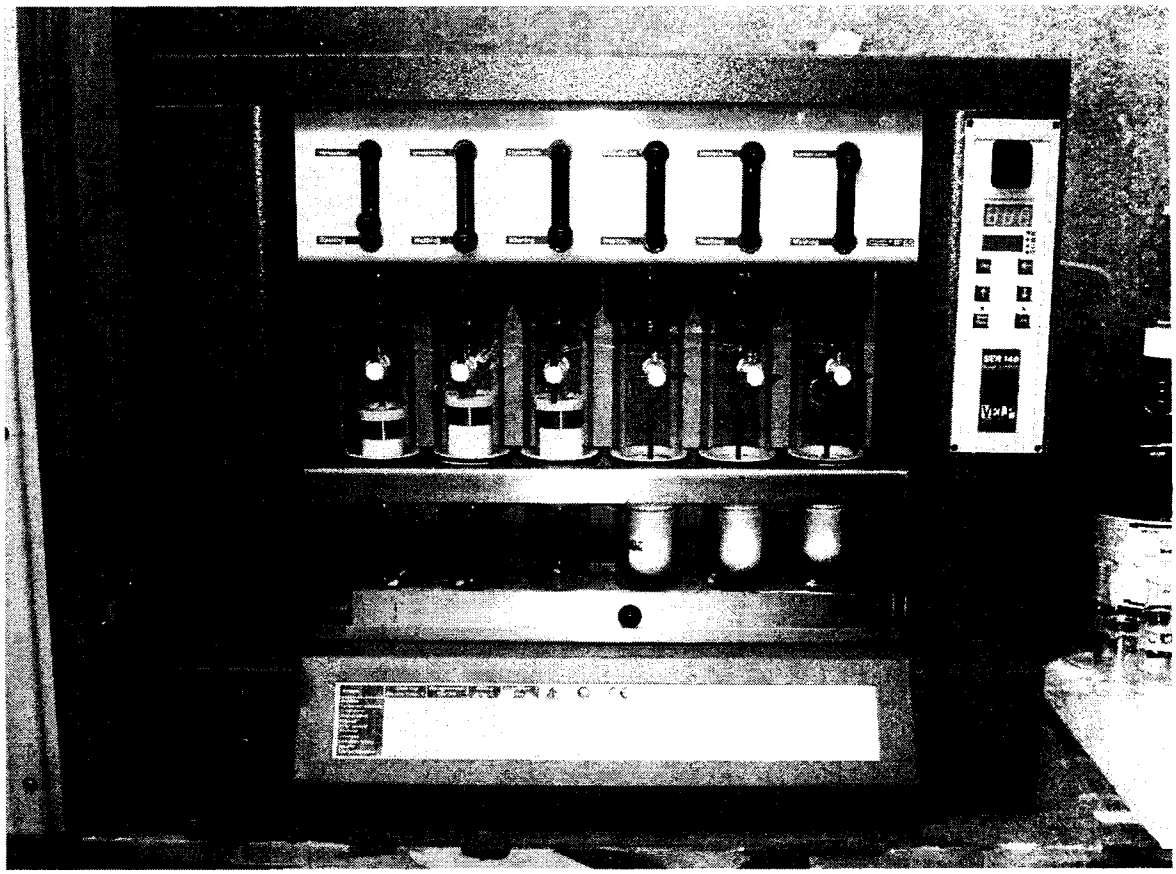


Figure 2.2. Velp Scientifica extractor, model SER 148

The solvent used for lipid extractions was the same azeotrope described for the Soxhlet procedure. Prior to extractions with the Velp extractor, the unit containing empty cellulose extraction thimbles was rinsed twice with ~30 mL MeOH, then twice with ~30 mL $CHCl_3$. Immediately prior to extraction the unit was rinsed with ~30 mL azeotrope solution. The temperature of the hotplate was set at 150°C for all rinses, and was programmed to run for 20 min. Boiling stones were added to all extraction vessels during the washing procedure.

2.4.7 Cleanup

An optional cleanup procedure could be undertaken to remove any non-lipid precipitate that may be present in the solvent extracts. The extracts were transferred to a 10 mL test tube and distilled water was added to half its volume. The mixture was vortexed and subsequently centrifuged for 3 min. at 3000 rpm. The lower, organic layer was removed and transferred to a clean vial and the pipet was rinsed well with $CHCl_3$. This rinse procedure was repeated three times, the extracts were pooled and evaporated under N_2 to their desired concentration.

2.4.8 Derivatization - Fatty Acid Methyl Esterification

Aliquots of extracts containing up to 20 mg total lipid were evaporated to dryness under N_2 and resuspended in 1.5 mL hexane and 0.5 mL 10% BF_3 in MeOH. The mixture was vortexed, sonicated for 4 min., flushed with N_2 and placed at 85°C for 90 min. The

resulting fatty acid methyl esters (FAMEs) were extracted by adding 0.5 mL hexane and 1.5 mL water. The upper, organic layer was removed and placed in a 2 mL vial, dried under a gentle flow of N_2, resuspended in hexane and frozen at -20°C until gas chromatographic analysis.

2.4.9 Detection Methods

2.4.9.1 Thin-Layer Chromatography with Flame Ionization Detection

Crude total lipid extracts were separated into individual lipid classes based on their relative polarities using an Iatroscan TLC/FID procedure developed by Parrish et al. (1987). The stationary phase, composed of silica bonded to thin quartz rods (Chromarods), is passed directly through the flame of the FID detector. The rods are aligned in groups of ten on a rack, and it is generally feasible to work with two racks at one time; thus up to 20 samples can be analysed at once. The rods are developed in four different solvent systems, and then partially scanned and the scans are combined to provide a full spectrum of the different lipid classes. The experimental procedure is outlined in Figure 2.2.

Analysis was performed with an Iatroscan MK V (Iatron Laboratories, Japan). The flow rate of the FID combustion gases, air and hydrogen, were set to 200 mL min^{-1} and 20 mL min^{-1}, respectively. Chromatograms were combined and integrated using T Data Scan Chromatography Analysis program (RSS, Bemis, TN, U.S.A).

2.4.9.2 Gas Chromatographic Detection of Fatty Acid Methyl Esters

FAMEs were analyzed using a Varian Model 3400 equipped with a model 8100 autosampler and an FID. The analytical column (30 m, 0.32 mm ID) was coated with Omegawax 320 (0.25 μm film thickness; Supelco, PA, U.S.A.). The carrier gas, hydrogen was set at a flow rate of 2 mL min^{-1}. The injection temperature was programmed to rise from 150°C to 250°C at 200°C $min.^{-1}$, where it was held for 10 min. The oven temperature was set to rise from 65°C to 195°C at 40°C $min.^{-1}$, where it was held for 15 min. and then ramped at 2°C $min.^{-1}$ to 215°C and held there for 1.25 min. The FID temperature was 260°C and the combustion gases were set at 30 mL $min.^{-1}$ (hydrogen) and 300 mL $min.^{-1}$ (air). Data acquisition, baseline subtraction and chromatogram plotting was performed using Varian GC Star Workstation software.

2.4.10 Data Handling and Statistical Analysis

Data presented are means ± standard deviations. All measurements were in triplicate unless otherwise stated. The statistical significance of differences between means of 2 groups ($p \leq 0.05$) was determined by Student's t-test. Comparisons of 3 or more groups were determined by one-way analysis of variance (ANOVA). When ANOVA revealed significant differences between groups, post hoc Tukey tests were performed to establish pairwise comparisons. All data evaluation was performed using SigmaStat software, version 3.1 (SPSS Inc.).

1. Spot sample with 25 µL Hamilton syringe equipped with automatic 0.5 µL repeating dispenser
2. Dry in constant humidity chamber 5 min. (chamber contains water saturated with calcium chloride)
3. Focus bands twice with 100% acetone
4. Dry in constant humidity chamber 5 min.
5. Develop twice in hexane:diethyl ether:formic acid (99:1:0.05; 25 min. and 20 min., respectively)
6. Partial scan (first scan detects HC, KET and SE/WE)
7. Dry in constant humidity chamber 5 min.
8. Develop in hexane:diethyl ether:formic acid (79:20:1; 40 min.)
9. Partial scan (second scan detects TAG, FFA, ALC, ST, DAG)
10. Dry in constant humidity chamber 5 min.
11. Develop twice in 100% acetone (15 min. each time)
12. Dry in constant humidity chamber 5 min.
13. Develop twice in chloroform:methanol:water (5:4:1; 10 min. each time)
14. Fully scan rods (third scan detects AMPL and PL)

Figure 2.3. Procedure for the detection of lipid classes using Iatroscan TLC/FID

2.5 Results and Discussion

2.5.1 Optimization of the Randall Procedure

It has been well documented that significant variability in lipid extraction efficiency can occur depending on the procedure used (Manirakiza et al., 2001; Iverson et al., 2001). For instance, many laboratories reportedly use the Bligh & Dyer procedure, however, as previously mentioned, modifications are frequently made in order to simplify the original extraction protocol. The nature of the modifications is rarely mentioned, although they can have an impact on the quantitative yield of extracted lipid. Often, modified methods of traditional extraction procedures may break down at large sample sizes and require large volumes of organic solvents, however the Randall method can easily accommodate large sample sizes and solvent can be recovered after extraction and possibly reused.

The objectives of this research were to develop a procedure that could reasonably be carried out in one day with minimal handling. Although the entire procedure takes approximately 6 hr to complete (including sample homogenization), other tasks can be carried out during the extraction process with very little active commitment from the analyst to extraction process.

Parameters for temperature programming, optimal submersion and washing times and the cycles required for quantitative extraction were explored. As well, sample capacity and the effect of water on the extraction efficiency of marine algae was examined. A rapid cleanup procedure was also developed.

For the most part, the marine alga *C. crispus* (Irish moss) was chosen for initial optimisation using the Randall method. Marine algae are known to contain a low percentage of total lipid with a high proportion of the polar lipids AMPL and PL. As well, the lipids in marine macrophytes contain a high level of omega-3 (ω3) fatty acids (Cheung et al., 1998), which are prone to oxidation and potential thermal instability, thus the analysis of their FA profiles would be very beneficial in determining if the heat required for extraction was causing any degradation. As well, the high content of pigments provided a very useful visual guide for monitoring the extraction efficiency, although the appearance of colour does not necessarily imply a lipid-rich sample. To test the efficiency of the optimized procedure, the optimization steps outlined below were compared to the modified Folch procedure, which is commonly used in our lab. Further, the procedure was investigated in two fish species, *G. morhua* and *M. villosus*. The optimized procedure was subsequently compared to the traditional Folch et al., Bligh & Dyer, and Soxhlet methods, using both macrophytes and animal tissues.

2.5.1.1 Solvent

The solvent used for extractions was an azeotrope composed of $CHCl_3$:MeOH:H_2O (90.5:8.2:1.3 wt%; Lide, 1992). The advantage of using this azeotropic solvent system is due to its low boiling point (52.3°C), compared to 61°C for $CHCl_3$ and 65°C for MeOH. This lower temperature may be required to help preserve the more labile thermosensitive lipids (i.e. PUFA), which have been reported to undergo

degradation using extraction methods such as Soxhlet (Cheung et al., 1998). Further, this monophasic solvent system, which contains water, is suitable for extracting wet samples.

Although there are environmental and health concerns associated with the use of halogenated solvents, $CHCl_3$ and MeOH based solvent systems are still considered to be the best option for routine lipid extraction (Parrish, 1999). No other solvents were tested since it has been well established with the Bligh & Dyer and the Folch et al. methods that $CHCl_3$ and MeOH are very effective for extracting both the polar and neutral lipid fractions in marine samples, with the water in the sample as the ternary component.

2.5.1.2 Sample Preparation

Initially, untreated (not rinsed) lyophilised *C. crispus* was extracted with the modified Folch procedure or with the Randall method to see if this could be a viable method for the preparation of samples. It has been reported that lyophilization can lead to a decrease in lipid yield if the sample is not rehydrated prior to extraction, particularly affecting the TAG fraction (Dunstan et al., 1993), thus the appropriate amount of water was added to all samples, and the samples were rehydrated for 20 min prior to extraction.

The results indicate that a significantly higher amount ($p<0.001$) of total lipid was extracted with lyophilised algae extracted via the modified Folch procedure compared to the Randall method (samples were immersed in boiling solvent for 30 min, followed by a 60 min rinse at 150°C; optimal wash and rinse times were not yet developed), however

there were no significant differences in relative proportions of the major individual lipid classes extracted (Figure 2.4).

With both procedures, lyophilization led to a significant decrease in the yield of the AMPL (51% decrease for Randall method and a 44% decrease for modified Folch), corresponding to a subsequent increase in the TAG fraction.

The reasons for this decrease in AMPL are unclear, however it has been reported that lyophilised tissues can be difficult to extract (Christie, 1973). Further, it has also been reported that some pigments can be degraded during lyophilization (Çinar, 2004) due to their inherent instability. While Dunstan et al. (1993) did not find any loss in polar lipids while examining the effects of lyophilization on the extraction of oysters, the nature of the polar lipids was not described and it is likely that oysters would not contain a significant amount of AMPL relative to TAG or PL.

It was concluded that lyophilization could lead to inaccurate lipid estimations. As a consequence of these findings, wet samples were used for all subsequent extractions.

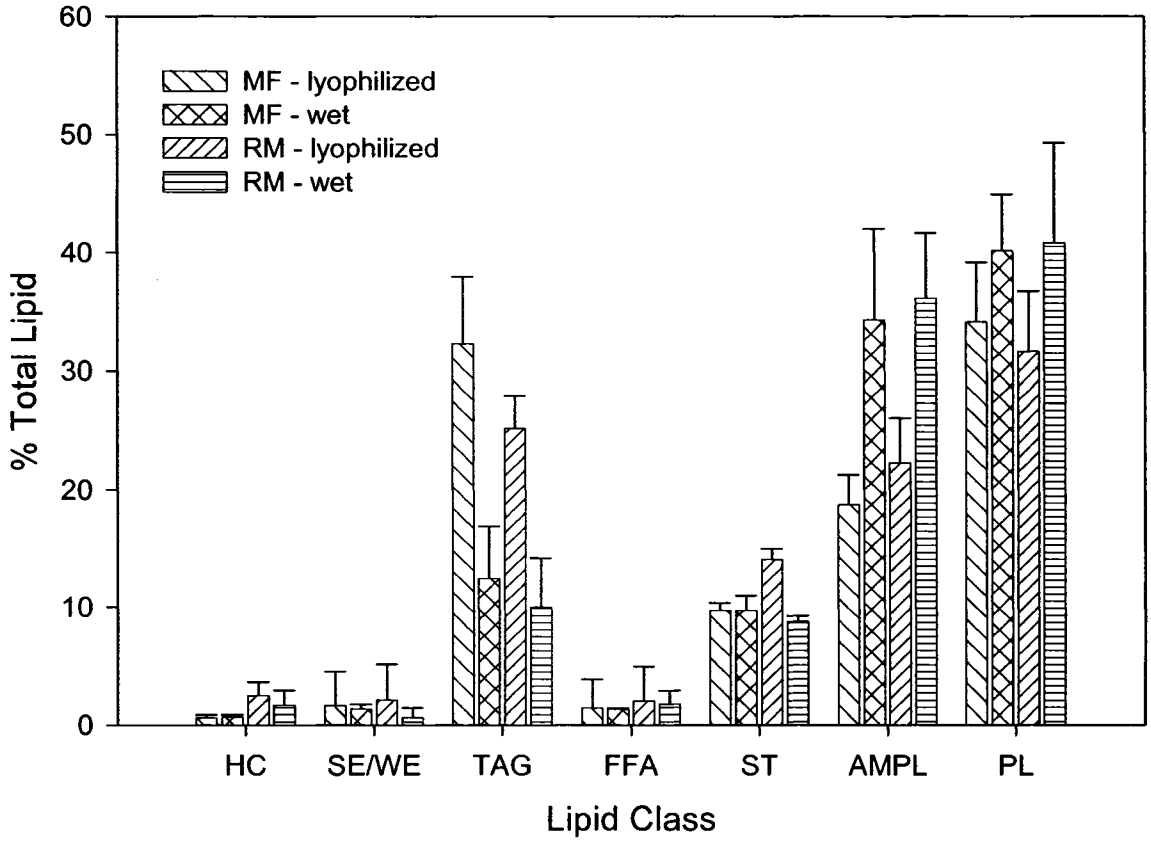


Figure 2.4. The effect of lyophilization on the extraction of major lipid classes in *C. crispus*, either lyophilised for 42 hr or left untreated. Lyophilized samples were rehydrated with water for 20 min. prior to extraction. MF: modified Folch; RM: Randall method. Error bars represent SD, n=3.

2.5.1.3 Sample Amount

Optimization of the Randall method was performed with 1 g and 4 g samples, although samples up to 15 g were extracted with success. Sample size did not appear to have a significant effect on the extraction efficiency. There was however, considerable dependence on sample amount and extraction efficiency when using the modified Folch procedure, and this will be discussed in further detail in section 2.7.

2.5.1.4 Solvent Volume

Samples were extracted in 100 mL of solvent volume. Although smaller volumes did not appear to have an effect on the extraction efficiency of *C. crispus*, this volume was chosen as a standard for all optimization experiments because it was enough solvent to sufficiently cover samples of various sizes (1 – 15 g) during immersion. Initial investigations using 75, 80 and 100 mL showed no significant difference in the extraction efficiency of algae samples weighing 4 g or less.

2.5.1.5 The Effect of Water

The azeotrope used for extractions should theoretically contain 1.3% water w/w (1.8 mL per 100 mL of solvent). Since wet samples were chosen for extractions, with a water content of approximately 80%, this solvent system would be limited to small samples (~ 2.5 g sample per 100 mL solvent volume) unless excess water could be tolerated in the system. Excess water would come out of solution, forming an aqueous

layer on top of the azeotrope. For Randall extractions with samples over 2.5 g, the water component of the azeotrope was omitted, with the assumption that the water required for the correct solvent proportions would come from the sample. For samples smaller than 2.5 g, the appropriate amount of water was added to the sample prior to homogenization.

In order to investigate the effect of water on the boiling point of the azeotrope, aliquots of freshly prepared monophasic solution were boiled while temperature measurements were taken repeatedly during evaporation. Modified solutions were also prepared, either containing no water ($CHCl_3$: MeOH, 85.5:14.5 v/v), or with increasing amounts of water added to the azeotrope (Table 2.1), and the boiling points of the different solvent mixtures were measured. It was readily apparent when the water saturated the solution, which could be visually seen as a clear colourless layer forming on top of the organic phase. This layer became more prominent with increasing amounts of water. The temperature of the unmodified solution both before and after distillation, as well as modified forms (either without any added water or with excess water), was in good agreement with the theoretical boiling point (52.3°C).

The boiling point of the recovered azeotrope was also measured after the extraction of mussel samples which weighed 2.7 – 14.2 g wet weight (samples 1 –5; Table 2.1). All solutions were clear and monophasic after recovery, except for the two largest samples (weighing 9.98 g and 14.2 g), in which there was a resulting biphasic solution characterized by the presence of a thin (~ 1 mm) layer of clear solution on top, which is likely excess water from the sample, not soluble in the azeotrope solution. The boiling points of these analyses, which varied between 52.7 – 54.7°C, did not appear to

be dependent on sample size, and were also in relatively good agreement with the theoretical boiling point of the azeotrope. It is unclear why these boiling temperature values are all slightly higher than the theoretical value, possibly the solution contains some non-lipid, volatile compounds. The recovered solvent was analysed via Iatroscan TLC/FID, however there was no sign of contaminating lipid species.

In a further investigation, extractions were carried out on *C. crispus* in which wet samples weighing 1.49 ± 0.24 g were extracted in either the azeotrope or a modified form of it (omitting the water portion; the resulting solution contained $CHCl_3$: MeOH, 85.5:14.5 v/v). No additional water was added to the samples. The data indicate that there were no significant differences between these two treatments (students T-test; $p = 0.709$) and all lipid classes were extracted in the same relative proportion and the same amount.

2.5.1.6 Immersion

The first step in the extraction procedure is the immersion of the sample directly in boiling solvent. Although the boiling point of the extracting solvent is 52.3°C, the temperature setting of the hotplate had to be set much higher in order to overcome the resistance of the glass extraction vessels to heat transmission. During the immersion step, the temperature of the hotplate was set at either 120°C or 150°C; both temperatures provided enough energy to produce a rapid boil. There was no significant difference in

Table 2.1. The boiling point of the azeotrope solution, and related solvents, under different conditions. Theoretical boiling point of the solution is 52.3°C. Data represent mean ± SD from a minimum of duplicate measurements.
*A.: azeotrope

Freshly prepared solution with modifications		Recovered solvent after mussel extractions	
	Temperature (°C)		Temperature (°C)
A.* - no treatment	52.7 ± 0.3	Sample 1 (14.2 g) †	54.5 ± 1.1
A. - after distillation	51.4 ± 0.2	Sample 2 (4.44 g)	53.7 ± 0.5
A. + 2% H_2O	51.2 ± 0.2	Sample 3 (9.98 g) †	52.7 ± 0.6
A. + 3% H_2O†	51.5 ± 0.5	Sample 4 (5.36 g)	54.7 ± 0.4
A. + 4% H_2O†	50.7 ± 0.3	Sample 5 (2.73 g)	53.8 ± 0.8
A. + 6% H_2O†	50.8 ± 0.0	Sample 6 (Blank)	53.8 ± 0.8
$CHCl_3$	60.5 ± 0.0		
$CHCl_3$/MeOH (85.5/14.5, v/v)	52.9 ± 0.3		

† Visible layer on top of lower solvent mixture

the lipid extracted between the two temperatures ($p = 0.251$), thus the lower 120°C was chosen for all subsequent experiments.

