DEVELOPMENT OF A RAPID *IN SITU* TRANSESTERIFICATION METHOD FOR FATTY ACID ANALYSIS IN MICROALGAE

by

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For Donna, Anne, and Sue

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ABSTRACT

The FAME yield from microalgae of two *in situ* transesterification methods were compared to a typical Folch et al. (1957) extraction followed by transesterification using the Hilditch et al. (1964) procedure. A method based on Park & Goins (1994), utilizing 0.5 N NaOH in methanol, then 14 % BCl_3 in methanol, was found to be superior to a method based on Lepage & Roy (1986), utilizing acetyl chloride in methanol. The Park & Goins (1994) method was equivalent to the traditional method and was, therefore, selected for further study. In establishing the parameters of the method, water contents up to 0.55 mL were not found to inhibit the reaction within the maximum lipid load, conservatively assessed at ~1 mg. The reaction time and temperature required to produce a maximum FAME yield was 10 min at 90 °C for the BCl_3-catalyzed reaction, while the NaOH-catalyzed reaction happened instantaneously at ambient temperature.

LIST OF ABBREVIATIONS USED

AOCS	American Oil Chemists' Society
BCl_3	Boron trichloride
BF_3	Boron trifluoride
CO_2	Carbon dioxide
DAG	Diacylglycerol
DEM	Diethoxymethane
DHA	Docosahexaenoic acid
DMF	Dimethylformamide
EE	Ethyl ether
EPA	Eicosapentaenoic acid
F&H	Folch et al. & Hilditch et al.
FA	Fatty acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FoA	Formic acid
GC	Gas chromatography
GFC	Glass fiber "C" (filter paper)
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
He	Helium
Hex	Hexane

HPLC	High performance liquid chromatography
KOH	Potassium hydroxide
L&R	Lepage & Roy
MAG	Monoacylglycerol
$NaOCH_3$	Sodium methoxide
NaOH	Sodium hydroxide
P&G	Park & Goins
PA	Peak area
PE	Petroleum ether
PL	Phospholipids
PUFA	Polyunsaturated fatty acid
SFE	Supercritical Fluid Extraction
TAG	Triacylglycerol
TLC-FID	Thin layer chromatography and flame ionization detection
UVSFS	UV sterilized and filtered seawater

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CHAPTER 1: INTRODUCTION

Long-chain omega-3 FAs have recently gained attention because they are essential nutrients for all vertebrates, including humans. Two of these fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), reduce inflammation and can help manage autoimmune diseases (Simopoulos, 2002) and aid in ocular and cerebral tissue development, particularly in infants (Simopoulos, 2011), respectively. A common dietary source for these acids are marine fish, such as salmon and trout, although both EPA and DHA can be synthesized in the human body in limited amounts from alpha-linolenic acid (Tu et al., 2010). Humans usually acquire these FAs through the consumption of fish and other marine species, but their initial source in the marine food web is phytoplankton.

The use of microalgae as a direct source of omega-3 FAs is gaining popularity in commercial processes for health supplementation (Mercer et al., 2011). Supplementation can take place either indirectly by using microalgal oils as supplements in farmed fish diets to improve their FA profiles—which will ultimately be consumed as fish oil—or directly, by harvesting large quantities of oleaginous microalgal biomass to be used in human diet supplementation. When growing microalgae for biomass and lipid production, a number of parameters need to be monitored to ensure productivity of the culture. For instance, to monitor biomass of an microalgal culture, dry cell mass can be determined through filtration or freeze-drying, or the optical density can be measured with a spectrophotometer (Uduman et al., 2010). Monitoring microalgal lipid production and identifying the lipids

produced in the culture involve more challenging techniques; knowledge of these characteristics are necessary to identify suitable microalgal cultures with desirable lipid profiles, as well as to monitor lipid content during the lifetime of a culture to establishing ideal harvesting times.