To test the optimal immersion time, duplicate samples of *C. crispus* (4 g) were boiled, using a hotplate setting of 120°C, for either 30 or 60 min., followed by a 2 hr rinse time, at a hotplate setting of 190°C.

The results indicate that there were no statistically significant differences in the total amount of lipids extracted ($p = 0.129$), or in the relative proportion of any of the individual lipid classes. Similarly, FAME analysis revealed that there were no statistically significant differences between the profiles under either condition. However, there appeared to be a higher amount of all lipid classes extracted with the longer immersion time. There was an apparent increase in the relative yield of PUFA (comprising 44% of total FA with a 60 min. immersion, compared to 40% with a 30 min. immersion).

In light of these results, it was concluded that 1 hr would be suitable for the first immersion. Although differences between the 30 min. and 60 min. immersion were not statistically significant regarding the total lipid extracted or in the relative yield of PUFA, the absolute amounts of total lipid and PUFA were apparently higher with the 1 hr immersion. Considering there was no visible degradation of PUFA after a 60 min., a longer immersion time was assumed to be better for maximal lipid extraction.

2.5.1.7 Rinse

Following the immersion of the sample in boiling solvent, the sample must be rinsed with freshly distilled solvent to ensure complete lipid recovery. This is achieved by raising the thimbles above the solvent level, allowing for cold refluxed solvent to continuously pass through the thimble. Initial investigations held the hotplate rinsing temperature at 150°C. Although the solvent was boiling, the drip rate averaged 1.5 – 2 drops $sec.^{-1}$ and the extraction efficiency of total lipid extracted was only 65% compared to similar modified Folch extractions. There was slight variation in the drip rate depending on the location of the sample cup on the heating plate, thus samples were always placed randomly to avoid any bias.

The temperature of the hotplate was raised to 190°C, which resulted in a marked increased in the reflux rate, and the drip rate increased to the point where there was a continuous or near-continuous stream of solvent always passing through the thimble (>1 drip $sec.^{-1}$). It is likely that the increased temperature was able to increase the solvent boiling rate inside the extraction vessel, thus increasing the reflux rate. For the extraction of *C. crispus*, after an immersion time of 30 min., with a 2 hr rinse at 190°C, between 94.9% and 105% total lipid was recovered compared to the modified Folch procedure, depending on the algal treatment. The neutral fraction, in particular TAG and ST, were ≥100% recovered compared to modified Folch, however recovery of the more polar lipids, in particular PL, ranged between 54.3% and 80.9%.

A second cycle of immersion/rinse was performed involving a 30 min. immersion followed by a 1 hr rinse, and the recovery of PL increased significantly, ranging from 101.2% to 111.8% extracted, relative to the modified Folch procedure. In a parallel investigation using the marine alga *A. cribrosum*, similar results were found, however the amount of PL extracted after 1 cycle of immersion/rinse was much higher (>150% recovered compared to modified Folch).

Longer rinse times (> 2 hr) were not tested since one of the primary aims in developing the protocol was to develop a method that could reasonably be carried out in one day. In an attempt to optimize the rinse temperature further, *C. crispus* was rinsed for 2 hr at either 190°C or 210°C, although the results revealed there were no significant differences in the relative proportions of any of the lipid classes or in the total lipid extracted ($p = 0.333$). FAME analysis revealed there were no significant differences in the FA profiles at either of the temperatures. It was concluded that 190°C was a suitable hotplate temperature for the wash cycle.

2.5.1.8 Cycles

The number of immersion/rinse cycles required for quantitative lipid recovery was evaluated as a final step in the development of the Randall method for the extraction of marine lipids. Similarly, the number of rinses required for the modified Folch procedure was also investigated.

During the modified Folch procedure, lipids were extracted once followed by three rinses; *C. crispus* and *A. cribrosum* were both extracted in this way. The total lipid was determined by summing these 4 fractions together. To determine whether any lipid remained, both of the algal samples were then rinsed a fourth time, and this rinse was transferred to a separate vial. All lipid extracts were quantified by TLC/FID. Although there was still some faint green colour in the extracts after the fourth rinse, as is common with algal samples, this fourth rinse only accounted for ~2.5% of the total lipid extract for *A. cribrosum* and ~4% for *C. crispus,* thus it was concluded that one extraction followed by 3 rinses is adequate for the quantitative extraction of all lipid classes, with a recovery of over 95% of total lipids.

For the Randall extractions, up to three immersion/rinse cycles were carried out, however the actual immersion/rinse times were reduced for the second and third cycles. The second cycle involved a 30 min. immersion followed by a 1 hr wash, and the third cycle (if applicable) involved a 30 min. immersion followed by a 30 min. wash.

The number of cycles required for optimal extraction efficiency was evaluated with three tissues: *C. crispus*, *G. morhua* (a lean fish, <2% lipid) and *M. villosus* (a fatty fish, >2% lipid). The total lipid (TL) extracted was determined by adding up the amount of lipid components extracted after the three cycles. The amount recovered from each cycle was determined by dividing each individual cycle by this total.

Two cycles were found to be sufficient for the recovery of >95% of all lipid classes from both plant and animal tissues (Figure 2.5). The second cycle accounted for 10.1% TL (*C. crispus),* 4.4% TL *(G. morhua)* and 3.1% TL for *M. villosus*. The third

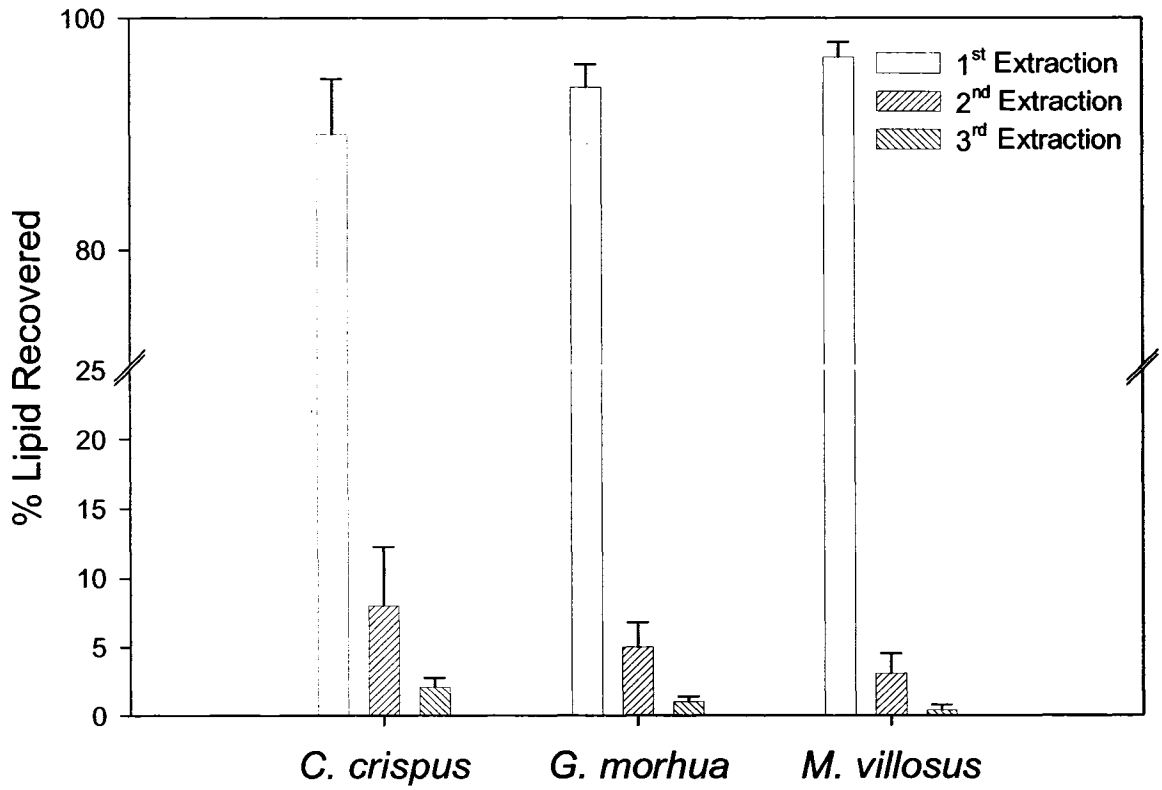


Figure 2.5. The contribution of 3 individual immersion/rinse cycles to the total lipid extracted in *C. crispus*, *G. morhua* and *M. villosus*. Totals were determined from all three cycles combined. Error bars represent SD, n=3.

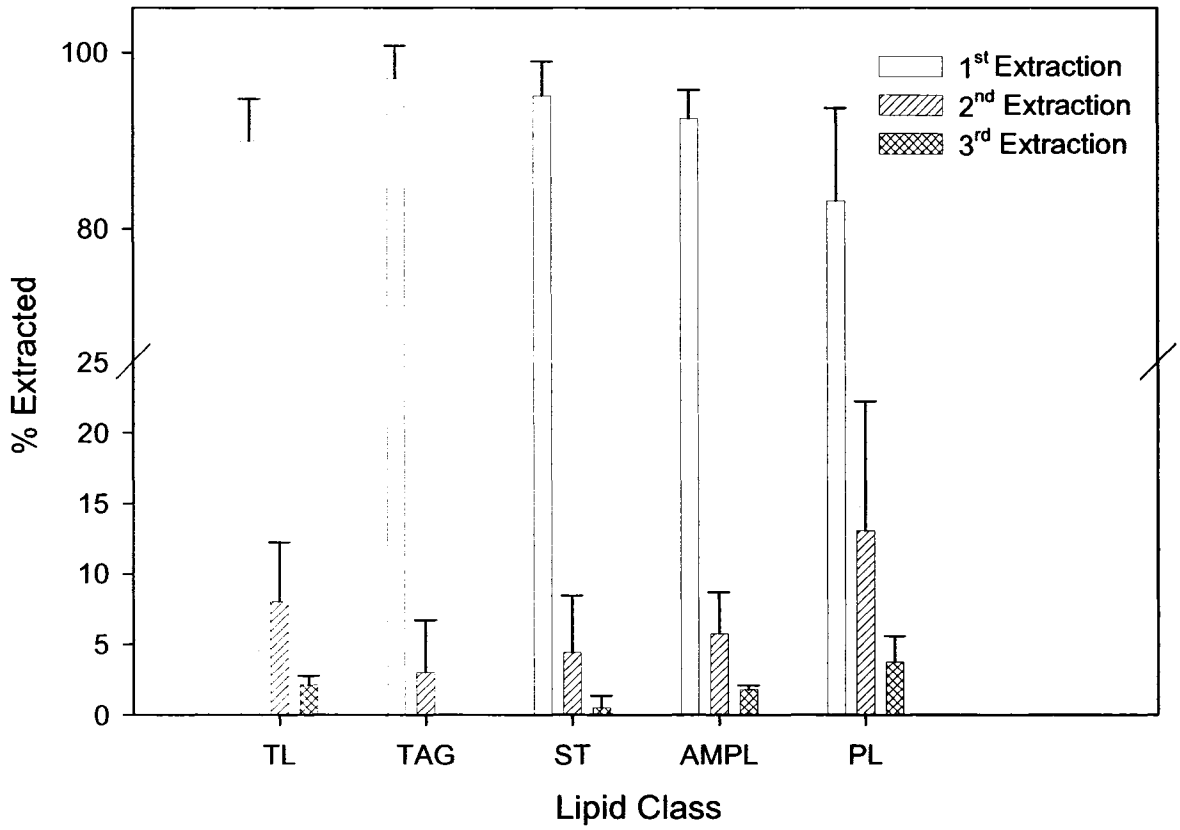


Figure 2.6. The contribution of 3 individual immersion/rinse cycles to the extraction of individual lipid classes extracted in *C. crispus*. Totals were determined from all three cycles combined. Error bars represent SD, n=3

cycle accounted for 2.4%, 0.9% and 0.3% TL for *C. crispus*, *G. morhua and M. villosus*, respectively.

Samples with a higher proportion of TAG, such as *M. villosus*, appeared to have a higher extraction efficiency during the first wash, which was expected since TAG are storage lipids and are generally not as difficult to extract as the more polar lipids. As presented in Figure 2.6, the polar lipids account for the major lipid classes extracted in *C. crispus* during the second and third cycles; the major neutral lipids, TAG and ST, were almost completely extracted during the first cycle (>97% TAG extracted and >94% ST extracted). Thus, for the quantitative recovery of all lipid classes, as well as for accurate estimation of individual lipid classes, a second extraction cycle is recommended.

G. morhua and *C. crispus,* which contain a high proportion of polar lipids, were also extracted at 4 g, and the results are displayed in Table 2.2. The extraction efficiency did not appear to be dependent on the size of the sample. Regardless of the sample size, total lipids in *C. crispus* were ≥96% extracted after 2 cycles and for *G. morhua,* ≥98% of the total lipids were extracted after 2 cycles.

The resulting extraction procedure, including 2 cycles, takes 4.5 hr actual extraction time, and with the preparation and recovery step the entire procedure can normally be conducted in less than 6 hr. If a third cycle is desired, the extraction time is 6 hr, and with sample preparation and solvent recovery, approximately 7.5 hr is required.

2.5.1.9 Cleanup

Water is frequently used to clean up crude lipid extracts (e.g.: Folch et al., 1957; Bligh & Dyer, 1959). However, washing the extracts with water can lead to some selective lipid loss, particularly of the more polar species such as gangliosides (Christie, 1973; Perkins, 1991).

Randall extractions generally produced final extracts that were free of insoluble material, however sometimes some particulate matter was present, particularly in the plant extracts, which seemed to emerge in a concentration dependent manner (larger samples had more particulate matter). A rapid cleanup investigation was undertaken to see if washing the crude extract would greatly reduce the amount of lipid yield.

Two treatments were performed, both in duplicate. Concentrated lipid extracts from both *C. crispus* and horse mussel, free of any particulate matter, were diluted to 10 mL with $CHCl_3$, and 1.00 mL aliquots were removed with a volumetric pipet and placed in a 10 mL centrifuge tube. The extracts were subsequently washed with twice their volume with either H_2O or a mixture of MeOH and H_2O (1:1). The samples were vortexed and centrifuged and the lower $CHCl_3$ layer was removed. The extracts were then rinsed three times with $CHCl_3$ and the pooled extracts were concentrated under N_2. These rinsed extracts were compared to 1.00 mL aliquots of the crude extract and individual lipid classes were quantified via TLC/FID.

Table 2.2. The percent recovered after three immersion/rinse cycles for *C. crispus* and *G. morhua* at 1.0 g and 4.0 g, and *M. villosus* at 1.0 g. 1st cycle: 60 min. immersion/ 120 min. wash; 2nd cycle: 30 min. immersion/60 min. wash; 3rd cycle: 30 min. immersion/ 30 min. wash. Values represent mean ± SD, n=3

Sample	Mass (g)	% Extracted			Total Lipid (mg/g)
		1st cycle	2nd cycle	3rd cycle	
C. crispus	1.0	89.9 ± 4.8	8.0 ± 4.3	2.1 ± 0.6	9.4 ± 0.4
C. crispus	4.0	87.7 ± 3.6	9.5 ± 2.8	2.8 ± 0.9	9.0 ± 1.8
G. morhua	1.0	93.9 ± 2.0	5.0 ± 1.8	1.0 ± 0.4	6.7 ± 1.4
G. morhua	4.0	86.5 ± 11.0	12.3 ± 10.1	1.3 ± 0.9	8.1 ± 5.2†
M. villosus	1.0	96.6 ± 1.3	3.1 ± 1.5	0.4 ± 0.4	33.4 ± 8.5

†Large SD is attributed to one sample that had a particularly high amount of PL extracted during the 1st immersion, however these results could not be rejected by q-test

The results indicate there was no significant differences in either the total lipid yield ($p = 0.533$ and 0.480 for horse mussel and *C. crispus*, respectively, as determined by one-way ANOVA) with either of the treatments compared to the untreated sample. Similarly, there were no significant differences in the relative proportion of any of the individual lipid classes. Thus it was concluded that a rapid rinse with water could be undertaken if required, without significantly affecting the quantitative yield of lipid extracted.

2.5.2 Sample Preparation: Rinsing Algae with Boiling Water

It has been reported that the action of lipolytic enzymes cannot be completely destroyed upon freezing at –20°C or by immersion in some organic solvents (Christie, 1973). In plant samples, enzymes such as phospholipase D can be activated leading to the hydrolysis of PL and a subsequent increase in the phosphatidic acid and FFA content. For the extraction of plant lipids, Nichols (1963) recommends replacing methanol with isopropanol during sample homogenization, as this solvent is effective at inhibiting the lipase activity. It has also been suggested that quick treatment of the samples with boiling water can deactivate these enzymes (Christie, 1973; Budge & Parrish, 1999).

An initial investigation was undertaken to see if isopropanol could be used in place of methanol for the entire extraction procedure, however during the extraction of *C. crispus* using this method, the AMPL fraction was significantly reduced ($p = 0.005$), as well there was an apparent decrease in the TAG and ST fractions. Using MeOH as the

extracting solvent exhibited no significant increase in the FFA content ($p = 0.866$), which accounted for less than 1% of the total lipid extract when using either methanol or isopropanol.

Budge & Parrish (1999) reported that treating microalgae with boiling water led to a significant decrease in the FFA and AMPL content in the total lipid extracts. To test this method with macroalgae, samples of *C. crispus* and *A. cribrosum*, previously frozen at –20°C, were immersed in distilled water at either 22°C or 100°C for *ca* 60 sec. and extracted with either the described Randall protocol or with the modified Folch procedure.

2.5.2.1 *Agarum cribrosum*

The treatment of *A. cribrosum* with boiling water showed no statistically significant differences ($p > 0.05$) in the lipid classes extracted with either treatment. The level of FFA was quite high in all samples (7.3 – 8.2%), which is likely due to prolonged storage of the algae at –20°C rather than due to sample treatment. Although, with both extraction procedures, there was a slight decrease in the level of FFA with the boiling water treatment, these differences were not statistically significant.

There was a difference however in the total lipid extracted between the two methods, with the Randall procedure extracting more total lipid than the modified Folch procedure, although the relative yields of the individual lipid classes did not change (Figure 2.7). The algal samples weighed between 1.3 – 2.1 g in size, thus the matter of

size limits for quantitative extraction using the modified Folch procedure were questioned, which will be discussed in greater detail in section 2.7.

2.5.2.2 *Chondrus crispus*

C. crispus was treated in the same way as *A. cribrosum*, however when *C. crispus* was rinsed with boiling water, a very sticky residue coated the samples, which is likely as a result of the release of carrageenan, which is commonly found in this alga (Saito and Idler, 1966). The presence of this residue did not appear to affect the extractions nor was there any difference in the dry weight measurements of samples treated with boiling or with 22°C water.

Results obtained for *C. crispus* were similar to those obtained with *A. cribrosum* in that the absolute yield of lipid was much higher for algae extracted using the Randall method compared to the modified Folch procedure. Notably, much more ST (>50%) was extracted with the Randall procedure compared to the modified Folch procedure, regardless of treatment (Figure 2.8). There was no difference in the FFA content with either treatment, which was always <5%. Alcohols were present in samples extracted using the Randall method; the alcohol content was <1% for samples rinsed in boiling water, however when alga was rinsed in 22°C water, the alcohol content accounted for 5.6 ± 3.2% of the total lipid extracted. There appeared to be a slight increase in the AMPL fraction with the boiling water treatment, with both extraction methods, however these differences were not statistically significantly different. The boiling water treatment did appear to lead to a slight increase in the PL fraction.

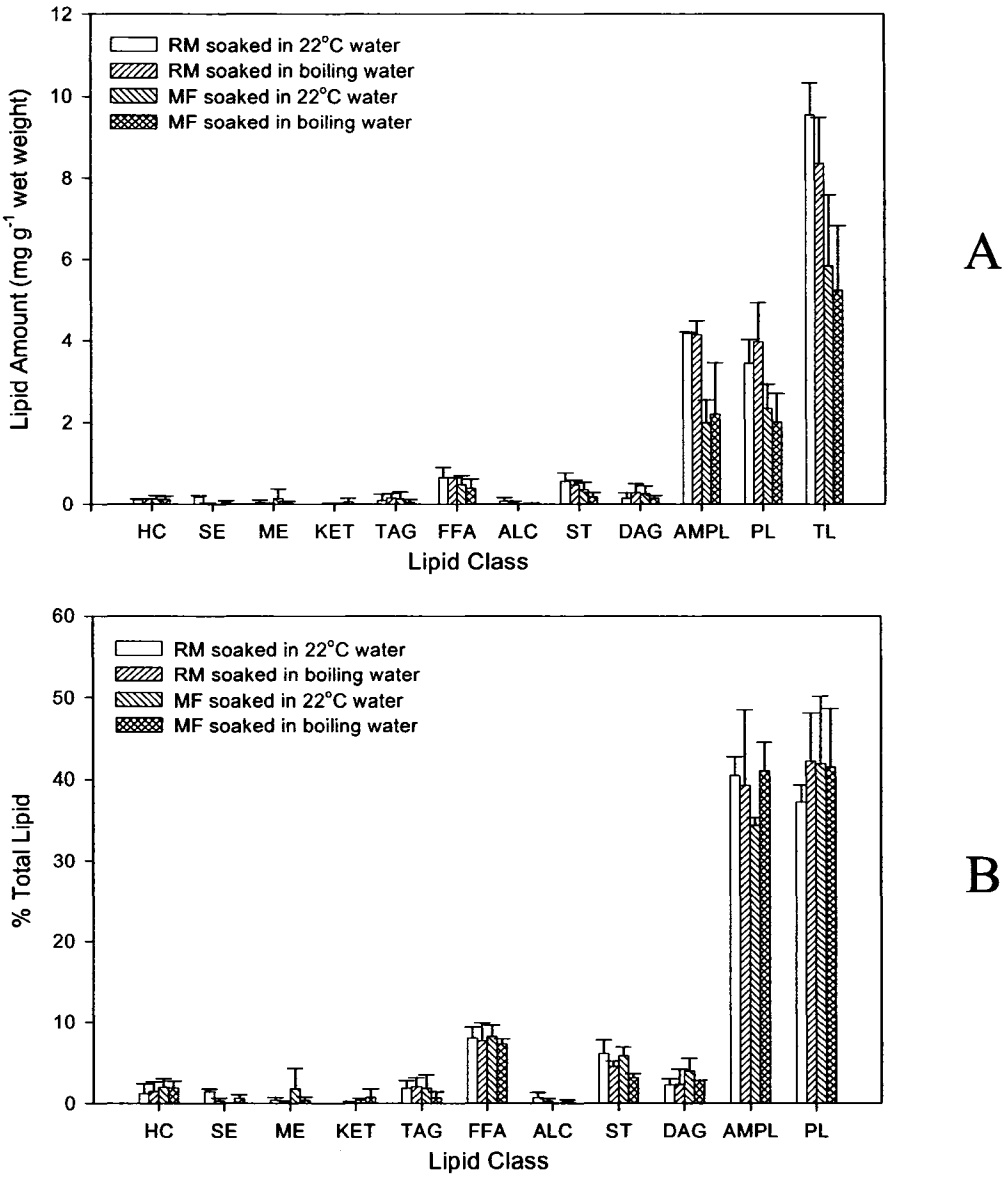


Figure 2.7. The extraction of lipids in *A. cribrosum* by the modified Folch (MF) procedure or the Randall method (RM). (A) Total lipids in mg g^{-1} wet weight; (B) The relative yield of individual lipid classes. The alga was rinsed with boiling water or 22°C water prior to extraction. Error bars represent SD, n=3

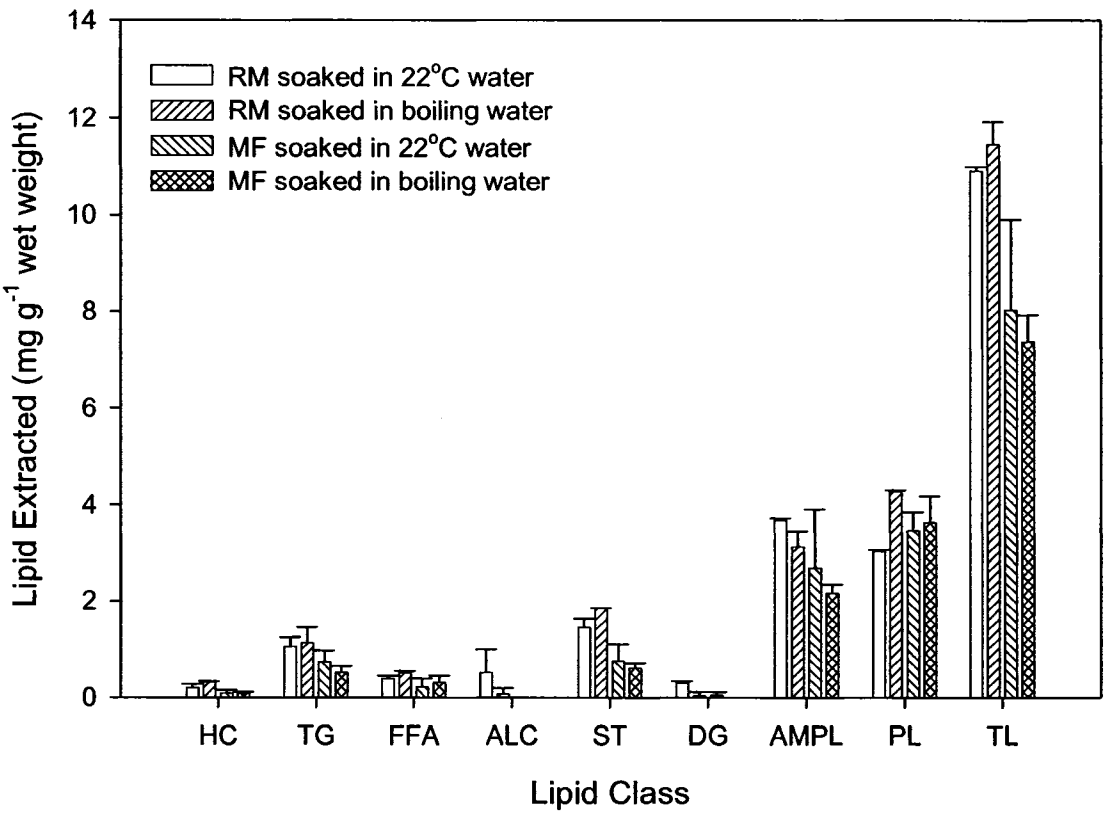


Figure 2.8. The extraction of lipids, in mg g^{-1} wet weight, in *C. crispus* by the modified Folch (MF) procedure or the Randall method (RM). The alga was rinsed with boiling water or 22°C water prior to extraction. Error bars represent SD, n=3.

2.5.3 The Modified Folch Procedure and Effect of Sample Size

As discussed in section 2.6.1, Randall extractions of *A. cribrosum* and *C. crispus* resulted in significantly higher yields of total extracted lipid compared to the modified Folch procedure (up to 134 –175% more TL extracted depending on treatment). An investigation was undertaken to determine if this was due to the Randall method being a more efficient extraction method or because the efficiency of the modified Folch procedure breaks down at higher sample loads. This procedure, originally designed for samples weighing 10 – 150 mg dry weight (Parrish, 1998), is routinely used for larger samples, with solvent scale up, due to its ease of use and because the extraction can easily be carried out in a 50 mL (or smaller) test tube, allowing for extraction, sonication and centrifugation to be easily carried out.

Samples of *C. crispus* weighing 0.5, 1.0 and 4.0 g were taken in triplicate and extractions were carried out with the modified Folch procedure. Dry weight measurements, taken by drying wet samples in the oven at 80°C until a constant mass was achieved, revealed a water content of approximately 70%. The alga was rinsed for ~60 sec with boiling water prior to extractions. Similarly, algal samples weighing 1.0 and 4.0 g were also extracted via the Randall method.

For the modified Folch extractions, all samples were extracted in 15 mL of 2:1 $CHCl_3$:MeOH, after which another 5 mL of 2:1 was added followed by 2.5 mL H_2O (or more for the 0.5 g and 1.0 g samples). For all of the extractions, the final solvent ratio was 8:4:3 $CHCl_3$:MeOH:H_2O, including the water in the sample, which was the same as

the critical Folch ratios, required for full lipid extraction. The samples were extracted once and rinsed a minimum of 3 times, however for the 4.0 g sample, extracts were rinsed 6 times, and the seventh rinse was kept separate. This seventh rinse accounted for ~4% of the total lipid.

The results for total lipid classes extracted via the modified Folch procedure, in mg g^{-1} wet weight, are presented in Table 2.3, as well as relative proportions of lipid classes in Table 2.4. One way analysis of variance (ANOVA) between the three different masses indicate that there was significantly less total lipid extracted with increasing sample mass, particularly at 4.0 g, owing in particular to significant differences in the ST ($p = 0.011$), AMPL ($p = 0.003$) and PL ($p < 0.001$) fractions. There was also significantly more PL extracted at 0.5 g compared to 1.0 g. The total percent lipid extracted was also significantly less with the 4.0 g samples, however the relative proportion of individual lipid classes was quite similar with the exception of ST ($p < 0.001$) and AMPL ($P = 0.052$).

In the above-described procedure, the 4.0 g samples, assuming a dry weight of 1.2 g based on dry weight measurements, would correspond to minimum of 24 mL solvent required, however the actual solvent ($CHCL_3$ and MeOH only) was only 20 mL. This would not have been a factor with the smaller mass samples. It is likely that with these large samples, the solvent-to-sample ratio is not great enough, possibly leading to a water barrier between the solvent and tissue, causing incomplete extraction.

It was also mentioned that a seventh wash of the 4.0 g sample accounted for ~4% of the total lipid extracted. As mentioned in section 2.5.7, where a fourth rinse from both

A. cribrosum and *C. crispus* extracts (samples weighing 3-5 g) was kept separate and quantified, and the contribution of this fourth rinse ranged between 2.5 and 4%. It seems likely that for large samples in particular, if the modified Folch procedure is to be used, more than seven rinses are required, or the ratio of solvent-to-sample must be scaled up.

The original Folch procedure (1957) describes using a solvent-to-sample ratio of 20:1, and although not explicitly stated it is assumed this ratio is based on wet weight measurements. The Bligh and Dyer (1959) procedure however requires a solvent-to-(wet) sample ratio of at least 4:1. According to Parrish (1998), not only should the final critical solvent ratios be 8:4:3 $CHCl_3$:MeOH:H_2O, but the solvent to sample ratio should be at least 3 mL per 150 mg dry weight (thus a ratio of 20:1 solvent-to-(dry) sample). Assuming a water content in the wet sample of approximately 80%, this would correspond to a solvent-to-(wet) sample ratio of approximately 5:1 (3 mL solvent to 0.75 g wet weight). It is probable that with these larger samples, the ratio of solvent-to-sample is not great enough and likely that 7 rinses would not be required if solvent volumes were increased. This does however pose problems when extracting samples in 50 mL test tubes, which are used because they can easily be centrifuged and manipulated.

In contrast to the modified Folch extractions of *C. crispus*, Randall extractions were carried out at 1.0 and 4.0 g under optimized conditions. When compared to the modified Folch procedure at 1.0 g, there was a very high correlation in the extraction efficiency between the two methods for all major lipid classes (Figure 2.9). The slope of the curve is 1.04 ± 0.06, while the y intercept is 0.19 ± 0.21, resulting in a notable 1:1 linear relationship between the two methods at 1 g. All individual lipid classes were

extracted in a similar proportion, with the exception of ST, which was extracted more efficiently with the Randall method ($p = 0.004$), at 1 g.

At 4.0 g, however, the Randall method proved to be much more efficient than the modified Folch procedure for the extraction of all lipid classes, in particular, ST ($p = 0.017$) and PL ($p = 0.003$), as illustrated in Figure 2.9. The slope of the curve is 1.93 ± 0.09, while the y intercept is -0.27 ± 0.14, resulting a near 2:1 relationship in the quantity of lipids extracted via the Randall method compared to the modified Folch procedure. It is possible that the heat involved with the Randall procedure is very effective at extracting the membrane lipids. As well, the Randall method uses more solvent (100 mL, leading to a 25:1 solvent-to-sample ratio). Samples smaller than 1.0 g were not tested because it is unlikely that the Randall procedure would be used for samples smaller than this size, especially in light of the fact the modified Folch procedure, which uses much less volume for small samples (<1 g), is very effective for the quantitative removal of lipids.

Table 2.3. Lipid class concentration (mg g^{-1} wet weight) of C. crispus extracted via the modified Folch procedure at 3 different sample amounts. The alga was treated with boiling water prior to extraction. Values with different superscripts represent a significant difference between values, $p < 0.05$. ND: not detected.

	4.0 g n=3	1.0 g n=3	0.5 g n=3
Total Lipid	$8.54^{a} \pm 1.45$	$15.95^{b} \pm 2.33$	$20.57^{b} \pm 3.06$
Hydrocarbons	0.19 ± 0.04	0.16 ± 0.08	0.31 ± 0.10
Steryl esters/wax esters	0.04 ± 0.08	ND	ND
Glycerol ethers	ND	ND	0.17 ± 0.30
Triacylglycerols	1.98 ± 0.63	3.45 ± 1.40	4.04 ± 1.93
Free fatty acids	0.06 ± 0.07	0.08 ± 0.15	0.07 ± 0.13
Alcohols	0.00 ± 0.01	ND	ND
Sterols	$1.01^{a} \pm 0.19$	$1.53^{b} \pm 0.18$	$1.68^{b} \pm 0.19$
Diacylglycerols	0.19 ± 0.14	0.12 ± 0.13	0.52 ± 0.28
Acetone mobile polar lipids	$1.65^{a} \pm 0.39$	$3.54^{b} \pm 0.34$	$4.10^{b} \pm 0.75$
Phospholipids	$3.41^{a} \pm 0.37$	$7.06^{b} \pm 0.43$	$9.68^{c} \pm 0.55$

Table 2.4. Lipid class content (% wet weight) of *C. crispus* extracted via the modified Folch procedure at 3 different sample amounts. The alga was treated with boiling water prior to extraction. Values with different superscripts represent a significant difference between values, $p < 0.05$. ND: not detected

	4.0 g n=3	1.0 g n=3	0.5 g n=3
Total Lipid (%)	$0.85^{a} \pm 0.14$	$1.60^{b} \pm 0.23$	$2.06^{b} \pm 0.31$
Hydrocarbons	$2.28^{a} \pm 0.26$	$1.01^{b} \pm 0.32$	$1.56^{ab} \pm 0.73$
Steryl Esters/Wax Esters	0.58 ± 1.00	ND	ND
Glycerol Ethers	0.01 ± 0.03	ND	0.75 ± 1.30
Triacylglycerols	22.84 ± 3.30	21.08 ± 5.89	18.97 ± 7.17
Free Fatty Acids	0.81 ± 0.92	0.62 ± 1.07	0.43 ± 0.75
Alcohols	0.04 ± 0.06	0.00 ± 0.00	ND
Sterols	$11.75^{a} \pm 0.37$	$9.65^{b} \pm 0.64$	$8.18^{c} \pm 0.34$
Diacylglycerols	2.37 ± 1.94	0.70 ± 0.70	2.71 ± 1.91
Acetone Mobile Polar Lipids	19.18 ± 1.41	22.32 ± 1.21	19.87 ± 1.19
Phospholipids	40.14 ± 2.37	44.63 ± 3.92	47.53 ± 4.70

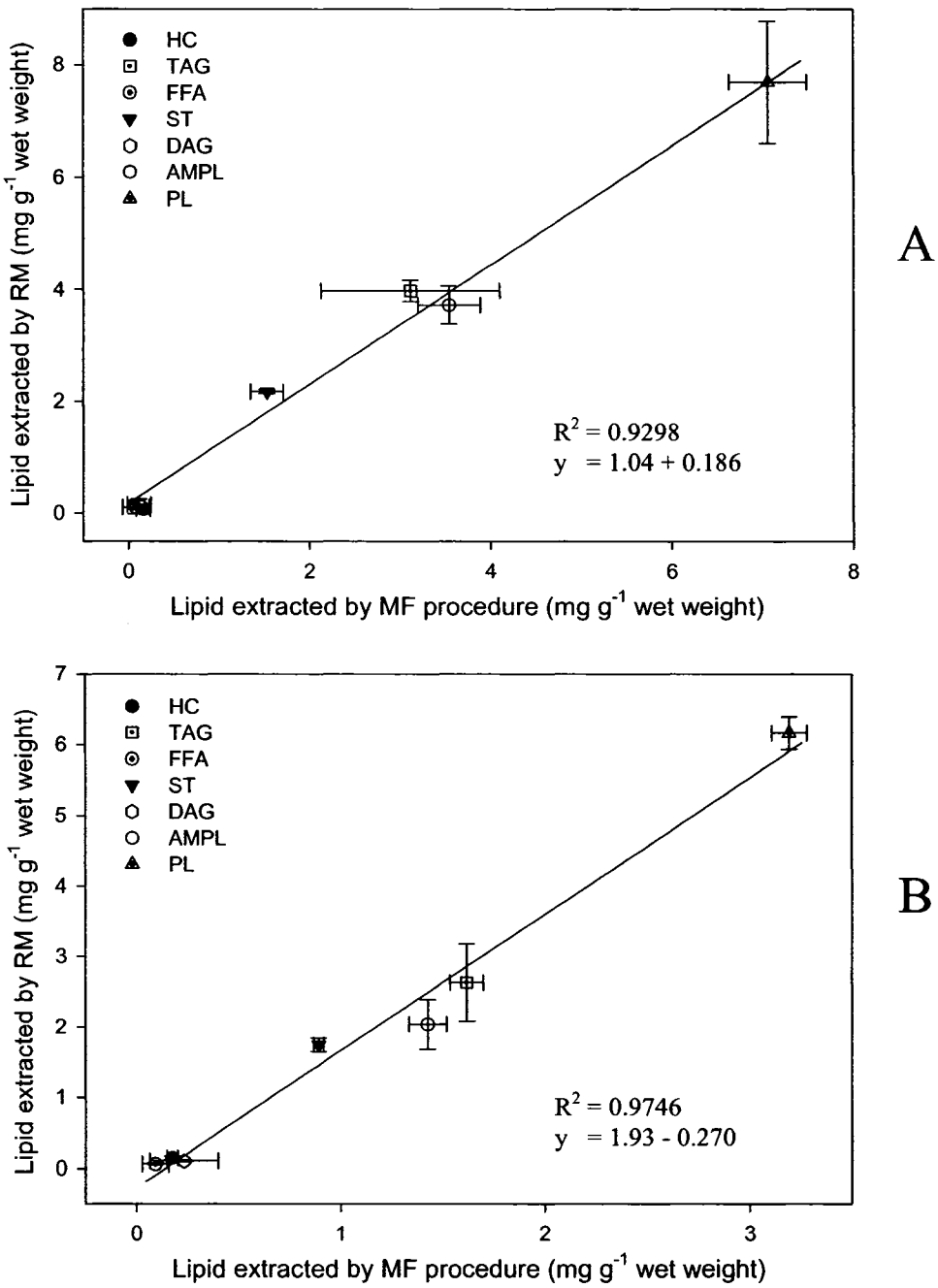


Figure 2.9. Linear correlations between Randall and modified Folch extractions of *C. crispus* at 2 different sample loads. (A) 1 g and (B) 4 g. Error bars represent SD, n=3. The alga was treated with boiling water prior to extraction.

2.5.4 Comparison of Different Extraction Methods

It is clear that the extraction procedure used can have an effect on total lipid extracted. As previously stated, most established extraction procedures, based on the Folch et al. and the Bligh & Dyer protocols, are modified in order to simplify the original protocols. In the current investigation, a cross-comparison study was undertaken to compare the Randall method to four different extraction procedures: the original Folch et al. and Bligh & Dyer methods, Soxhlet and the modified Folch procedure.

Marine samples used for this comparison were *C. crispus*, a typical marine macroalgae, *G. morhua*, a lean North Atlantic fish with a high proportion of PL, and *M. villosus*, which is a relatively fatty fish with a high proportion of TAG. *C. crispus* was first soaked in 22°C water prior to extraction to remove excess grit and salts. Samples were all 1 g in size. Samples were extracted according to their respective procedure and the lipids were quantified via Iatroscan TLC/FID; in this way, the proportions of specific lipid classes could be determined. The FA composition, detected as FAME derivatives by GC/FID, was also examined.

2.5.4.1 *Chondrus crispus*

2.5.4.1.1 Total lipids

Of all the extraction methods tested, the Bligh & Dyer posed the most serious problems. This procedure, which involves homogenizing the sample in a solvent system composed of 2:1 MeOH:$CHCl_3$ followed by the stepwise addition of $CHCl_3$ and water, posed a specific problem for the extraction of *C. crispus*. After the water is added, as a

washing step, the sample is further homogenized for 30 sec. However, when the sample was further homogenized, a very thick suspension formed, preventing the sample from being filtered. To alleviate this problem, many attempts were made at using different sample sizes as well as larger Buchner funnels, however this problem could not be overcome. It was not possible to recover any significant amount of the lipid-containing organic phase by using the original Bligh & Dyer procedure, thus it was concluded that this method, according to its originally published form, was not a suitable extraction method for algal samples. Modified forms of this procedure often skip the filtration step in favor of centrifugation, or filter the material prior to the addition of the water wash, however since these were procedures were modifications and diversions from the original protocol, they were not attempted. No further attempts were made to extract plant samples with this method.

The modified Folch extractions, previously discussed in some detail throughout this thesis, proved to be unsatisfactory for the direct comparison of extraction techniques presented in this study. The original notion was that results from previous examinations could easily be compiled within this investigation, however, as can be seen in Table 2.5, the results from earlier investigations performed in our lab were quite different from those in the current study, although they corresponded well with Randall extractions carried out at the same time.

Directly comparing the modified Folch results here could be misleading for two reasons. First, as stated, 1.0 g samples that were extracted using the modified Folch procedure were originally treated with boiling water, while for the other methods the

algae was treated with 22°C water (this was an oversight on my part). Although, as previously discussed, it is possible that samples treated with boiling water could extract more total lipid, previous studies showed that this was inconclusive and results were not statistically significant. Boiling water treatment did not appear to have a substantial effect on the extraction of *C. crispus*. Second, samples that previously extracted according to the modified Folch procedure were prepared from freshly gathered algae (not frozen), however for the other extraction procedures, algal samples had been frozen for 7 weeks at –20°C prior to preparation. It is possible that freezing could have an affect on lipid extraction, since freezing permanently disrupts the cell membranes due to osmotic shock (Christie, 1973). Ohman (1996) investigated the effect of freezing copepods and found no significant differences in total lipid yield after storage, however those samples were frozen immediately in liquid nitrogen, where in the present study, algal samples were stored, untreated, at –20°C prior to sample preparation. It has been documented that a storage temperature of –20°C is not great enough to prevent the action of lipases (Ohman, 1996), however since the aim of this investigation was to compare different extraction methods, degradation, although undesirable, was not a critical concern. It would have been interesting to examine the effects of freezing on the extraction efficiency of *C. crispus*, however due to time constraints this was not possible.