Traditional microalgae lipid testing is a two-step process: lipid extraction followed by derivatization. This method first extracts the lipids from the microalgae sample with successive grinding and washes with organic solvents. The extracted lipids are then condensed by evaporating the organic solvents before derivatizing them into volatile fatty acid methyl esters (FAME) to be analyzed by gas chromatography (GC). Although thorough, these methods involving frequent transfers between many pieces of glassware can promote sample losses, have long reaction times, and use copious amounts of toxic organic solvents. However, the presence of water within biological samples often inhibits the derivatizing reagent's potency in traditional methods, causing side-reactions and producing off-results (Suter et al., 1997). Thus, isolation of lipids from the aqueous matrix is often necessary to ensure the efficacy of the derivatization reaction.

A proposed solution to these setbacks is the use of an *in situ* derivatization method, where small samples of microalgae are directly derivatized within one reaction vessel without the need to first extract their lipids. Section 3.0 will provide a review of current derivatization techniques and how they can best be applied to microalgal cultures.

CHAPTER 2: THESIS OBJECTIVES

The objective of this study was to adapt an existing *in situ* transesterification method to convert the lipids of microalgae into fatty acid methyl esters to be analyzed by gas chromatography. To achieve this objective, the following steps were implemented: 1) select an existing *in situ* transesterification method and assess its candidacy for applications with microalgae; 2) assess the maximum water and lipid load of the method; and 3) optimize two aspects of the method, length of reaction and reaction temperature, to make the method as rapid, yet precise, as possible.

CHAPTER 3: LITERATURE REVIEW

3.1 Microalgal Lipids

Algae are a group of organisms found in many diverse aquatic environments around the world. They range in size from single cells to long seaweeds and act as one of the fundamental food sources at the base of the marine food web (Gachon et al., 2010). Unicellular algae, or microalgae, typically grow photosynthetically in nature but some species also possesses the ability to grow heterotrophically, relying on organic carbon substrates for energy. Their growth conditions alter the organism's ability to accumulate storage products such as starches and lipids, making certain species desirable for the production of biodiesel and nutraceuticals (Imhoff et al., 2011). The typical growth curve of microalgae starts with the initial lag period, followed by accelerated growth during its exponential phase, and finally ceasing replication due to nutrient depletion during its stationary phase (Fig. 3.1).

The lipid classes of microalgal cultures are primarily composed of phospholipids (PL), which act as the structural lipids of the cell, comprising the cell wall. These lipids are in greatest abundance as the culture is developing biomass, during its exponential growth phase, as the cells are replicating (Zhu et al., 1997b). During the stationary phase of growth, cellular division begins to slow down and the cells mature, activating their ability to convert energy to storage lipids. Depending on the species and cultural conditions, the primary storage lipids in a culture are triacylglycerols (TAG) (Su et al., 2011), although diacylglycerols (DAG), and monoacylglycerols (MAG) may also be identified (He et al., 2011, Fig. 3.2).