The Folch, Soxhlet and Randall methods were highly comparable, with the exception that the Folch procedure extracted significantly more AMPL than the other methods, however the relative proportions of all lipid classes were similar. It is likely that the large solvent to sample ratio is very effective for the removal of AMPL, thus the

Folch procedure appears to be very effective for the extraction of macrophytes. The Soxhlet extractions, showed a significantly higher level of HC, and it is most likely that this is from some kind of contamination; relatively high levels of HC were present in all samples extracted with the Soxhlet procedure, including the blanks. During extraction, the Soxhlet apparatus was flushed with N_2 continuously during the extraction procedure, N_2 was blown through the top of the condenser tube, from a glass pipet attached to rubber tubing. It is possible that the HC from the tubing managed to contaminate the samples although the tubing itself had no direct contact with the extracts.

2.5.4.1.2 Fatty acids

The FA profiles were very similar for the Randall, Folch and Soxhlet methods. FA data is not available for the modified Folch procedure. As expected, PUFA accounted for the majority of FAMEs (>46%), with 20:4ω6 and 20:5ω3 accounting for over 20% of the FA profile. The results have been included in appendix A.

Table 2.5. Lipid class content (mg g^{-1} wet weight) of *C. crispus* extracted using different methods. Samples were all 1 g. *Algae was stored at –20°C for 7 weeks prior to preparation and rinsed with 22°C water. ** Algae was treated with boiling water and prepared fresh. Values represent average ± SD, n=3. Values with different superscripts represent a significant difference between values, $p < 0.05$. ND: not detected.

	Randall*	Soxhlet*	Folch*	Randall**	Modified Folch**
Total Lipids (mg g^{-1} wet wt)	10.47 ± 0.53	10.87 ± 4.42	13.35 ± 2.06	17.89 ± 1.01	15.95 ± 2.33
Hydrocarbons	0.11 ± 0.04^{a}	0.41 ± 0.20^{b}	0.09 ± 0.03^{a}	0.07 ± 0.07	0.16 ± 0.08
Steryl Esters/Wax Esters	0.00 ± 0.01	0.01 ± 0.02	0.15 ± 0.15	ND	ND
Ethyl Esters	0.04 ± 0.07	0.02 ± 0.04	ND	ND	ND
Methyl Esters	0.04 ± 0.07	ND	ND	ND	ND
Methyl Ketones	0.03 ± 0.06	0.05 ± 0.07	ND	ND	ND
Triacylglycerols	2.06 ± 0.53	3.05 ± 2.57	2.69 ± 0.62	3.97 ± 0.19	3.45 ± 1.40
Free Fatty Acids	0.08 ± 0.03	0.29 ± 0.14	0.09 ± 0.11	0.10 ± 0.12	0.08 ± 0.15
Alcohols	0.08 ± 0.14	0.10 ± 0.09	ND	ND	
Sterols	1.05 ± 0.30	0.88 ± 0.28	0.88 ± 0.03	2.17 ± 0.04^{x}	1.53 ± 0.18^{y}
Diacylglycerols	0.20 ± 0.07	0.14 ± 0.15	0.22 ± 0.03	0.16 ± 0.09	0.12 ± 0.13
Acetone Mobile Polar Lipids	2.73 ± 0.81^{a}	3.11 ± 0.64^{ab}	5.69 ± 1.72^{b}	3.72 ± 0.34	3.54 ± 0.34
Phospholipids	4.05 ± 1.11	2.82 ± 0.60	3.55 ± 0.31	7.69 ± 1.09	7.06 ± 0.43

Table 2.6. Lipid class content (% wet weight) of *C. crispus* extracted using different methods. Samples were all 1 g. *Algae was stored at –20°C for 7 weeks prior to preparation and rinsed with 22°C water. ** Algae was treated with boiling water and prepared fresh. Values represent average ± SD, n=3. Values with different superscripts represent a significant difference between values, $p < 0.05$. ND: not detected

	Randall*	Soxhlet*	Folch*	Randall**	Modified Folch**
Total Lipids (% wet wt)	1.07 ± 0.04	1.09 ± 0.44	1.34 ± 0.21	1.79 ± 0.10	1.60 ± 0.23
Hydrocarbons	1.02 ± 0.40[a]	3.78 ± 1.60[b]	0.70 ± 0.33[a]	0.42 ± 0.37	1.01 ± 0.32
Steryl Esters/Wax Esters	0.04 ± 0.07	0.11 ± 0.20	1.24 ± 1.46	ND	ND
Ethyl Esters	0.35 ± 0.61	0.35 ± 0.60	ND	ND	ND
Methyl Esters	0.38 ± 0.66	ND	ND	ND	ND
Methyl Ketones	0.32 ± 0.56	0.32 ± 0.43	ND	ND	ND
Triacylglycerols	19.68 ± 5.03	24.92 ± 11.55	20.09 ± 2.45	22.24 ± 0.79	21.08 ± 5.89
Free Fatty Acids	0.74 ± 0.26	2.63 ± 1.02	0.76 ± 1.00	0.61 ± 0.72	0.62 ± 1.07
Alcohols	0.72 ± 1.25	0.83 ± 0.88	ND	ND	ND
Sterols	10.02 ± 2.99	8.54 ± 3.08	6.68 ± 1.18	12.15 ± 0.49	9.65 ± 0.64
Diacylglycerols	1.91 ± 0.67	1.14 ± 0.82	1.69 ± 0.34	0.87 ± 0.49	0.70 ± 0.70
Acetone Mobile Polar Lipids	25.90 ± 6.30[a]	30.09 ± 5.72[ab]	41.87 ± 7.05[b]	20.84 ± 2.35	22.32 ± 1.21
Phospholipids	38.92 ± 11.73[ab]	27.29 ± 4.96[ab]	26.96 ± 4.19[a]	42.88 ± 4.18	44.63 ± 3.92

2.5.4.2 *Gadus morhua*

2.5.4.2.1 Total lipids

G. morhua is a lean fish, characterized by a high proportion of PL in relation to TAG, as well by a high level of PUFA. According to all extraction methods, the main lipid classes extracted were PL, ST and FFA. The high proportion of FFA is likely due to enzymatic degradation, possibly due to prolonged storage prior to extraction. Although the tissue was stored at –80°C after the muscle samples were removed from the whole fish, the time between sampling and sample excision likely resulted in some degradation, resulting in an increase in FFA; samples from the same fish were originally extracted according to the modified Folch procedure by J. Wells approximately 18 months prior to the current study and those results also indicate a significant level of FFA (14.6 ± 2.4% total lipid compared to 8.3 ± 4.9% total lipid in the current study). The samples did not appear to undergo any further breakdown after storage for 18 months at –80°C.

The Bligh & Dyer method, originally developed for the extraction of cod flesh, extracted a significantly lower level of total lipid compared to the Folch, modified Folch and the Soxhlet method (Table 2.7), however the relative proportions of lipid extracted were quite similar for all extraction methods (Table 2.8).

Sample sizes were all 1 g, however when the samples were prepared they were stored in 5 mL of $CHCl_3$ in order to adequately cover the tissue. It has been reported that samples immersed in chloroform and stored under a blanket of nitrogen can be stored frozen for months without any significant changes in their lipid composition (Sasaki &

Capuzzo, 1984). In order to produce the required MeOH:$CHCl_3$:H_2O ratios of 2:1:0.8, including water present in the sample, excess water (>3 mL) had to be added to the extraction vessels prior to homogenization. Although the solvent ratios were correct, it is possible that this excess of water led to a decrease in the lipid extracted.

To verify this, the Bligh & Dyer procedure was repeated with 4 g samples of *G. morhua*, using the same initial solvent volumes of MeOH and $CHCl_3$ as with the 1 g samples. Very little water (<1 mL) had to be added to the samples to ensure the proper solvent ratios. In this instance, the total lipid extracted with the 4 g samples was significantly higher than with the 1 g samples (6.7 ± 0.26 mg g^{-1} wet weight at 4 g versus 4.40 ± 0.18 mg g^{-1} wet weight at 4 g; $p < 0.001$), however there were no significant differences in relative proportions of any of the major lipid classes extracted. Further, the results for the 4 g samples were consistent with the normalized amount extracted at 1 g for all of the other extraction methods. Possibly, exogenous water added to the extraction vessel leads to a water barrier between the solvent and the tissue. It seems important that the water required for the proper solvent ratios come from the sample, thus it appears that as little exogenous water as possible should be added to ensure effective extraction efficiency.

2.5.4.2.2 Fatty Acids

The major FA's (accounting for > 5% of total) were 16:0, 18:1ω9, 20:5ω3 and 22:6ω3, the latter accounting for ~ 30% of the total FA present. The FA profiles were evaluated and the results are presented in appendix A.

Although there was a significant underestimation in the total lipid content according to the Bligh & Dyer procedure, this did not appear to affect the FA profiles. There was significantly more total PUFA extracted via the Bligh & Dyer procedure than with Soxhlet ($p = 0.021$), indicating that the extracts may have somehow been altered during the long extraction procedure. This may account for the relatively higher levels of MUFA and SFA extracted with Soxhlet, although the differences were not statistically significantly different. In general, all extraction procedures extracted similar proportions of all major FA.

With the Randall method, the extracts are removed after each extracting step, concentrated and promptly frozen under N_2. During the Soxhlet procedure however, the extracts are heated during the entire extraction (6 hr); N_2 was gently blown through the top of the condenser of the Soxhlet extractor during the entire extraction procedure to avoid oxygen entering the extraction vessel.

Table 2.7. Lipid class content (mg g^{-1} wet weight) of *G. morhua,* caught from the coastal waters at Bonne Bay, NL in July 2003, extracted using 5 different methods. Samples were all 1g. Values represent average ± SD, (n=2, modified Folch and Soxhlet; n=3, Bligh & Dyer, Randall and Folch). Values with different superscripts represent a significant difference between values, $p < 0.05$. ND: not detected.

	Bligh & Dyer n=3	Modified Folch n=2	Randall n=3	Soxhlet n=2	Folch n=3
Total Lipid (mg g^{-1})	4.40 ± 0.18^{b}	7.07 ± 0.66^{a}	7.06 ± 1.46^{ab}	7.83 ± 0.47^{a}	6.19 ± 1.01^{a}
Hydrocarbons	0.02 ± 0.03	0.01 ± 0.02	0.10 ± 0.02	0.20 ± 0.18	0.15 ± 0.07
Steryl Esters/Wax Esters	ND	ND	0.04 ± 0.07	0.02 ± 0.03	0.08 ± 0.15
Ethyl Esters	ND	ND	0.06 ± 0.08	0.06 ± 0.02	ND
Methyl Esters	ND	0.10 ± 0.08	0.02 ± 0.04	ND	ND
Ketones	ND	0.17 ± 0.30	0.02 ± 0.03	0.10 ± 0.09	ND
Triacylglycerols	0.02 ± 0.04^{a}	0.27 ± 0.14^{b}	0.19 ± 0.18^{ab}	0.00 ± 0.00^{a}	0.05 ± 0.05^{a}
Free Fatty Acids	0.93 ± 0.14	0.67 ± 0.40	0.66 ± 0.56	0.80 ± 0.38	1.00 ± 0.17
Sterols	0.44 ± 0.04	0.57 ± 0.20	0.64 ± 0.29	0.63 ± 0.17	0.63 ± 0.23
Diacylglycerols	0.06 ± 0.02	0.04 ± 0.07	0.05 ± 0.03	0.16 ± 0.09	0.12 ± 0.03
Acetone Mobile Polar Lipids	0.09 ± 0.09	0.17 ± 0.10	0.48 ± 0.25	0.43 ± 0.51	0.25 ± 0.07
Phospholipids	2.84 ± 0.43^{b}	5.90 ± 1.01^{a}	4.78 ± 0.76^{ab}	5.44 ± 0.19^{a}	3.91 ± 1.13^{ab}

Table 2.8. Lipid class content (% wet weight) of *G. morhua,* caught from the coastal waters at Bonne Bay, NL in July 2003, extracted using 5 different methods. Samples were all 1g. Values represent average ± SD, (n=2, modified Folch and Soxhlet; n=3, Bligh & Dyer, Randall and Folch). Values with different superscripts represent a significant difference between values, $p < 0.05$. ND: not detected.

	Bligh & Dyer n=3	Modified Folch n=2	Randall n=3	Soxhlet n=2	Folch n=3
Total Lipids (% wet weight)	0.44 ± 0.02^{a}	0.79 ± 0.15^{b}	0.71 ± 0.15^{ab}	0.78 ± 0.05^{b}	0.62 ± 0.10^{ab}
Hydrocarbons	0.40 ± 0.70	0.13 ± 0.22	1.46 ± 0.29	2.44 ± 2.18	2.33 ± 0.78
Steryl Esters/Wax Esters	ND	ND	0.73 ± 1.26	0.32 ± 0.46	1.68 ± 2.91
Ethyl Esters	ND	ND	1.13 ± 1.56	0.73 ± 0.28	ND
Methyl Esters	ND	1.37 ± 1.22	0.43 ± 0.75	ND	ND
Ketones	ND	1.79 ± 3.11	0.20 ± 0.34	1.23 ± 1.12	ND
Triacylglycerols	0.59 ± 1.02^{a}	3.38 ± 1.56^{a}	3.23 ± 3.61^{ab}	0.00 ± 0.00^{b}	0.95 ± 0.97^{ab}
Free Fatty Acids	21.26 ± 3.87^{a}	8.26 ± 4.92^{b}	8.37 ± 6.99^{ab}	10.35 ± 5.47^{ab}	16.87 ± 6.10^{ab}
Sterols	10.08 ± 0.81	7.21 ± 2.23	8.89 ± 2.69	8.10 ± 2.65	9.96 ± 2.52
Diacylglycerols	1.37 ± 0.62	0.51 ± 0.88	0.72 ± 0.32	2.05 ± 1.06	1.90 ± 0.28
Acetone Mobile Polar Lipids	2.06 ± 2.07	2.29 ± 1.73	6.54 ± 2.61	5.28 ± 6.21	4.09 ± 0.87
Phospholipids	64.22 ± 7.56	75.06 ± 5.16	68.29 ± 3.94	69.51 ± 1.69	62.23 ± 9.57

The Randall method did not show any significant decrease in the amount of PUFA, thus it appears that boiling the extracts alone does not damage the integrity of the lipid extracts, even with long-chain PUFA.

2.5.4.3 *Mallotus villosus*

For all of the extraction methods, the precision of the results was quite low (SD >15%), which could be due to improper mincing/mixing of the samples during sample preparation; perhaps bits of skin could have been attached to the muscle tissue. Further, *M. villosus* is a very lipid rich fish containing a high proportion of the storage lipid TAG, which could be distributed unevenly throughout the flesh, thus any heterogeneity between samples could result in a large variation in the final lipid content.

In hindsight, to get around this variability, it would have been better to take subsamples from a larger homogenous sample. The difference in results between extraction methods is probably much less than differences encountered with species variability.

2.5.4.3.1 Total Lipids

For all of the extraction methods, there were virtually no significant differences in the lipid class composition, although these results could potentially be misleading due to large standard deviations. TAG accounted for between 60-72% of the total lipid composition, with Soxhlet extracting the most (28.1 ± 6.4 mg g^{-1} wet weight) and Bligh

and Dyer extracting the least (22.6 ± 7.2 mg g^{-1} wet weight). The proportion of PL was found to be highest using the Randall method, (~30% of total lipid) and lowest for the Bligh and Dyer procedure (~18% total lipid). The FFA content was quite low (> 4% total lipid).

As with *G. morhua*, samples from the same fish were extracted (by J. Wells) via the modified Folch procedure 18 months prior to this study, and the results from that study are very similar to those obtained in the present analysis. This corroborates well with the *G. morhua* results, indicating that samples stored at –80°C are not subject to significant breakdown or changes in their lipid composition.

Similarly to the Bligh & Dyer extractions of *G. morhua*, the extractions of *M. villosus* required >3 mL of water to be added to the sample prior to homogenization, and although the Bligh and Dyer procedure extracted less lipids, these differences were not statistically significant different. Total lipid data is presented in appendix A.

2.5.4.3.2 Fatty Acids

There was good agreement among the fatty acid profiles found in *M. villosus*. The major FA's (> 5%) were determined to be 16:0, 16:1ω7, 20:1ω9, 22:1ω9, 20:5ω3 and 22:6ω3. The data are presented in appendix A.

Overall, it appeared that the yield of PUFA in the Bligh & Dyer and Folch extractions was less than the other three methods, corresponding to a higher proportion of MUFA. However, these results are not statistically significant and ANOVA results revealed all FA were extracted in the same proportion.

CHAPTER 3

SHORT COLUMN GAS CHROMATOGRAPHIC PROFILING OF LIPIDS IN MACROPHYTES AND INDIVIDUAL FISH LARVAE

3.1 Introduction

Lipid profiling via GC was initially investigated by A. Kuksis and co-workers over 30 years ago in blood plasma samples using packed columns (Kuksis et al., 1967). The application to neutral lipids in cold-water marine samples was explored by Yang et al. (1996), who developed a rapid method for the separation of neutral lipids based on their polarity and carbon number using a short (5.5 m) analytical column. Recently, this method was further expanded to include the analysis of phospholipids (PL), achieved via enzymatic dephosphorylation. In this method, the polar head group is hydrolyzed, producing diacylglycerol (DAG; Kehoe, 2003), which can be readily analyzed with GC/FID.

Short-column GC/FID is advantageous over Iatroscan TLC/FID for a number of reasons: individual lipid components are separated according to their carbon number instead of being pooled in their individual class, it is more sensitive and precise and has

lower detection limits than Iatroscan. Furthermore, it is easy to use, relatively cheap to run, reproducible and readily automated.

The analysis of crude lipid extracts containing a high level of acetone mobile polar lipids (AMPL) is generally not feasible due to, as the name implies, the relatively polar nature of these molecules. This category of molecules is a complex mixture of pigments, glycolipids and monoacylglycerols (MAG), and most species within this group are not amenable to routine lipid profiling via GC, with the exception of the latter. For example, analysis of macrophytes, which contain a large AMPL fraction, via short column GC/FID has not been achieved due to the polar nature of the headgroups of these compounds, resulting in low volatility as well as low affinity for the relatively non-polar stationary phase of the DB-5 analytical column. The result of running samples containing a large AMPL fraction is the likely build up on these species on the analytical column.

Enzymatic digestion of the major species in the AMPL fraction such as chlorophyll-*a* and the glycosyldiacylglycerols was originally postulated to be a route for analyzing this class of molecules via GC. Enzymes such as β-galactosidase and chlorophyllase could be used in a similar manner to the phospholipases, however these procedures would involve very long and costly preparatory time and it was unlikely that the enzymes could be used simultaneously, rather the digestions would have to occur sequentially involving purification between each step. As well, chlorophyllase, which cleaves chlorophyll into phytol and the magnesium-containing chlorophyllide, is not commercially available, thus it would have to be carefully purified prior to digestion.

The addition of a guard column, or retention gap, to the analytical column was explored as a solution to the AMPL problem. A guard column would protect the analytical column and allow for the routine analysis of crude plant extracts. However, a longer column would affect factors such as retention time, flow rates as well as weight correction factors, which are required for accurate quantitative GC analysis. These factors were explored in the current study.

The current research, which not only explored the feasibility of GC/FID for the detection of lipids in macrophytes, was further expanded for the analysis of neutral lipids, namely triacylglycerols (TAG) and sterols (ST), in very small samples such as individual fish larvae.

3.2 Methods for Lipid Analysis and Detection

3.2.1 Thin Layer Chromatography with Flame Ionization Detection

Thin layer chromatography with flame ionization detection (TLC/FID) is an analytical method commonly used for the separation and quantification of individual lipid classes. Please refer to Section 2.3.1 for a more detailed description of this method.

3.2.2 Supercritical Fluid Chromatography (SFC)

This separation method is useful for separating lipid classes and has the advantage of rapid processing time, minimal use of harmful solvents and it is very practical for temperature-sensitive compounds (Christie et al., 1998). It is compatible with many types of detectors such as MS, electron capture (ECD) and most commonly, FID (although

caution must be used when choosing an appropriate modifier, as not all are compatible with this type of detector (McDonald & Mossoba, 1997).

3.2.3 High-Performance Liquid Chromatography (HPLC)

This method is suitable for temperature sensitive compounds and for the analysis of high molecular weight lipids such as PL and conjugated lipids (Shulka, 1988). This method separates lipid classes by using solvents of varying polarities run through a solid phase column at high pressures, as analytes are eluted based on their affinity for the mobile phase. HPLC, coupled with evaporative light-scattering detection has been used to separate total plant lipid extracts (Christie et al., 1998).

3.2.4 Nuclear Magnetic Resonance (NMR)

Low-field NMR is an accurate, rapid and solvent–free method for detecting total lipid (Toussaint et al., 2001). One experiment that compared lipids detected via NMR to and those detected after Soxhlet extraction (in petroleum ether at 155°C) found a good correlation between the two techniques ($R^2 = 0.98$, std error 3 g kg^{-1}; Toussaint et al., 2001). This method is suitable for detecting the more simple amorphous lipids such as TAG, although the more polar lipids such as PL can potentially cause erroneous signals. One major disadvantage of using NMR is due the fact that samples must be completely dried before analysis, due to the interference in the signal given off by any water (Toussaint et al., 2001).

3.2.5 Short Column Gas Chromatography

This profiling method groups compounds in each class according to their carbon number (Parrish et al., 2000). This method is advantageous because it can be applied to a wide range of marine samples, is readily automated and has high sensitivity (Yang et al., 1996). Further, GC is relatively cheap to run, and has rapid analysis times. The procedure has been developed for the profiling of neutral lipids (Yang et al., 1996), and has recently been optimised to include PL (Kehoe, 2003).

3.3 Experimental

3.3.1 Glassware and Chemicals

All solvents used were of analytical or chromatographic grade. Standards used for calibration and verification, prepared from chromatographically purified materials, were supplied by Sigma (St. Louis, MO, U.S.A.). For further details, please refer to section 2.4.1.