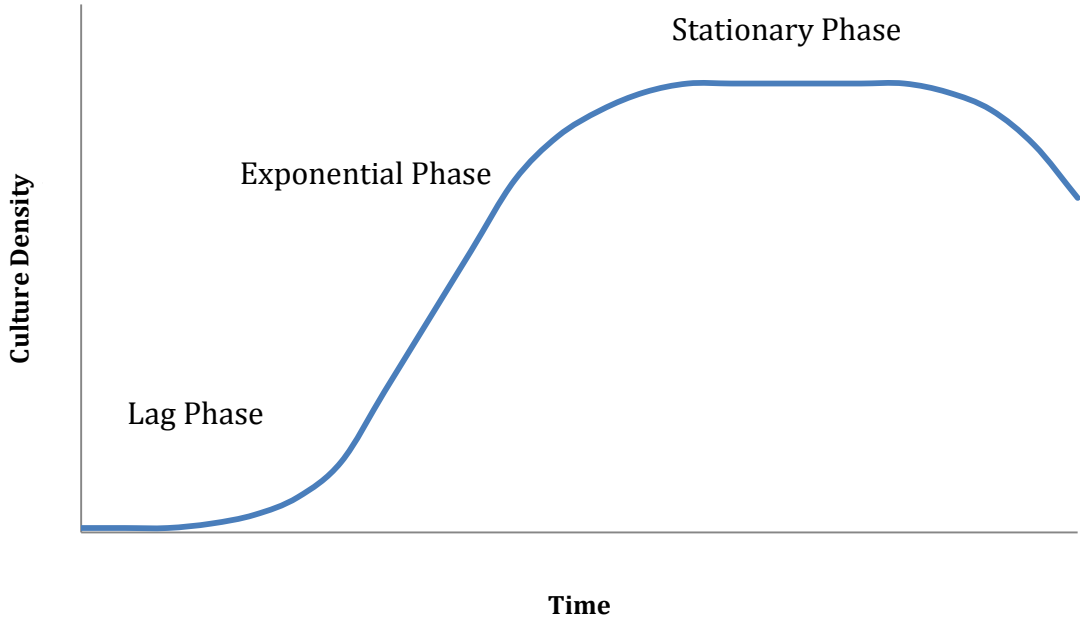

Figure 3.1. Typical growth curve of microalgae.

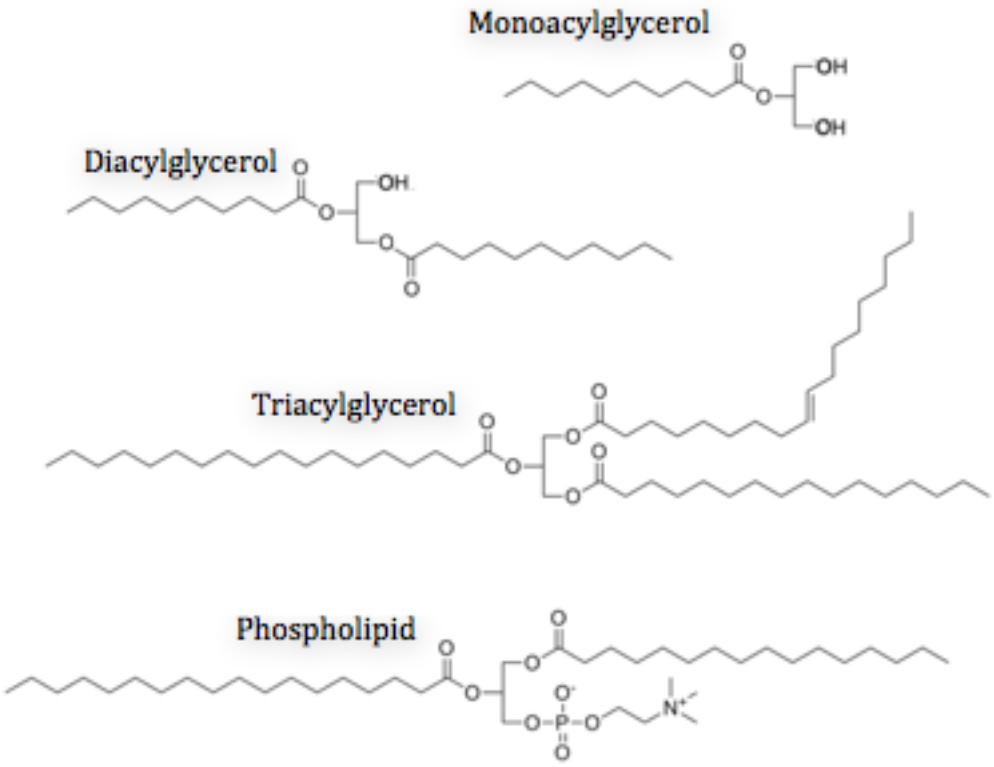

Figure 3.2. Molecular structures of MAG, DAG, TAG, and PL.

3.2 Dewatering Microalgal Samples

Testing unicellular microalgae for lipid content and composition can be a challenge as its culture density in a photoautotrophic batch process is very low, usually in the range of 1-10 g culture/L (Kim et al., 2011). Thus, a key preliminary step is the removal of water to concentrate the cells. Carvalho et al. (2011) used passive settling and flocculation of their microalgal culture to increase the cellular density prior to derivatization; however, passive settling is time consuming and requires that the culture not be disturbed. A faster technique uses centrifugation, where the microalgal culture is spun in a centrifuge at high RPM until a dense slurry of cells is collected at the bottom of the centrifuge container. The supernatant, composed of the aqueous media, is then carefully decanted from the pellet, leaving just the slurry of cells. Though this technique is efficient, it is difficult to assure the reproducibility of the composition of a culture's slurry, as more or less water could be decanted in separate batches, or cells could be lost in decanting the supernatant (Roush et al., 2008).

Another dewatering technique is filtration, where measured quantities of microalgal culture are deposited onto a filter while the aqueous growth media is pulled through the filter with suction. This method allows for more control over the amount of microalgae recovered in a sample and produces very consistent results (Prepas et al., 1988). Filter paper acts as an efficient vehicle for transferring dewatered culture from a preparation area to the reaction vessel. The culture can also be washed to remove residual material on its surface by rinsing the culture on the filter paper with an aqueous solution similar to its growth media (such as buffer,

salt water, etc). Rinsing with a solution of a different osmotic pressure than the growth media can cause cells to shrink or burst on the filter paper, potentially losing the storage products contained within the cell.

There exist many different kinds of filter paper, which are appropriate for different purposes. Cellulose paper is relatively inexpensive; it, however, carries a higher proportion of organic compounds compared to glass filter paper (Whatman, 2009). Glass filters, comprised exclusively of borosilicate fibers, have a smaller pore size compared to cellulose, and can also be ashed in a muffle furnace to thoroughly oxidize any potential organic compounds, assuring no contamination from external sources (Zhu et al., 1997a). This is especially important when analyzing small amounts of microalgae, which would typically have no more than 60 % lipid per dry mass (Griffiths et al., 2009). Using filter paper, however, yields less control over the content of water being added to the reaction as up to 0.5 mL of water can be retained by the filter (Cheng et al., 2010).

3.3 Lipid Extraction

Analysis of FAs in organic matter traditionally involves two preparation stages: extraction followed by derivatization (Carrapiso et al., 2000). The extraction process isolates lipids from organic matter through repeated solvent washings and mechanical disruption of cells. The derivatization process is a conversion of the FA in the extracted lipids into a volatile form to be analyzed by gas chromatography (GC) for quantification. These two processes are often performed independently of

each other in biological samples, where the abundant water present can be disruptive to the catalysts used in derivatization reactions (Bautista et al., 2009).

A number of extraction methods have been implemented with microalgae where samples are first concentrated and then dried. This facilitates contact between the non-polar solvents and the sample lipids and is typically done through bulk water removal and subsequent drying of the cells to remove intra- and extra-cellular water. Convection oven drying on pre-weighed metal trays has been occasionally used, although the heat of the oven may cause oxidation of lipids (Sukhija et al., 1988). Freeze drying is the preferred method, where the lack of heat and application of vacuum provides an environment to maintain lipid integrity (Aguilera et al., 2003). Thoroughly drying samples with freeze-drying methods usually takes between 12-24 hr, depending on the instrument used and the thickness (density and size) of the sample (Gutierrez et al., 2008).

After drying, the cells are typically mechanically lysed to liberate as much lipid as possible. Mechanical grinding is a common approach, achieved simply with a mortar and pestle or in the presence of solvent with a small lysing blender. The solvent system used to extract the lipids can be comprised of mostly non-polar organic solvents when extracting from a desiccated sample or can be a combination of more polar solvents, such as alcohols, esters, and ethers to ensure the most thorough solubilization of a range of lipids (Christie, 1993). If all lipid classes are desired, for quantification or isolation, a broader combination of solvents that range in polarity should be used, and a typical combination is a 2:1 ratio of chloroform:methanol (Folch et al., 1957). Lee et al. (1998) utilized this common

solvent system in a proposed extraction method for lipid determination in the green microalgae *Botryococcus braunii,* whereby microalgal samples were centrifuged into pellets and had their cell walls disrupted mechanically in a solvent system of 2:1 chloroform:methanol.

The Soxhlet method is a popular automated technique, where dry samples are placed in a porous cup within the Soxhlet apparatus and solvent is refluxed to repeatedly pass though the sample (Leray et al., 1995). This method is limited to a single solvent, as multiple solvents are unlikely to reflux at the same temperature. Thus, the diversity of lipids present in biological samples requires multiple solvents used in series due to the relative solubility of different lipid classes in organic solvents.