3.3.2 Marine Samples and Sample Storage

Larval samples of *Cyclopterus lumpus* (lumpfish) and *Myoxocephalus scorpius* (shorthorn sculpin) were cultured at the Ocean Sciences Centre, Memorial University of Newfoundland. Fresh samples, weighing less than 10 mg wet weight, were filtered, weighed, placed in 1 mL of $CHCl_3$ and frozen at $-20^{\circ}C$ until extraction.

Algal samples were collected in September 2003 and prepared as described in section 2.4.3.

3.3.3 Total System Blanks

Procedural blanks, which involved all manipulations used for experimental samples, were carried out and background subtracted where appropriate.

3.3.4 Extractions

Larval samples were extracted according to the modified Folch procedure, as described in section 2.4.6.2.

3.3.5 Phospholipid Digestion

Phospholipids were dephosphorylated according to the procedure developed by Kehoe (2003). In a lipid cleaned 25 mL test tube, 4 mL Tris buffer (pH 7.3), 1.3 mL of 1% $CaCl_2$ and 50 units (or less) of phospholipase-C (PL-C) were added and the mixture was mixed well. One unit of protein, purchased as a lyophilized powder (Sigma, St. Louis, U.S.A.) is defined as the amount of enzyme that will liberate 1.0 μmole of water soluble organic phosphate from L-α-phosphatidylcholine per min. at 37°C. To the mixture, 2 mL of diethyl ether and add 0.5 mL sample (or less) were added and the mixture was vortexed and sonicated at 37°C for 2 hr. To stop the enzymatic digestion, 5

drops of 0.1N HCl was added and the mixture vortexed. The solution was centrifuged for 2 min. at 1000 rpm, and the upper organic layer was placed in a 10 mL vial.

3.3.6 Hydrogenation

Enzyme-digested extracts were hydrogenated using platinum as the catalyst. Approximately 5 mg of PtO_2 was added to a 15 mL vial containing about 5 mL of lipid extract (containing ~3 mg total lipid suspended in $CHCl_3$) and a stir bar. The solution was bubbled with a gently stream of H_2 for 20 min., and the vial was capped and stirred for 2 hr. To remove the catalyst, the solution was vacuum-filtered through a GF/F filter and the filtrate was transferred to a clean 15 mL vial.

3.3.7 Derivatization - Trimethylsilylation

Aliquots from total lipid extracts were evaporated to dryness under nitrogen and the lipids were dissolved in ~2 drops of N,O-bis(trimethylsilyl)-acetamide (BSA) and ~2 drops of N,O-bis(trimethylysilyl)-trifluoroacetamide (BTSFA). The derivatives were flushed with N_2, capped and placed at 85°C for 15 min. Excess reagents were removed by evaporation with N_2 and the extracts were re-suspended in $CHCl_3$ and analysed via GC/FID. In another TMS derivatization procedure, aliquots from total lipid extracts were evaporated to dryness under N_2 and the lipids were dissolved in ~ 4 drops of BTSFA. The derivatives were flushed with N_2, capped and placed at 85°C for 15 min. Excess reagents

were removed by evaporation with N_2 and the extracts were re-suspended in $CHCl_3$ and analysed via GC/FID.

3.3.8 Chromatographic Methods

3.3.8.1 Column Chromatography

Column chromatography was used for the isolation of neutral lipid fractions. Pasteur pipettes were plugged with a small amount of glass wool and heated in a muffle furnace for over 4 hr at 450°C. Approximately 2 g of Florisil was added to the columns, which was then activated by heating it to 110°C for at least 30 min. The columns were then cooled in a desiccator for 30 min. prior to chromatography. The activated Florisil was washed with 3 mL of MeOH followed by 3 mL $CHCl_3$, after which the sample was added and the 2 mL vial containing the extract was rinsed three times with a small amount of $CHCl_3$. The neutral lipids were eluted with 5 mL of a solvent mixture composed of $CHCl_3$:MeOH:HCOOH (99:1:1) and collected in a 15 mL vial.

3.3.8.2 Short Column GC/FID

Lipid profiles were analysed with a Hewlett Packard 6890 Plus gas chromatograph equipped with a HP 7863 automated injector and a flame ionization detector (FID), using cool on-column injection. The stationary phase of the 5.5 m DB-5 fused silica analytical column (0.32 mm ID, 0.25 μm film thickness) was coated with cross-linked 5% phenylmethyl-silicone (Supelco, Bellefonte, U.S.A), and was attached

via a butt connector to an intermediate polarity guard column of the same diameter. The injector was programmed to rise from an initial injection temperature of 61°C to 360°C, where it was held for the rest of the run. The oven temperature program was set to rise to 115°C from an initial temperature of 60°C at a rate of 4°C min^{-1}, then from 115°C to 225°C at 25°C min^{-1} to 280°C at 15°C $min.^{-1}$, then to a final temperature of 340°C at 5°C min^{-1} where it was held for 10.58 min. The FID temperature was 345°C. The flow rates of the detector gases were 30 mL $min.^{-1}$ (hydrogen) and 300 mL min^{-1} for air. The carrier gas, He, was set to a constant flow rate of 5.8 mL min^{-1}.

The above-mentioned GC operating parameters were optimized by Yang et al. (1996), however some minor modifications were made in the current study. The addition of a guard column required the adjustment of carrier gas flow, which was altered from an original program of 10 psi column head pressure to a constant flow of 5.8 mL min^{-1}.

Lipid samples were freshly TMS derivatized prior to analysis and 1 µL aliquots were injected directly onto the column, either automatically or manually, and the entire run lasted 32 min.

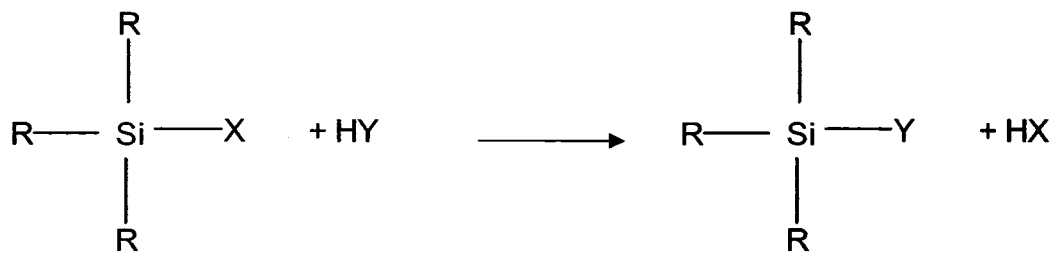
3.3.8.3 Thin Layer Chromatography with Flame Ionization Detection

Crude total lipid extracts were separated into individual lipid classes based on their relative polarities using an Iatroscan TLC/FID procedure developed by Parrish et al. (1987). For further details on this procedure, please refer to section 2.4.9.1.

3.4 Results and Discussion

3.4.1 TMS Derivatization

Prior to gas chromatographic analysis, lipid species with active hydrogen functions, such as those containing free hydroxyl and free carboxyl functional groups, must be derivatized in order to increase their molecular thermal stability and volatility; examples of these species include free fatty acids (FFA), sterols (ST) and diacylglycerols (DAG). Derivatization is commonly achieved via a silylation reaction (Poole, 1978):



R = alkyl or halocarbon

There are many derivatization reagents available; the method currently used in our lab for the derivatization of marine samples involves the addition of an equal volume of N,O-bis(trimethylsilyl)-acetamide (BSA) and N,O-bis(trimethylysilyl)-trifluoroacetamide (BTSFA). Both of the reagents are considered to have strong silyl donor ability, however, the combustion products of the two reagents differ; BSA can oxidize to form silicon dioxide, which can foul the FID detector, however the combustion

product of BTSFA is silicon tetrafluoride, which does not foul the detector. Further, BSTFA by-products are generally more volatile than those for BSA, thus there is less likeliness of interference from impurities in the GC chromatogram, which can pose a particular problem with BSA (Poole, 1978).

An investigation was undertaken to see if BSTFA alone could sufficiently derivatize the lipid samples. A synthetic 14 component standard, containing approximately 100 ng each of all representative neutral lipid classes (hydrocarbon (HC), ketone (KET), FFA, ST, DAG, wax ester (WE), steryl ester (SE) and TAG), was derivatized with either 4 drops BSA, 4 drops BTSFA or 2 drops BSA and 2 drops BTSFA. Samples were evaporated to dryness prior to adding the derivatizing reagents, flushed with N_2, sealed and placed at 85°C for 15 min. Samples were all run in triplicate.

By visual inspection, it was observed that the all three of the chromatograms looked nearly identical, although there appeared to be less noise in the lower MW end of the chromatogram when using only BTSFA (Figure 3.1). It was concluded that BTSFA alone should be used for all subsequent derivatizations since there was less interference from contaminating species in the lower MW region of the chromatogram, which could be mistaken for, or overlap with, analyte peaks. As well, the frequency of FID maintenance, which was routinely carried out, could be reduced due to fewer deposits from contaminating species and combustion products, which was visibly seen as a white residue on the detector.

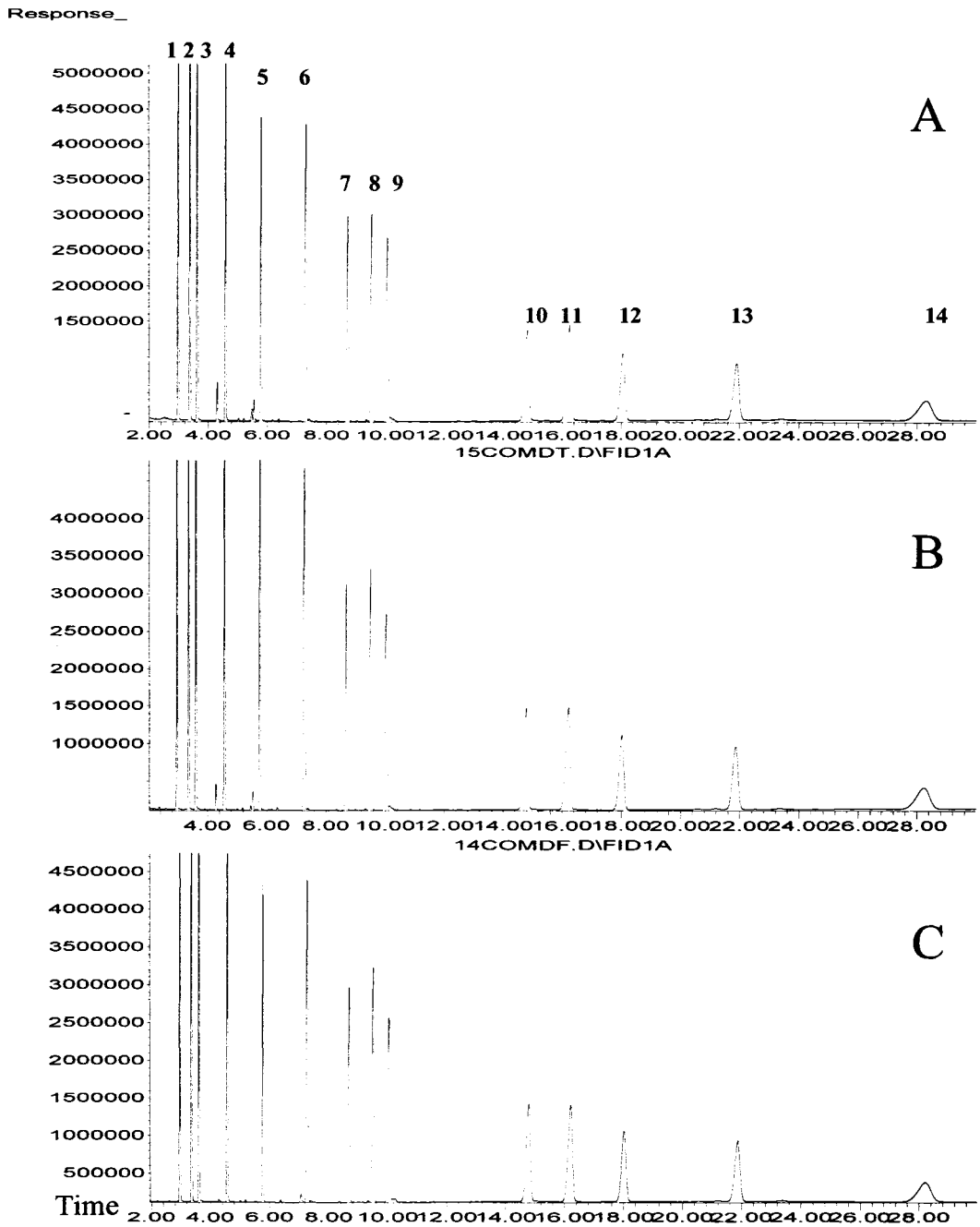


Figure 3.1. The effect of TMS derivatization with (A) BSA/BSTFA, (B) BSA and (C) BSTFA as the derivatizing agents, on a 14 component synthetic standard solution. 1-C16 KET; 2-C19 HC; 3- C16 ALC; 4- C18 FFA; 6- C22 FFA; 7- C27 ST; 8- C36 WE; 9- 36:0 DAG; 10- C43 SE; 11- C45 SE; 12- 48:0 TAG; 13- 54:0 TAG; 14- 60:0 TAG.

The derivatization temperature for BSTFA was also investigated at both 70°C and 85°C, and it was found that there was no difference in the derivatization efficiency, after 15 min., at either temperature.

3.4.2 Guard Column

As previously mentioned, acetone mobile polar lipids, found in particularly high levels in plant tissues, are not readily amenable to GC analysis due to their complexity and relatively polar nature. In marine micro- and macroalgae, the presence of AMPL can contribute a significant amount to the total lipid content, mostly due to the presence of pigments, in particular chlorophyll-*a*.

It was postulated that a guard column could solve the problem by "trapping" the polar AMPL molecules prior to any interaction with the active sites of the analytical column. Thus, the parts of the guard column with visible residue on it could be routinely cut as required, while preserving the integrity and lifetime of the analytical column.

In order to test whether a guard column could allow for the analysis of samples containing AMPL, crude *C. crispus* lipid extracts were dephosphorylated, hydrogenated, derivatized, and run repeatedly on the short column GC. Figure 3.2 reveals that the same sample, after the 1st, 20th and 40th run, shows no sign of any differences in the retention times between the spectra. Hexane was run as a blank every 5 samples, however there was no sign of carryover. After the 41st injection, the column was visually inspected, and it could be seen that there was a faint dark band ~10 cm from the injector end, which is

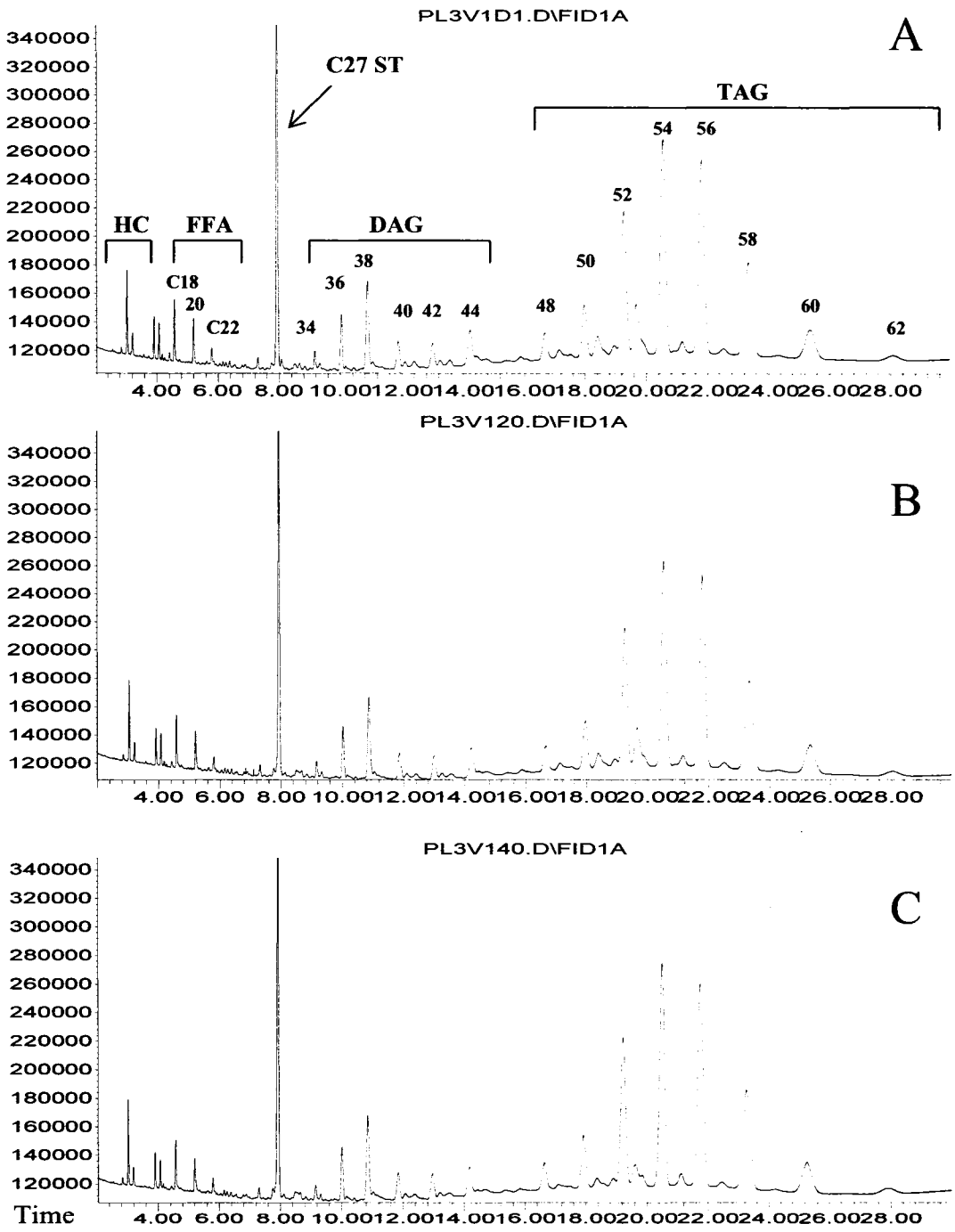


Figure 3.2. Chromatograms of dephosphorylated, hydrogenated, and derivatized extracts of *C. crispus*, after the (A) 1st, (B) 20th and (C) 40th injection. For FFA and ST, numbers represent carbon number; for TAG and DAG, numbers represent acyl carbon number.

likely the result of build-up from non-volatile AMPL species. When necessary, this portion could be removed, thus preserving the longevity of the analytical column.

A pure glycolipid standard, digalactosyldiacylglycerol, which contains two galactose residues connected to a diacylglycerol backbone, was TMS derivatized and injected onto the GC column, however the chromatograms revealed no trace of analyte; it is likely that this molecule is much too large due to the bulky sugar residues, thus derivatization would not be possible due to stearic hindrance. Pure chlorophyll-*a* was also derivatized and injected onto the GC, however there was no trace of analyte in the chromatogram. These compounds are probably not volatile enough for GC analysis.

Although GC is not a viable method for the detection of most individual AMPL components, with the exception of MAG, a guard column permits for the analysis of the other neutral components found in plant samples. As an example, extracts of *C. crispus* were extracted by either the modified Folch procedure or Randall method, and were subsequently dephosphorylated, hydrogenated, derivatized and run on the short column GC, containing a 1 m guard column. As Figure 3.3 reveals, not only does the GC trace reveal that the lipid classes were extracted in the same proportion, it also reveals detailed insight into the compounds present.

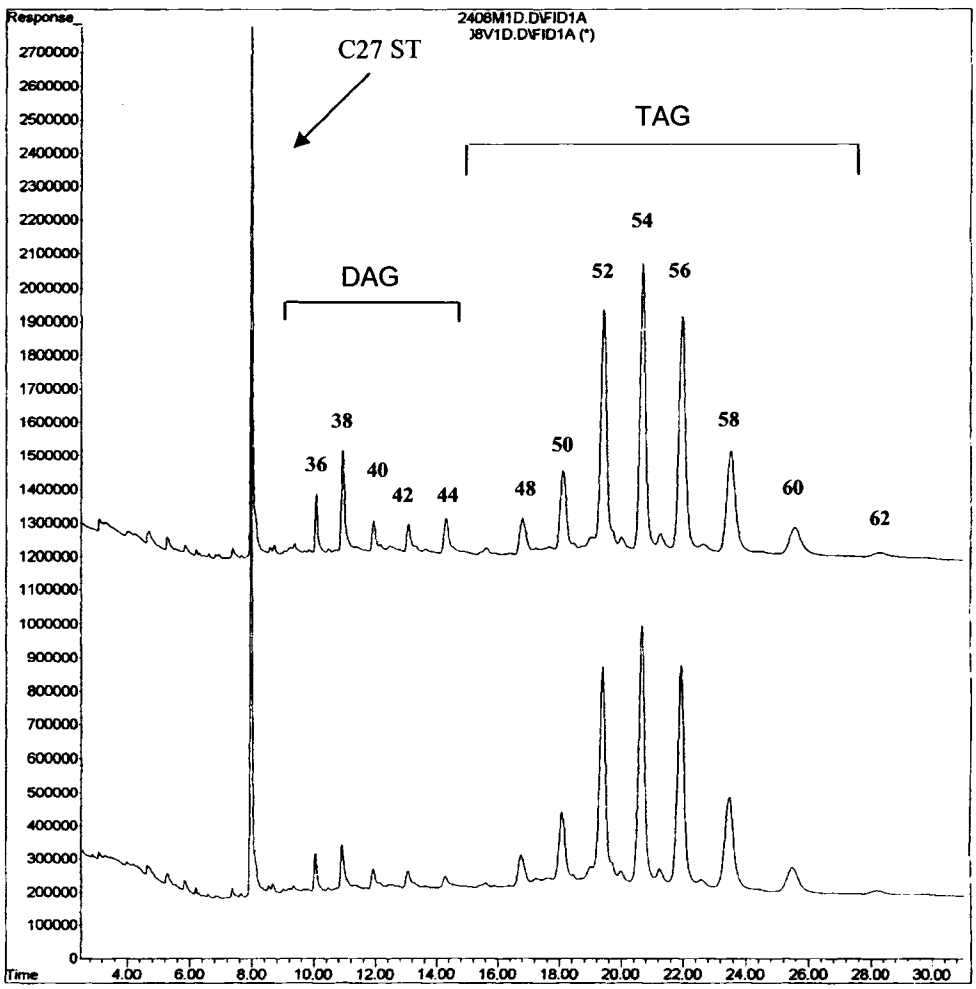


Figure 3.3. Comparison of GC chromatograms for *C. crispus*, dephosphorylated, hydrogenated and derivatized, extracted via the (A) Randall method and (B) the modified Folch procedure. For DAG and TAG, numbers represent acyl carbon numbers

3.4.3 Quantitative Analysis of Lipid Components

The response of the FID can vary according to the molecular species present, thus weight correction factors (f_w) must be used for accurate quantitative determination of lipids using short column GC. All f_w were determined using Equation 1 (Kuksis, 1975):

$$f_w = (A_{IS}/A_X)*(M_X/M_{IS}) \quad (1)$$

A_{IS} and A_X are the peak areas of the internal standard and analyte, respectively, and M_X in the mass of analyte and M_{IS} is the mass of internal standard. Tridecanoin, a short-chain triacylglycerol (30:0 TAG), was chosen as a suitable internal standard because it is unlikely for this species to be found in marine samples, and its retention time does not interfere with other lipid components. As well, it can be assumed that the recovery of tridecanoin on the GC column is 100% complete, thus losses of other higher molecular weight compounds such as long-chain TAG can be measured relative to this species (Christie, 1973).

The f_w, under the same conditions, should be highly reproducible, however factors such as column length and age, carrier gas flow rate and sample load can all have an effect on the precision and accuracy of f_w over a given concentration range of analyte (Mares et al., 1978). For this reason, the f_w should be routinely checked due to day-to-day variations in the GC system.

An investigation was undertaken to see how the f_w varies according to column length for selected neutral lipids. For each trial, the analytical column was 5.5 m and the carrier gas flow rate was kept at a constant 5.8 mL $min.^{-1}$, but the guard column length varied between 0.7, 1.0 and 2 m. The f_w was also compared between a new analytical column attached to a 1 m guard column and an older analytical column (~300 samples already run on it), also attached to a 1 m guard column.

A 14 component standard solution, containing HC, KET, FFA, ST, DAG, WE, SE and TAG, was prepared and the f_w for each species were determined at various concentrations ranging from 0.1 – 500 ng μL^{-1}. Solutions of various concentrations were made from serial dilutions of a 1 mg mL^{-1} stock standard solution. It was assumed that the internal standard, 30:0 TAG, was fully recovered under all conditions.

The f_w at higher concentrations (500 ng per species per injection) did not change considerably regardless of the column length or age. This is consistent with the literature (Mares et al., 1978); the f_w should remain fairly constant with large sample loads. The coefficient of variation (C.V.) for all 14 components, when compared under all conditions, was less than 4% with the exception of behenic acid (C22 FFA; C.V. = 8.8%) and 60:0 TAG (C.V. = 9.3%).

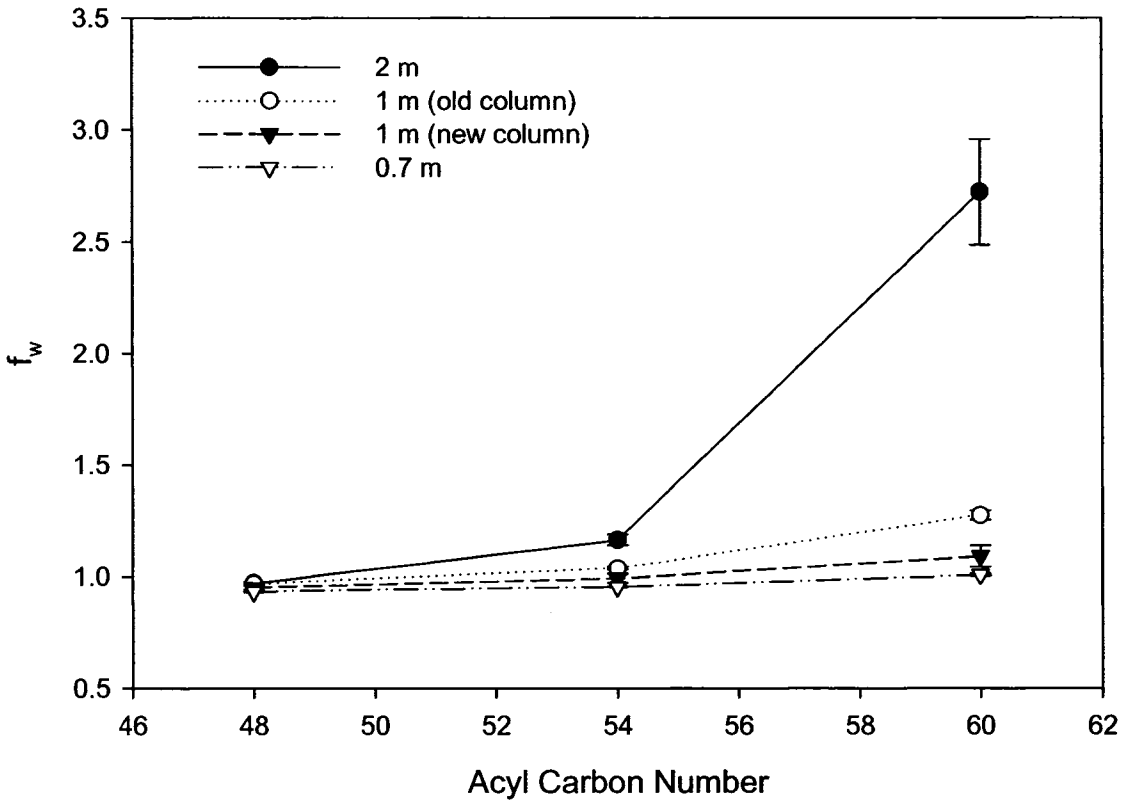


Figure 3.4. The dependence of f_w on carbon number. 100 ng of 48:0 TAG, 54:0 TAG and 60:0 TAG were injected in triplicate on a 5.5 m analytical column with a variable guard column length. Error bars represent SD, n=3

At lower concentrations, however, larger fluctuations in the f_w were found, particularly in the higher M.W. TAG. Large molecular weight compounds such as tristearin (54:0 TAG) and triarachadin (60:0 TAG), which require very high temperatures to be detected (~340°C), are prone to breakdown due to pyrolysis or reaction with the column's stationary phase (Christie, 1973), thus variation in the f_w is to be expected. At 0.1 ng, 60:0 TAG was not detectable under any conditions. Similarly, f_w for 48:0, 54:0 and 60:0 TAG were dependent on the column length, as can be seen in Figure 3.4. The age of the column also appeared to have an effect on the f_w, which increased significantly at small sample loads (≤10 ng). For instance, 60:0 TAG, which was not detectable with the older column at 1 ng, was clearly visible with the newer column at the sample amount.

It was concluded that a maximum guard column length of 0.7 m was ideal for routine quantitative analysis. This length provided detection limits in the range of 0.1 – 500 ng, with the exception of very large M.W. species such as 60:0 TAG, which was only detectable at ≥1 ng. As can be seen in Figure 3.5, with a 0.7 m column, the f_w for 60:0 TAG is linear between 1- 500 ng (C.V. = 1.7%), although this linearity is lost with longer column lengths. Further, the f_w remained relatively constant for large M.W. TAG with 48 – 60 acyl carbons. Table 3.1 summarizes the f_w values with a 0.7 m column, while Table 3.2 summarizes the linearity of f_w among different concentration ranges.

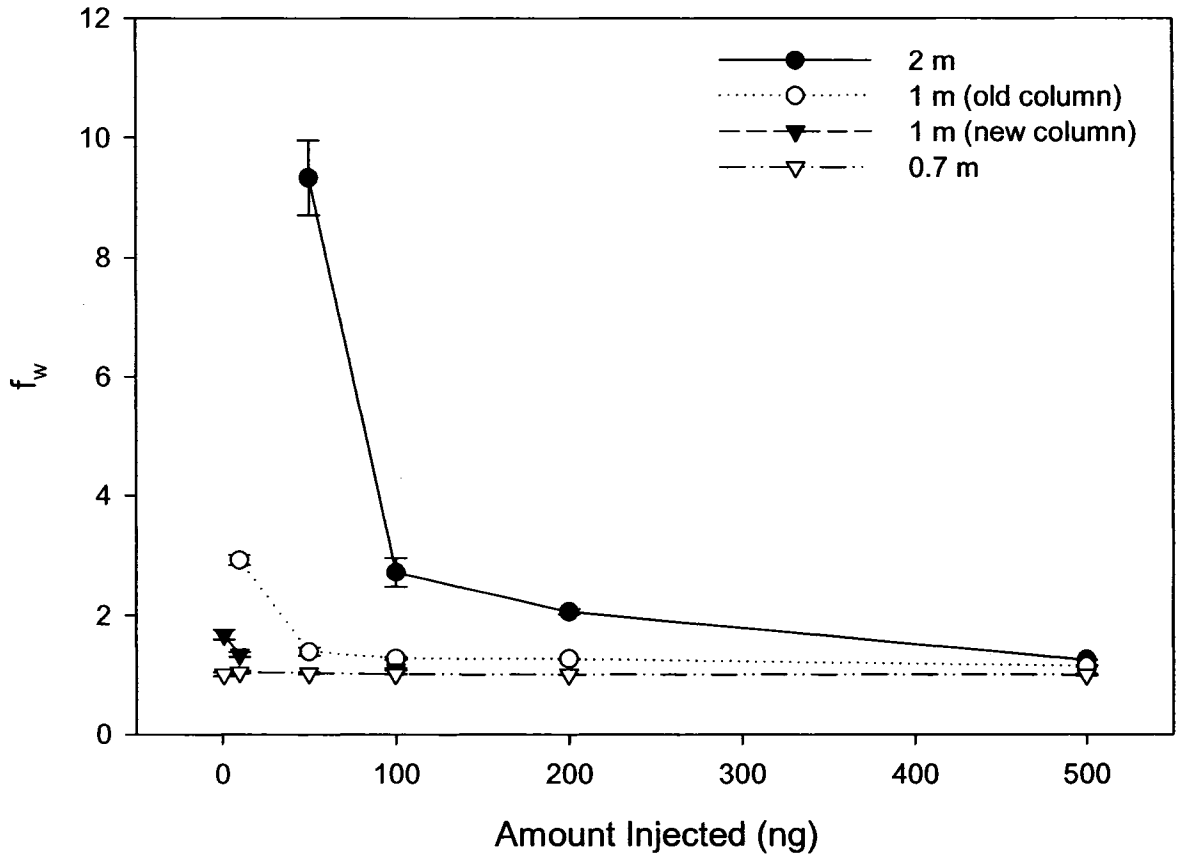


Figure 3.5. The dependence of f_w on the amount of analyte injected, with different guard column lengths. 60:0 TAG was injected at 5 different concentrations between 1-500 ng. Error bars represent SD, n=3

Table 3.1. The f_w of various different lipid classes between 0.1-500 ng with a 5.5 m analytical column equipped with a 0.7 m medium polarity guard column. For operating parameters, see text. Values represent mean ± SD, n =4. [a]60:0 TAG was not detectable at 0.1 ng. [1]Summed carbon numbers are used. [2]Acyl carbon numbers are used.

Lipid Class	Amount (ng)				
	500	**100**	**10**	**1**	**0.1**
C16 KET[1]	0.964 ± 0.008	1.005 ± 0.003	1.120 ± 0.017	1.286 ± 0.165	1.796 ± 0.146
C19 HC[1]	0.678 ± 0.004	0.693 ± 0.002	0.715 ± 0.010	1.354 ± 0.031	1.147 ± 0.064
C16 ALC[1]	0.716 ± 0.005	0.725 ± 0.002	0.722 ± 0.010	0.823 ± 0.012	0.860 ± 0.020
C18 FFA[1]	0.772 ± 0.004	0.777 ± 0.001	0.797 ± 0.011	0.992 ± 0.017	0.909 ± 0.095
C22 FFA[1]	0.773 ± 0.003	0.785 ± 0.001	0.801 ± 0.007	1.037 ± 0.014	0.972 ± 0.092
C27 ST[1]	0.729 ± 0.001	0.729 ± 0.001	0.709 ± 0.005	0.713 ± 0.005	0.742 ± 0.024
C36 WE[1]	0.869 ± 0.002	0.873 ± 0.002	0.876 ± 0.002	0.870 ± 0.008	0.842 ± 0.045
32:0 DAG[2]	0.823 ± 0.000	0.824 ± 0.002	0.824 ± 0.003	0.824 ± 0.012	0.781 ± 0.054
C43 SE[1]	0.799 ± 0.005	0.799 ± 0.004	0.805 ± 0.007	0.797 ± 0.021	0.584 ± 0.078
C45 SE[1]	0.819 ± 0.004	0.820 ± 0.002	0.823 ± 0.009	0.830 ± 0.006	0.840 ± 0.258
48:0 TAG[2]	0.929 ± 0.002	0.934 ± 0.003	0.945 ± 0.009	0.918 ± 0.015	0.743 ± 0.250
54:0 TAG[2]	0.967 ± 0.028	0.953 ± 0.002	0.967 ± 0.006	0.932 ± 0.027	0.796 ± 0.107
60:0 TAG[2]	0.997 ± 0.013	1.007 ± 0.005	1.044 ± 0.023	1.008 ± 0.037	ND[a]

Table 3.2. Linearity of f_w among different concentration ranges, with a 5.5 m analytical column equipped with a 0.7 m medium polarity guard column. For operating parameters, see text. Values represent mean ± SD, n =4. [a]60:0 TAG was not detectable at 0.1 ng. [1]Summed carbon numbers are used. [2]Acyl carbon numbers are used.

Lipid Class	Average f_x (0.1-500 ng)	C.V.(%)	(1-500 ng)	C.V. (%)	(10-500 ng)	C.V. (%)
C16 KET[1]	1.234 ± 0.338	27.4	1.094 ± 0.144	13.2	1.030 ± 0.081	7.84
C19 HC[1]	0.918 ± 0.313	34.1	0.860 ± 0.330	38.3	0.696 ± 0.019	2.71
C16 ALC[1]	0.769 ± 0.068	8.79	0.746 ± 0.051	6.89	0.721 ± 0.005	0.68
C18 FFA[1]	0.849 ± 0.097	11.4	0.834 ± 0.105	12.6	0.782 ± 0.013	1.70
C22 FFA[1]	0.874 ± 0.122	14.0	0.849 ± 0.126	14.8	0.786 ± 0.014	1.78
C27 ST[1]	0.725 ± 0.013	1.83	0.720 ± 0.010	1.44	0.722 ± 0.011	1.57
C36 WE[1]	0.866 ± 0.014	1.60	0.872 ± 0.003	0.34	0.873 ± 0.003	0.39
32:0 DAG[2]	0.815 ± 0.019	2.32	0.823 ± 0.001	0.07	0.823 ± 0.001	0.08
C43 SE[1]	0.757 ± 0.097	12.8	0.800 ± 0.003	0.42	0.801 ± 0.003	0.43
C45 SE[1]	0.826 ± 0.009	1.08	0.823 ± 0.005	0.61	0.821 ± 0.002	0.23
48:0 TAG[2]	0.894 ± 0.085	9.47	0.931 ± 0.011	1.21	0.936 ± 0.008	0.86
54:0 TAG[2]	0.923 ± 0.073	7.87	0.955 ± 0.017	1.74	0.963 ± 0.008	0.84
60:0 TAG[2]	ND[a]		1.014 ± 0.020	2.01	1.016 ± 0.025	2.41

The linear range for all analytes, as determined by regression analysis, was between 0.1-500 ng ($R^2 > 0.99$), except for 60:0 TAG, which was 1-500 ng ($R^2 > 0.99$). Analyte concentrations less than 0.1 ng μL^{-1} or more than 500 ng μL^{-1} were not analyzed; however it is likely that some species would be detected at lower amounts. Amounts over 500 ng were not injected to prevent column overloading and peak tailing.

3.5 Neutral Lipid Profiling of Individual Fish Larvae

The TAG composition in individual fish larvae can give a measure of larval nutritional condition (Fraser, 1989), which can be beneficial in rearing studies, especially with increased interest in aquaculture. TAG, a storage lipid, is shown to decrease during times of stress and starvation; since exogenous sources of energy cannot be found, energy reserves are used up endogenously. The use of a TAG to ST ratio was proposed as a condition index to eliminate the size dependence of TAG, since larger larvae generally have a higher absolute amount of this lipid class (Fraser et al., 1998). ST, which is most commonly associated with cell membranes, could be used as a proxy for body size since it can be correlated with the wet weight of many eukaryotes (Nes, 1974).

The most common method for the detection of total lipids in individual larva is via Iatroscan TLC/FID (Lochmann et al., 1995; Harding & Fraser, 1999), however due to the very small size of these species, large quantities of the extract must often be applied to the silica rods in order to get an adequate response. GC/FID provides a useful alternative over TLC/FID due to its inherently lower detection limit. Further, additional information

concerning the presence of individual lipid profiles can be readily obtained via GC/FID. A comparison of Iatroscan TLC/FID and GC/FID chromatograms, showing the lipid content in an individual *M. scorpius* larva, is presented in Figure 3.6.

The detection limit of Iatroscan TLC/FID is approximately 50 ng (Parrish et al., 2000), and the detection limit for most species using GC/FID, in the present study, is ~ 0.1 ng, except for very large M.W. 60:0 TAG, which is ~1 ng. Although some species could be detected at lower amounts (as low as 0.01 ng by Yang et al., 1996), these levels are out of linear range and are not useful for practical quantitative analysis.

A comparative study was undertaken to compare GC to Iatroscan for the detection of TAG and ST in individual fish larvae. An outline of the procedure for comparing the two methods is presented in Figure 3.7. All samples were extracted using the modified Folch procedure, however reports in the literature have also suggested a passive method for the extraction of lipids in very small samples using a combination of dichloromethane and methanol (2:1 v/v; Lochmann et al., 1995). Due to time constraints, this method was not attempted.

The total volume of the crude lipid extract was brought to 10 mL in a volumetric flask. A 1.00 mL aliquot was removed and transferred to a 2 mL vial and the remaining 9 mL of extract was evaporated to near dryness and run on the Iatroscan without any further treatment. To the 1 mL aliquot, approximately 5 μg of surrogate standard (tridecanoin) was added and the extract was run through a Florisil column and the neutral lipids were isolated. The neutral lipids were TMS-derivatized and concentrated down to

<100 μL. The extracts were subsequently transferred to a 200 μL glass insert and then 1 μL was injected onto the GC.