3.4 Wet Extraction Techniques

Techniques for lipid extraction from desiccated material are usually quite thorough; however, the requirement for a drying stage can easily prolong the sample preparation time by as much as a full day. There exist several methods of lipid extraction that do not require dry samples, and instead extract lipids directly from a biological sample using solvents with a range of polarities, such as the methods of Folch et al. (1957) and Bligh & Dyer (1959). Folch et al. (1957) used a 8:4:3 ratio of chloroform:methanol:water at a 20:1 ratio of solvent to sample, while Bligh & Dyer (1959) used a ratio of 10:10:9 (or, traditionally, 2:2:1.8) chloroform:methanol:water at a 4:1 ratio of solvent:sample. Because of the excessive solvents used in the Folch et al. (1957) technique, the Bligh & Dyer (1959) method

was developed to extract polar lipid from lean fish (i.e. low fat samples) using far less solvent relative to the sample. In a direct comparison of the two methods, samples with <2 % fat produced identical lipid yields, whereas lipid content in high fat samples were consistently underestimated with the Bligh & Dyer (1959) method (Iverson et al., 2001). With the Folch et al. (1957) method, fresh samples were mechanically ground and homogenized with chloroform and methanol. For ideal extraction efficiency, these mixtures would sit for several hours or overnight. The mixtures were washed with water, centrifuged, and the phases separated (top, aqueous phase discarded). The bottom layer was dried over ~1 g anhydrous sodium sulfate and concentrated by nitrogen stream to vaporize all remaining solvents, resulting in pure extracted lipids. Bligh & Dyer (1959) used a similar technique though they incorporated a higher ratio of water in the reagents compared to Folch et al. (1957). The downsides to these methods are their long extraction times and use of excessive amounts of organic solvents.

Other techniques being developed for lipid extraction and derivatization include microwave irradiation as a means to disrupt cells. Preliminary studies indicate that microwaving samples for a short period can be useful for lipid extraction with a solvent system comprised of equal volumes of ethyl acetate and cyclohexane (Batista et al., 2001). This method generated lipid yields that compared to those achieved with the Bligh & Dyer (1959) method for fish tissues (Batista et al., 2001). These methods have been used with great success to quantify the lipids of food samples with varying lipid contents (i.e. meat, dairy, and egg samples) without prior desiccation of the sample (Paré et al., 1997).

There also exist methods of lipid extraction without the use of organic solvents, such as supercritical fluid extraction (SFE), where CO_2 is pressurized under moderate temperatures to achieve a supercritical fluid state and subsequently used to extract organic compounds from samples (Davarnejad et al., 2008). Specifically applied to microalgal samples, OriginOil (2012) used electromagnetic lysing and pH modulation with CO_2 incorporation to break down the microalgal cell walls, which liberated lipids, and allowed passive gravitational settling to separate the biomass, water, and lipid phases. Though this patented method requires no solvents, the initial costs of implementation may be prohibitive and applications require large quantities of culture. Currently, SFE methods are limited by a number of factors, such as a requirement for drier samples and lack of proficiency in dissolving polar lipids in CO_2 without the aid of organic solvents (Hammam, 1992).

3.5 Lipid Analysis and Derivatization

The myriad of instruments available to identify organic compounds include: thin layer chromatography with flame ionization (TLC-FID), high performance liquid chromatography (HPLC), and gas chromatography (GC). TLC-FID and HPLC are useful for determining lipid classes in a sample, such as relative amounts of TAG or PL. If further characterization of individual lipids is required, such as quantifying individual PUFA, GC is typically used.

Lipid samples must be derivatized into volatile compounds before they can be analyzed by GC for FA composition. When FAs are the primary lipids being analyzed, an appropriate derivative would be methyl esters. FAs are liberated from

their molecular structure through transesterification in the presence of methanol, producing a FA methyl ester (FAME). This reaction is mediated with an acidic or basic catalyst.