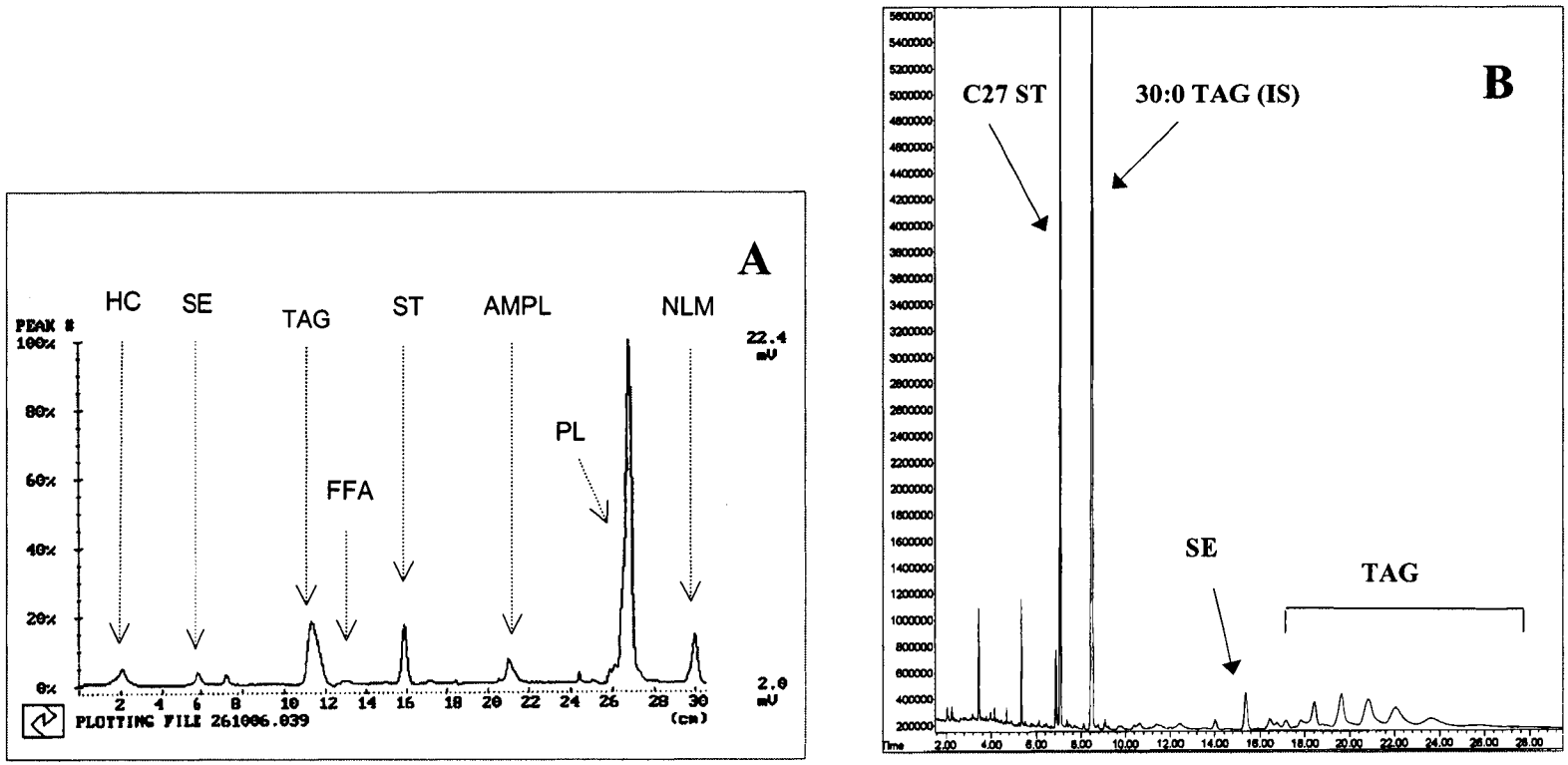


Figure 3.6. Comparison between (A) Iatroscan TLC/FID and (B) short column GC/FID chromatograms for the determination of neutral lipids from an individual sculpin *(Myoxocephalus scorpius)* larva

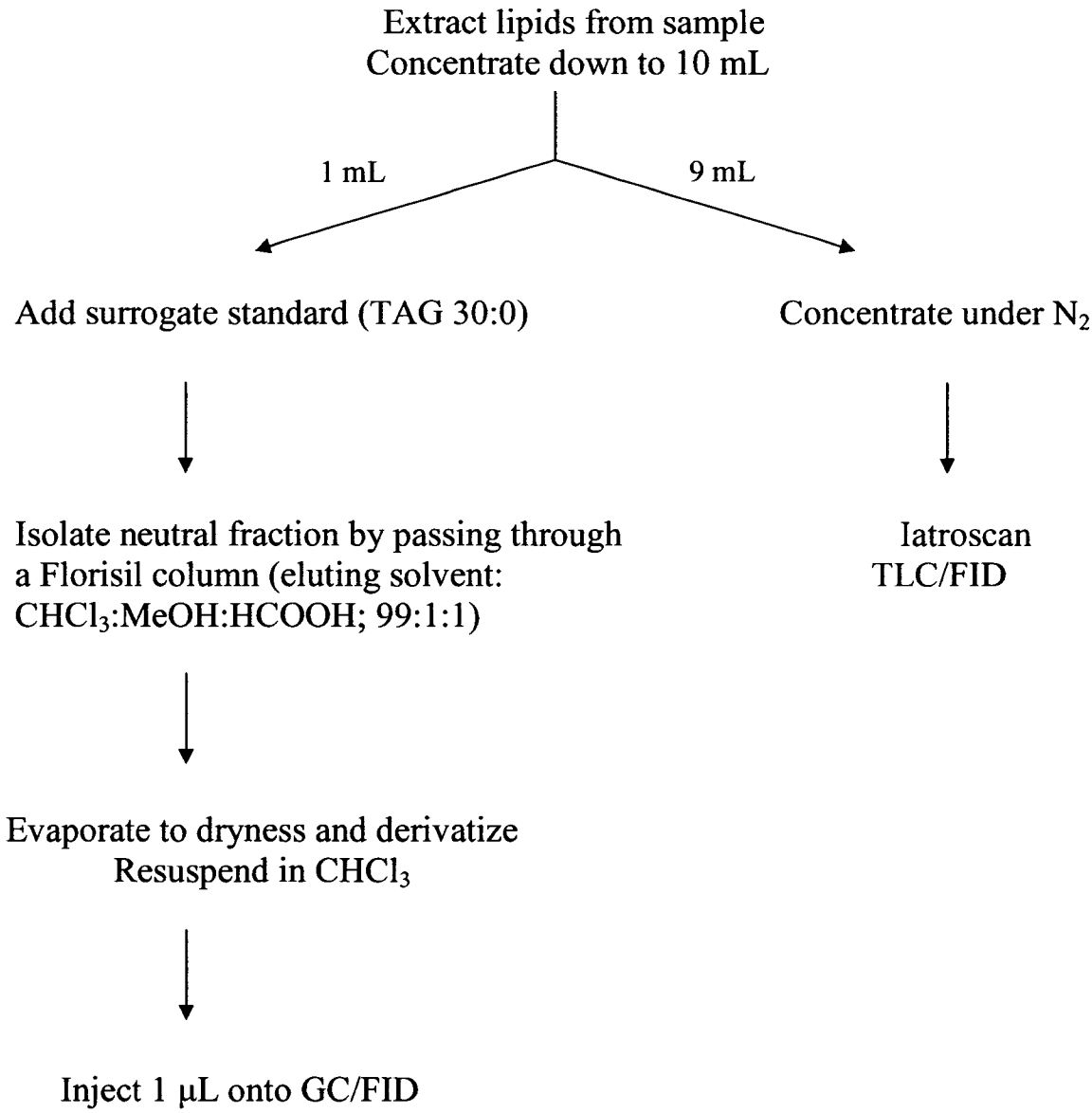


Figure 3.7. Outline the procedure used for the comparison of TLC/FID and short column GC/FID for the quantification of neutral lipids in individual fish larvae.

The results are presented in Figure 3.8, which shows a very good linear correlation between the two methods for the detection of sterols and triacylglycerols. Table 3.3 compares lipid percent data for ST and TAG, as detected by GC/FID and TLC/FID. One advantage of using GC over Iatroscan is that individual lipid components can be separated based on their carbon number rather than being pooled together in one class.

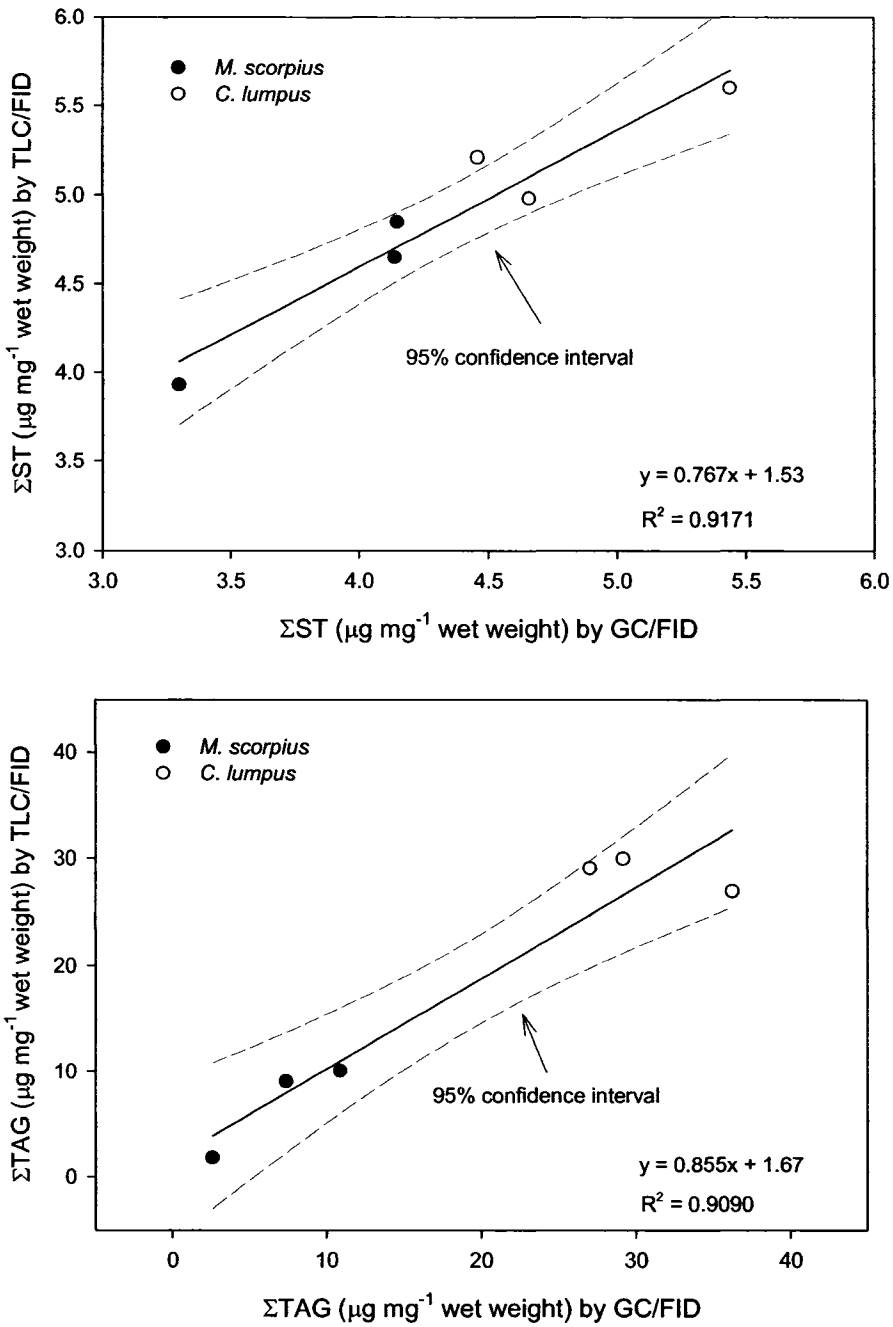


Figure 3.8. Correlation of (A) sterol and (B) triacylglycerol amounts, as determined by TLC/FID and GC/FID, in individual fish larva (<10 mg wet weight) of two marine fish species

Table 3.3. Percent lipid data for TAG and ST as determined by short column GC and Iatroscan in 3 individual larva from 2 marine species, *C. lumpus* and *M. scorpius.* For TAG, carbon numbers represent acyl carbons

M. scorpius

Larva 1	Carbon number	% Neutral Lipid GC	Iatroscan	*Larva 2*	Carbon number	% Neutral Lipid GC	Iatroscan	*Larva 3*	Carbon number	% Neutral Lipid GC	Iatroscan
ST	26	2.09		ST	26	1.78		ST	26	4.41	
	27	15.98			27	16.91			27	18.51	
	28	0.15			28	0.15			28	0.00	
	Σ ST	**18.22**	**16.54**		**Σ ST**	**18.84**	**21.35**		**Σ ST**	**22.92**	**22.64**
TAG	46	2.40		TAG	46	1.78		TAG	46	1.15	
	48	0.09			48	1.11			48	1.52	
	50	7.09			50	3.70			50	1.18	
	52	7.58			52	6.60			52	2.08	
	54	7.70			54	7.93			54	6.31	
	56	8.09			56	6.78			56	3.90	
	58	4.07			58	4.72			58	1.93	
	60	1.15			60	0.77			60	0.23	
	62	0.52			62	0.21			62	0.14	
	Σ TAG	**36.69**	**39.62**		**Σ TAG**	**33.61**	**38.42**		**Σ TAG**	**18.44**	**22.19**

C. lumpus

Larva 1	Carbon number	% Neutral Lipid GC	Iatroscan	*Larva 2*	Carbon number	% Neutral Lipid GC	Iatroscan	*Larva 3*	Carbon number	% Neutral Lipid GC	Iatroscan
ST	26	2.64		ST	26	1.44		ST	26	1.15	
	27	9.98			27	10.99			27	11.64	
	28	0.18			28	0.00			28	0.01	
	Σ ST	**12.80**	**11.38**		**Σ ST**	**12.43**	**11.96**		**Σ ST**	**12.79**	**12.89**
TAG	46	1.09		TAG	46	2.14		TAG	46	1.87	
	48	2.00			48	2.15			48	2.03	
	50	3.02			50	3.50			50	3.17	
	52	8.13			52	7.83			52	8.27	
	54	12.75			54	11.85			54	11.45	
	56	14.83			56	15.22			56	13.18	
	58	10.56			58	9.29			58	10.24	
	60	2.59			60	2.32			60	6.38	
	62	0.36			62	0.56			62	0.26	
	Σ TAG	**55.34**	**53.89**		**Σ TAG**	**54.85**	**58.50**		**Σ TAG**	**56.86**	**60.81**

The results reveal that there were no significant differences in the quantification of triacylglycerols or sterols in individual fish larvae ($p > 0.05$). From the data presented, it can be concluded that GC can be a viable and positive alternative over TLC/FID for the quantification of TAG and ST in individual larval samples. GC is particularly advantageous because it detects such a small sample amount in contrast to Iatroscan, where frequently a large aliquot of the sample must be spotted in order to get an adequate response. Although the Iatroscan procedure is more rapid since crude extracts can be run, GC has several other advantages over Iatroscan such as lower cost, reduced solvent waste and automation.

It would be interesting to apply the dephosphorylation procedure developed by Kehoe (2003) to the larval samples, thus supplemental phospholipid data could be obtained. As well, since such a small fraction of the sample is used compared to Iatroscan, further FA analysis could possibly be performed on the larval samples, however this was beyond the scope of the current research.

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

Due to the heterogeneous nature of lipids, their exhaustive extraction from marine plants and animals poses a complex analytical challenge. Extraction efficiency can be highly dependent on factors including sample matrix, sample size and the nature of the extracting solvent. Most current liquid extraction procedures, based on the Bligh & Dyer procedure and Folch methods, are time consuming and involve many manual manipulations. Modifications to traditional lipid extraction procedures are often made, however the nature of these modifications is rarely mentioned, although these adaptations can have an impact on the extraction efficiency.

The Randall procedure developed during this research project can be applied to the extraction of macrophytes and fish without destroying the integrity of the native lipid composition. It efficiency is comparable to traditional extraction methods such as the Folch and Bligh & Dyer procedures. The heat required for extraction does not affect the fatty acid profiles, in particular the polyunsaturated fatty acids; FA profiling is an integral part of most current lipid research.

Extraction methods such as the Randall procedure and Soxhlet commonly use non-polar solvents such as hexanes or diethyl ether, however these solvents are not

effective at extracting polar lipids, such as phospholipids, without harsh chemical pre-treatment. With the use of a chloroform/methanol/water azeotrope, it is possible to analyse wet samples and to quantitatively extract the polar fraction. This procedure can be carried out within a reasonable amount of time, generating reproducible results and it is suitable for large sample sizes. As well, this automated method requires very little active hands-on time by the analyst, thus there is less chance for variability in extraction efficiency depending on the skill of the analyst.

Short column gas chromatography with flame ionization detection is an effective analysis method for the detection of neutral lipids in very small samples such as individual fish larvae, and it is advantageous over Iatroscan thin-layer chromatography with flame ionization detection due to its inherently higher sensitivity and lower detection limits. By using weight correction factors, lipid classes such as triacylglycerols and sterols can be accurately quantified. As well, extra information on the individual lipid profiles, based on their carbon number, can easily be obtained. Further, a short guard column allows for routine analysis of crude plant extracts while preserving the integrity of analytical column. Although most acetone mobile polar lipids cannot be directly analysed via short column GC, much information can still be obtained of the individual lipid components in plant lipid extracts, particularly if the sample is dephosphorylated prior to analysis.

Future Work:

Until now, chloroform/methanol/water solvent systems have proven to be the most satisfactory for the removal of total lipids in marine species. For the Randall procedure, the azeotropic solvent system described in this research has been demonstrated to be very effective for the quantitative removal of all lipid classes; however it would be beneficial to try solvent systems that do not use chlorinated solvents. There are some serious health concerns with the use of chloroform, and a reduction in the amount of halogenated waste generated would be beneficial. Further, over time, the chlorinated solvents can cause corrosion to the metal parts in the interior of the solvent extractor (which can be removed with strong acids).

In another attempt to reduce the amount of solvent waste generated, it would also be useful to investigate in further detail the cleanliness of the recovered solvent after lipid extraction, so that it could possibly be reused.

The methods described here were tested on only a few sample types, thus it would be useful to further validate the method with different marine samples, such as bivalves and sediments. As well, it would be interesting to investigate whether freezing has an effect on the lipid extraction efficiency in plants.

For the GC analysis, it would of benefit to investigate passive extraction methods for individual larva and to see whether they are capable of exhaustively extracting all lipid components. Due to the small size of these samples (generally less than 10 mg wet weight), it is likely that the extraction procedure can be simplified.

Short column GC with mass spectrometric detection would be an interesting direction for the further development of the GC method; mass spectrometry would provide valuable insight into the fragmentation pattern of the molecular species present.

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APPENDIX A

FA profile for *C. crispus* extracted by 3 different methods

	Folch	Soxhlet	Randall
14:0	11.16 ± 0.73	10.35 ± 0.32	11.07 ± 0.52
15:0i	0.36 ± 0.11	0.32 ± 0.05	0.57 ± 0.05
15:0	0.58 ± 0.03	0.57 ± 0.01	0.54 ± 0.02
16:0i	0.16 ± 0.15	0.32 ± 0.01	0.38 ± 0.02
16:0ai?	0.31 ± 0.13	0.25 ± 0.13	0.27 ± 0.02
16:0	16.59 ± 0.49	17.29 ± 1.07	15.84 ± 1.20
17:0i	0.58 ± 0.11	0.64 ± 0.06	0.76 ± 0.12
17:0ai?	0.11 ± 0.03	0.06 ± 0.05	0.06 ± 0.06
17:0	0.23 ± 0.03	0.27 ± 0.06	0.29 ± 0.03
18:0	0.37 ± 0.02	0.45 ± 0.39	0.43 ± 0.02
19:0	0.00 ± 0.00	0.07 ± 0.12	0.00 ± 0.00
20:0	0.23 ± 0.04	0.27 ± 0.10	0.25 ± 0.07
21:0	0.00 ± 0.00	0.16 ± 0.04	0.09 ± 0.08
22:0	0.15 ± 0.07	0.45 ± 0.14	0.21 ± 0.09
23:0	0.04 ± 0.07	0.04 ± 0.07	0.00 ± 0.00
24:0	0.00 ± 0.00	0.98 ± 1.04	0.16 ± 0.04
Σ SFA	**30.86 ± 0.89**	**32.47 ± 1.33**	**30.93 ± 1.56**
14:1	0.22 ± 0.07	0.06 ± 0.10	0.06 ± 0.10
15:1	0.21 ± 0.06	0.26 ± 0.02	0.27 ± 0.00
16:1ω11?	0.00 ± 0.00	0.35 ± 0.60	0.00 ± 0.00
16:1ω9?	0.73 ± 0.57	0.39 ± 0.34	0.77 ± 0.06
16:1ω7	1.65 ± 0.06	1.63 ± 0.16	1.61 ± 0.03
16:1ω5	0.03 ± 0.05	0.00 ± 0.00	0.04 ± 0.07
17:1	0.52 ± 0.20	0.60 ± 0.06	0.40 ± 0.10
18:1ω11?	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
18:1ω9	16.51 ± 0.23	15.90 ± 3.13	15.85 ± 1.05
18:1ω6?	0.00 ± 0.00	0.04 ± 0.06	0.05 ± 0.09
18:1ω5?	0.07 ± 0.06	0.00 ± 0.00	0.03 ± 0.05
20:1ω11?	0.28 ± 0.04	0.19 ± 0.18	0.32 ± 0.10
20:1ω9	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.05
20:1ω7?	0.05 ± 0.09	0.00 ± 0.00	0.04 ± 0.08
22:1ω11(13)	0.43 ± 0.08	0.20 ± 0.17	0.28 ± 0.24
24:1	0.21 ± 0.09	0.22 ± 0.15	0.26 ± 0.12
Σ MUFA	**20.91 ± 0.34**	**19.83 ± 3.03**	**20.02 ± 1.02**

	Folch	Soxhlet	Randall
16:2ω4	0.27 ± 0.13	0.31 ± 0.07	0.19 ± 0.03
16:3ω4?	0.23 ± 0.20	0.10 ± 0.18	0.21 ± 0.18
16:4ω3?	0.13 ± 0.05	0.17 ± 0.08	0.22 ± 0.07
16:4ω1	0.14 ± 0.01	0.12 ± 0.03	0.11 ± 0.10
18:2ω6	8.00 ± 0.74	8.03 ± 0.25	8.30 ± 0.60
18:2ω4	0.09 ± 0.00	0.21 ± 0.04	0.08 ± 0.07
18:3ω6	0.55 ± 0.01	0.47 ± 0.05	0.55 ± 0.06
18:3ω4	0.08 ± 0.07	0.15 ± 0.10	0.03 ± 0.06
18:3ω3	6.69 ± 0.52	6.68 ± 0.77	6.76 ± 0.63
18:4ω3	5.10 ± 0.23	4.98 ± 0.75	5.64 ± 0.92
18:4ω1?	0.15 ± 0.05	0.03 ± 0.05	0.15 ± 0.16
18:5ω3	0.00 ± 0.00	0.10 ± 0.18	0.00 ± 0.00
20:2ω6	0.49 ± 0.06	0.44 ± 0.04	0.47 ± 0.10
20:3ω6	0.66 ± 0.15	0.58 ± 0.07	0.59 ± 0.04
20:4ω6	12.56 ± 0.32	12.99 ± 0.41	12.60 ± 0.45
20:3ω3	0.24 ± 0.06	0.19 ± 0.03	0.23 ± 0.02
20:4ω3	0.51 ± 0.16	0.44 ± 0.06	0.40 ± 0.04
20:5ω3	8.90 ± 0.85	8.58 ± 1.20	9.31 ± 0.92
22:4ω6?	0.07 ± 0.12	0.29 ± 0.19	0.17 ± 0.07
22:5ω6	0.15 ± 0.15	0.00 ± 0.00	0.09 ± 0.09
22:4ω3?	0.15 ± 0.15	0.00 ± 0.00	0.00 ± 0.00
22:5ω3	0.23 ± 0.08	0.04 ± 0.06	0.06 ± 0.10
22:6ω3	2.75 ± 0.16	1.98 ± 0.45	2.35 ± 0.32
Σ PUFA	**48.15 ± 0.86**	**46.87 ± 2.75**	**48.51 ± 2.00**
PUFA/SFA	1.56 ± 0.07	1.44 ± 0.08	1.57 ± 0.14
Σ ω3	24.71 ± 0.79	23.17 ± 2.81	24.98 ± 2.18
Σ ω6	22.48 ± 0.69	22.79 ± 0.69	22.77 ± 0.77
Σ ω3/ Σ ω6	1.10 ± 0.06	1.02 ± 0.14	1.10 ± 0.12

FA profile for *G. Morhua* extracted by 5 different methods

	Bligh & Dyer	Folch	Modified Folch	Soxhlet	Randall
14:0	1.54 ± 0.12	1.30 ± 0.20	1.39 ± 0.07	1.35 ± 0.21	1.38 ± 0.03
15:0i	0.14 ± 0.06	0.05 ± 0.05	0.08 ± 0.02	0.10 ± 0.14	0.08 ± 0.01
15:0ai	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
15:0	0.31 ± 0.01	0.29 ± 0.03	0.30 ± 0.01	0.36 ± 0.07	0.31 ± 0.02
16:0i	0.08 ± 0.02	0.04 ± 0.04	0.07 ± 0.01	0.08 ± 0.11	0.05 ± 0.04
16:0ai?	0.02 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16:0	17.88 ± 0.20	18.05 ± 0.21	18.15 ± 0.71	18.31 ± 0.68	18.17 ± 0.40
17:0i	0.31 ± 0.03	0.34 ± 0.03	0.35 ± 0.01	0.34 ± 0.01	0.34 ± 0.02
17:0ai?	0.14 ± 0.01	0.13 ± 0.00	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
17:0	0.15 ± 0.13	0.22 ± 0.01	0.22 ± 0.00	0.12 ± 0.17	0.21 ± 0.01
18:0	2.75 ± 0.08	3.47 ± 0.30	3.26 ± 0.17	3.23 ± 0.06	2.75 ± 0.33
20:0	0.05 ± 0.09	0.04 ± 0.06	0.06 ± 0.11	0.09 ± 0.02	0.02 ± 0.03
22:0	0.19 ± 0.12	0.19 ± 0.02	0.10 ± 0.03	0.57 ± 0.05	0.16 ± 0.05
23:0	0.16 ± 0.08	0.17 ± 0.04	0.19 ± 0.03	0.15 ± 0.00	0.14 ± 0.03
Σ SFA	**23.71 ± 0.12**	**24.33 ± 0.29**	**24.32 ± 0.77**	**24.83 ± 1.23**	**23.74 ± 0.59**
14:1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.16 ± 0.06	0.00 ± 0.00
15:1	0.15 ± 0.05	0.12 ± 0.03	0.15 ± 0.05	0.19 ± 0.07	0.13 ± 0.01
16:1ω11?	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.12
16:1ω9?	0.00 ± 0.00	0.05 ± 0.09	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16:1ω7	2.79 ± 0.11	2.43 ± 0.25	2.48 ± 0.06	2.32 ± 0.28	2.72 ± 0.21
16:1ω5	0.36 ± 0.03	0.36 ± 0.01	0.38 ± 0.03	0.39 ± 0.01	0.37 ± 0.02
17:1	0.00 ± 0.00	0.03 ± 0.06	0.04 ± 0.06	0.00 ± 0.00	0.02 ± 0.03
18:1ω9	7.19 ± 0.09	7.43 ± 0.24	7.85 ± 0.12	7.30 ± 0.04	7.40 ± 0.14
18:1ω7	3.71 ± 0.13	3.85 ± 0.27	3.73 ± 0.26	3.58 ± 0.02	3.70 ± 0.16
18:1ω6?	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.29	0.27 ± 0.38	0.00 ± 0.00
18:1ω5?	0.50 ± 0.01	0.53 ± 0.07	0.40 ± 0.29	0.58 ± 0.13	0.44 ± 0.04
20:1ω9	2.03 ± 0.15	2.25 ± 0.05	2.28 ± 0.12	2.07 ± 0.05	2.09 ± 0.17
20:1ω7?	0.27 ± 0.04	0.52 ± 0.40	0.30 ± 0.02	0.28 ± 0.01	0.27 ± 0.05
22:1ω11(13)	0.50 ± 0.10	0.43 ± 0.21	0.24 ± 0.16	1.02 ± 0.65	0.48 ± 0.15
22:1ω9	0.16 ± 0.02	0.12 ± 0.10	0.05 ± 0.08	0.16 ± 0.11	0.21 ± 0.05
22:1ω7	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.04	0.00 ± 0.00	0.11 ± 0.12
24:1	0.89 ± 0.13	1.04 ± 0.11	1.09 ± 0.13	0.89 ± 0.02	0.95 ± 0.12
Σ MUFA	**18.54 ± 0.28**	**19.17 ± 0.26**	**19.18 ± 0.38**	**19.21 ± 0.71**	**18.97 ± 0.55**

	Bligh & Dyer	Folch	Modified Folch	Soxhlet	Randall
16:2ω4	0.62 ± 0.12	0.49 ± 0.05	0.48 ± 0.06	0.50 ± 0.06	0.48 ± 0.05
16:3ω4?	0.35 ± 0.06	0.25 ± 0.01	0.28 ± 0.02	0.29 ± 0.01	0.26 ± 0.02
16:4ω3?	0.41 ± 0.10	0.17 ± 0.06	0.15 ± 0.07	0.38 ± 0.02	0.13 ± 0.03
16:4ω1	0.16 ± 0.03	0.11 ± 0.02	0.13 ± 0.01	0.13 ± 0.01	0.10 ± 0.02
18:2ω6	0.68 ± 0.04	0.65 ± 0.06	0.67 ± 0.01	0.40 ± 0.32	0.65 ± 0.06
18:2ω4	0.13 ± 0.01	0.11 ± 0.00	0.13 ± 0.01	0.09 ± 0.01	0.12 ± 0.03
18:3ω6	0.05 ± 0.04	0.00 ± 0.00	0.09 ± 0.02	0.00 ± 0.00	0.03 ± 0.05
18:3ω4	0.11 ± 0.05	0.08 ± 0.04	0.10 ± 0.06	0.13 ± 0.07	0.09 ± 0.02
18:3ω3	0.25 ± 0.02	0.24 ± 0.03	0.28 ± 0.02	0.23 ± 0.03	0.24 ± 0.02
18:4ω3	0.52 ± 0.04	0.47 ± 0.06	0.53 ± 0.04	0.42 ± 0.05	0.47 ± 0.03
18:4ω1?	0.08 ± 0.03	0.11 ± 0.03	0.14 ± 0.04	0.04 ± 0.05	0.06 ± 0.06
20:3ω6	0.04 ± 0.03	0.02 ± 0.04	0.00 ± 0.00	0.04 ± 0.06	0.06 ± 0.01
20:4ω6	2.47 ± 0.30	2.84 ± 0.24	1.81 ± 1.65	2.58 ± 0.43	2.65 ± 0.17
20:3ω3	0.09 ± 0.01	0.10 ± 0.02	0.08 ± 0.07	0.09 ± 0.01	0.11 ± 0.01
20:4ω3	0.37 ± 0.02	0.34 ± 0.05	0.43 ± 0.03	0.32 ± 0.00	0.37 ± 0.01
20:5ω3	18.44 ± 0.76	17.78 ± 0.51	18.26 ± 0.48	17.70 ± 0.03	18.39 ± 0.34
21:5ω3?	0.24 ± 0.00	0.26 ± 0.02	0.26 ± 0.02	0.30 ± 0.11	0.26 ± 0.01
22:4ω6?	0.05 ± 0.05	0.03 ± 0.05	0.04 ± 0.07	0.18 ± 0.10	0.03 ± 0.06
22:5ω6	0.40 ± 0.02	0.42 ± 0.07	0.35 ± 0.02	0.17 ± 0.24	0.38 ± 0.01
22:4ω3?	0.17 ± 0.16	0.06 ± 0.11	0.19 ± 0.17	0.50 ± 0.71	0.37 ± 0.55
22:5ω3	1.35 ± 0.10	1.44 ± 0.13	1.44 ± 0.11	1.33 ± 0.09	1.36 ± 0.19
22:6ω3	30.45 ± 0.84	30.13 ± 0.28	30.43 ± 0.58	29.55 ± 0.44	30.38 ± 0.30
Σ PUFA	**57.65 ± 0.18**	**56.35 ± 0.41**	**56.44 ± 1.05**	**55.57 ± 0.43**	**57.22 ± 0.25**
PUFA/SFA	2.43 ± 0.01	2.32 ± 0.04	2.32 ± 0.11	2.24 ± 0.13	2.41 ± 0.06
Σ ω3	52.29 ± 0.39	51.00 ± 0.71	52.06 ± 1.20	50.82 ± 1.30	52.09 ± 0.10
Σ ω6	3.91 ± 0.23	4.19 ± 0.23	3.13 ± 1.69	3.59 ± 0.92	4.02 ± 0.19
Σ ω3/ Σ ω6	13.40 ± 0.87	12.19 ± 0.87	22.21 ± 15.94	14.69 ± 4.11	12.98 ± 0.59

FA profile for *M. villosus* extracted by 5 different methods

	Bligh & Dyer	Folch	Modified Folcl	Soxhlet	Randall
14:0	4.03 ± 0.59	3.80 ± 0.75	3.33 ± 0.03	3.49 ± 0.09	3.56 ± 0.73
15:0i	0.12 ± 0.01	0.10 ± 0.03	0.09 ± 0.01	0.10 ± 0.00	0.11 ± 0.02
15:0ai	0.04 ± 0.04	0.04 ± 0.03	0.00 ± 0.00	0.03 ± 0.04	0.02 ± 0.04
15:0	0.28 ± 0.01	0.37 ± 0.11	0.26 ± 0.01	0.29 ± 0.04	0.32 ± 0.10
16:0i	0.02 ± 0.03	0.06 ± 0.06	0.00 ± 0.00	0.02 ± 0.03	0.05 ± 0.08
16:0ai?	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.05	0.04 ± 0.05	0.13 ± 0.07
16:0	11.89 ± 1.26	13.89 ± 2.30	12.78 ± 0.48	12.43 ± 0.30	11.72 ± 1.02
17:0i	0.18 ± 0.01	0.20 ± 0.03	0.25 ± 0.10	0.17 ± 0.00	0.20 ± 0.06
17:0ai?	0.15 ± 0.02	0.16 ± 0.01	0.07 ± 0.10	0.15 ± 0.01	0.17 ± 0.03
17:0	0.09 ± 0.00	0.11 ± 0.02	0.09 ± 0.00	0.09 ± 0.01	0.11 ± 0.04
18:0	0.98 ± 0.10	1.25 ± 0.18	1.12 ± 0.07	1.14 ± 0.09	1.04 ± 0.12
20:0	0.03 ± 0.05	0.07 ± 0.06	0.04 ± 0.06	0.10 ± 0.01	0.11 ± 0.02
21:0	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.05	0.00 ± 0.00	0.00 ± 0.00
22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.13	0.04 ± 0.04
23:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.05	0.00 ± 0.00
ΣSFA	**17.81 ± 0.81**	**20.05 ± 3.15**	**18.12 ± 0.38**	**18.17 ± 0.10**	**17.59 ± 0.63**
14:1	0.10 ± 0.02	0.09 ± 0.02	0.08 ± 0.01	0.08 ± 0.00	0.09 ± 0.02
15:1	0.12 ± 0.01	0.14 ± 0.07	0.13 ± 0.02	0.13 ± 0.00	0.16 ± 0.08
16:1ω9?	0.00 ± 0.00	0.03 ± 0.05	0.07 ± 0.10	0.00 ± 0.00	0.08 ± 0.07
16:1ω7	7.78 ± 0.97	7.77 ± 1.02	6.74 ± 0.18	7.05 ± 0.25	7.08 ± 0.70
16:1ω5	0.35 ± 0.03	0.31 ± 0.20	0.37 ± 0.02	0.37 ± 0.02	0.38 ± 0.03
17:1	0.05 ± 0.04	0.05 ± 0.05	0.07 ± 0.00	0.09 ± 0.03	0.04 ± 0.06
18:1ω9	4.55 ± 0.17	4.85 ± 0.69	4.47 ± 0.23	4.46 ± 0.26	4.34 ± 0.17
18:1ω7	2.34 ± 0.21	2.77 ± 0.59	2.55 ± 0.07	2.45 ± 0.05	2.46 ± 0.24
18:1ω5?	0.67 ± 0.03	0.78 ± 0.14	0.69 ± 0.05	0.66 ± 0.01	0.68 ± 0.08
20:1ω9	14.87 ± 2.47	14.50 ± 2.72	12.43 ± 0.96	12.26 ± 0.77	13.10 ± 3.03
20:1ω7?	0.60 ± 0.20	0.87 ± 0.78	0.53 ± 0.76	0.63 ± 0.81	1.16 ± 0.20
22:1ω9	16.24 ± 3.18	15.65 ± 3.34	13.02 ± 1.45	14.26 ± 0.48	13.80 ± 3.92
22:1ω7	0.48 ± 0.05	0.35 ± 0.33	0.78 ± 1.10	0.96 ± 0.79	0.45 ± 0.77
24:1	1.00 ± 0.08	1.01 ± 0.09	1.00 ± 0.01	1.09 ± 0.16	1.06 ± 0.06
ΣMUFA	**49.14 ± 6.32**	**49.19 ± 8.32**	**42.93 ± 0.92**	**44.49 ± 0.08**	**44.88 ± 6.90**

	Bligh & Dyer	Folch	Modified Folcl	Soxhlet	Randall
16:2ω4	0.66 ± 0.07	0.65 ± 0.04	0.59 ± 0.00	0.62 ± 0.02	0.63 ± 0.06
16:3ω4?	0.51 ± 0.07	0.41 ± 0.03	0.39 ± 0.03	0.43 ± 0.07	0.47 ± 0.09
16:4ω3?	0.10 ± 0.00	0.10 ± 0.01	0.12 ± 0.04	0.09 ± 0.01	0.10 ± 0.03
16:4ω1	0.75 ± 0.17	0.65 ± 0.11	0.62 ± 0.03	0.71 ± 0.09	0.77 ± 0.24
18:2ω6	0.59 ± 0.02	0.62 ± 0.03	0.63 ± 0.02	0.61 ± 0.01	0.62 ± 0.03
18:2ω4	0.12 ± 0.00	0.13 ± 0.02	0.12 ± 0.01	0.13 ± 0.00	0.19 ± 0.13
18:3ω6	0.04 ± 0.04	0.05 ± 0.04	0.03 ± 0.05	0.11 ± 0.06	0.09 ± 0.10
18:3ω4	0.09 ± 0.04	0.07 ± 0.01	0.10 ± 0.04	0.08 ± 0.00	0.09 ± 0.11
18:3ω3	0.29 ± 0.01	0.33 ± 0.05	0.34 ± 0.05	0.34 ± 0.02	0.34 ± 0.06
18:4ω3	1.08 ± 0.08	0.95 ± 0.18	1.01 ± 0.00	1.07 ± 0.12	1.07 ± 0.13
18:4ω1?	0.25 ± 0.01	0.25 ± 0.04	0.25 ± 0.01	0.26 ± 0.02	0.26 ± 0.06
18:5ω3	0.06 ± 0.05	0.03 ± 0.05	0.04 ± 0.06	0.00 ± 0.00	0.00 ± 0.00
20:2ω6	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.11 ± 0.01
20:3ω6	0.02 ± 0.03	0.02 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:4ω6	0.23 ± 0.03	0.24 ± 0.08	0.26 ± 0.01	0.27 ± 0.01	0.27 ± 0.04
20:3ω3	0.03 ± 0.03	0.04 ± 0.03	0.00 ± 0.00	0.03 ± 0.04	0.02 ± 0.03
20:4ω3	0.46 ± 0.13	0.40 ± 0.06	0.45 ± 0.09	0.38 ± 0.01	0.40 ± 0.04
20:5ω3	10.70 ± 1.64	9.80 ± 3.70	11.86 ± 0.52	11.86 ± 0.22	11.69 ± 1.84
21:5ω3?	0.30 ± 0.04	0.27 ± 0.09	0.33 ± 0.01	0.32 ± 0.02	0.34 ± 0.07
22:4ω6?	0.02 ± 0.04	0.03 ± 0.04	0.06 ± 0.00	0.03 ± 0.05	0.04 ± 0.04
22:5ω6	0.12 ± 0.02	0.14 ± 0.03	0.18 ± 0.02	0.16 ± 0.00	0.18 ± 0.04
22:4ω3?	0.04 ± 0.04	0.02 ± 0.04	0.09 ± 0.01	0.00 ± 0.00	0.03 ± 0.05
22:5ω3	1.36 ± 0.24	1.23 ± 0.40	1.63 ± 0.05	1.50 ± 0.07	1.55 ± 0.30
22:6ω3	14.87 ± 3.71	13.89 ± 6.39	19.14 ± 0.96	17.92 ± 0.53	17.69 ± 3.57
ΣPUFA	**32.78 ± 5.32**	**30.40 ± 10.99**	**38.38 ± 1.42**	**37.02 ± 0.16**	**36.97 ± 6.42**
PUFA/SFA	1.84 ± 0.23	1.59 ± 0.71	2.12 ± 0.12	2.04 ± 0.00	2.09 ± 0.30
Σ ω3	29.30 ± 5.61	27.06 ± 10.83	35.03 ± 1.49	33.52 ± 0.17	33.23 ± 5.86
Σ ω6	1.11 ± 0.02	1.19 ± 0.06	1.28 ± 0.02	1.27 ± 0.12	1.31 ± 0.17
Σ ω3/ Σ ω6	26.28 ± 4.52	22.72 ± 8.69	27.49 ± 1.66	26.55 ± 2.63	25.19 ± 1.35

Lipid composition (mg g^{-1} wet weight) of *M. villosus*, extracted by 5 different methods

	Bligh & Dyer	Folch	Modified Folch	Soxhlet	Randall
Total Lipid	30.88 ± 8.55	32.22 ± 6.35	33.33 ± 6.41	41.28 ± 11.23	34.59 ± 8.58
Hydrocarbons	0.40 ± 0.12	0.28 ± 0.06	0.18 ± 0.18	0.21 ± 0.20	0.35 ± 0.05
Steryl Esters/Wax Esters	0.13 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.02	0.00 ± 0.00
Ethyl Esters	0.07 ± 0.07	0.00 ± 0.00	0.16 ± 0.03	0.16 ± 0.05	0.07 ± 0.12
Methyl Ketones	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.07
Triacylglycerols	22.57 ± 7.20	23.13 ± 5.37	23.39 ± 7.48	28.09 ± 6.41	20.86 ± 5.68
Free Fatty Acids	0.85 ± 0.19	0.88 ± 0.37	1.08 ± 0.30	1.06 ± 0.36	0.67 ± 0.24
Alcohols	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.21 ± 0.04	0.13 ± 0.23
Sterols	0.63 ± 0.19	0.82 ± 0.19	0.61 ± 0.05	0.90 ± 0.28	0.78 ± 0.14
Diacylglycerols	0.05 ± 0.04	0.00 ± 0.00	0.06 ± 0.05	0.03 ± 0.04	0.09 ± 0.15
Acetone Mobile Polar Lipids	0.69 ± 0.16	1.14 ± 1.28	0.85 ± 0.18	1.31 ± 0.76	1.48 ± 1.11
Phospholipids	5.49 ± 2.66	5.97 ± 0.58	7.00 ± 1.09	9.15 ± 3.18	10.13 ± 1.79

Lipid composition (% wet weight) of *M. villosus*, extracted by 5 different methods

	Bligh & Dyer	Folch	Modified Folch	Soxhlet	Randall
Total Lipid	3.09 ± 0.86	3.22 ± 0.64	3.33 ± 0.64	4.13 ± 1.12	3.46 ± 0.86
Hydrocarbons	1.32 ± 0.33	0.90 ± 0.23	0.51 ± 0.44	0.45 ± 0.37	1.02 ± 0.29
Steryl Esters/Wax Esters	0.46 ± 0.40	0.00 ± 0.00	0.00 ± 0.00	0.38 ± 0.15	0.00 ± 0.00
Ethyl Esters	0.20 ± 0.19	0.00 ± 0.00	0.49 ± 0.18	0.38 ± 0.03	0.20 ± 0.35
Methyl Ketones	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.17
Triacylglycerols	72.59 ± 7.63	71.55 ± 4.24	69.29 ± 9.10	68.48 ± 3.11	60.12 ± 2.16
Free Fatty Acids	2.89 ± 0.86	2.70 ± 0.72	3.40 ± 1.56	2.53 ± 0.17	1.93 ± 0.36
Alcohols	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.55 ± 0.25	0.49 ± 0.85
Sterols	2.13 ± 0.68	2.55 ± 0.21	1.87 ± 0.52	2.17 ± 0.09	2.33 ± 0.60
Diacylglycerols	0.19 ± 0.21	0.00 ± 0.00	0.18 ± 0.12	0.06 ± 0.09	0.20 ± 0.35
Acetone Mobile Polar Lipids	2.31 ± 0.58	3.50 ± 3.74	2.54 ± 0.04	3.05 ± 1.02	3.90 ± 2.22
Phospholipids	17.92 ± 6.35	18.81 ± 2.17	21.72 ± 7.45	21.94 ± 1.75	29.67 ± 3.16