Acidic catalysts are more widely used as they both transesterify FAs bound to alcohol groups as well as esterify FFA, although their reactions take place at higher temperatures and require long reaction times (Carrapiso et al., 2000). The American Oil Chemists' Society (AOCS) uses BF_3 in methanol as their primary acid-catalyzed transesterification reaction, although BF_3 is known to produce artifacts when analyzed by GC (Ackman, 1998). An equivalent substitution of BCl_3 in methanol works as well and avoids artifact formation (Ackman, 1998). Methanolic HCl is another commonly used acidic catalyst, whose popularity is likely due to its compatibility with both desiccated and aqueous samples (Meier et al., 2006). Methanolic H_2SO_4 has also been used as an acidic catalyst, although BF_3 has a higher transesterifying power than these latter two acids (Garcés et al., 1993).

Basic catalysts are also commonly used, as their reactions are carried out at ambient temperatures and are relatively quick, derivatizing samples within a few minutes. Bases only catalyze transesterification; they are unable to esterify FFA (Suter et al., 1997). Base-catalyzed reactions are also more sensitive to the presence of water as saponification may occur (Bautista et al., 2009). When a basic catalyst transesterifies FAs of a TAG molecule in the presence of methanol, the anticipated products are FAME and free glycerol (Fig. 3.3). If water is also present and the reaction is heated, hydrolysis may occur, generating carboxylic acid salts (see structure 10 in Fig. 3.1, where R" is replaced with the alkali metal from the base

catalyst), and free glycerol (Ackman, 1998). In the formation of undesirable byproducts, the basic catalyst is quickly consumed, rather than regenerated, and the reaction ends prematurely, resulting in partial derivatization. The most widely used basic catalyst for FAME preparation is sodium methoxide ($NaOCH_3$). Potassium hydroxide (KOH) in methanol and sodium hydroxide (NaOH) in methanol are also used, although both are more prone to saponification reactions compared to sodium methoxide (Christie, 1993).

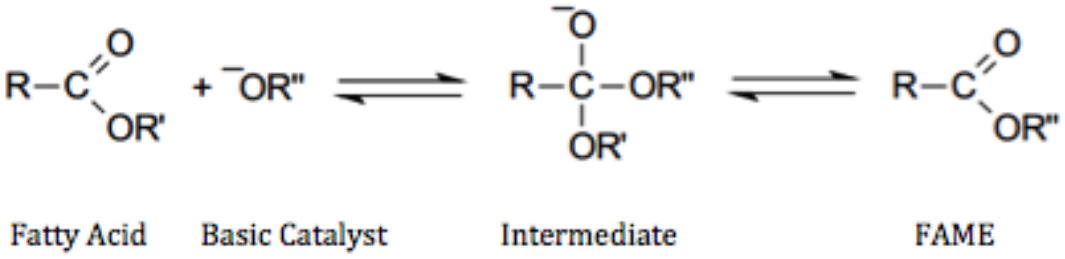

Figure 3.3. Base-catalysed transesterification. Source: Christie (2011).

3.6 *In situ* Derivatization

In the interest of creating the most rapid method of lipid extraction and FAME formation, a number of *in situ* methods that combine both of those steps have been proposed. *In situ* methods avoid the cumbersome step of first extracting the lipids from their organic matrix to then be derivatized by acidic or basic catalyst, as well as removing the risk of sample loss through excessive sample handling. Instead, the derivatization step happens *in situ*; reagents are incorporated with the sample directly, which simultaneously extracts lipids into solution and derivatizes them.

Aside from saving time and solvent, there is evidence that acidic/basic *in situ* reactions may contribute to higher FAME yields (Lepage et al., 1986). This may be due to a minimization of sample losses from solvent washings, and the transferring and evaporating of solvents. However, Lepage & Roy (1986) also suggested that polar lipids require an additional hydrolysis step before they can be fully removed from intracellular water.

The main limitation of *in situ* reactions compared to separately extracting lipids is the water tolerance of the derivatization reaction. Studies suggest that water contents as low as 10 % (v/v) for base-catalyzed reactions and 20 % (v/v) for acid-catalysed reactions may interfere in derivatization reactions (Carrapiso et al., 2000). Acidic catalysts have a higher tolerance for water, though water can dissociate one of the derivatization intermediates (see Intermediate 2 in Fig. 3.4), into a free acid (Christie, 1993). Though basic catalysts seem more sensitive to the presence of water due to saponification, studies suggest that saponification reactions are far slower than transesterification reactions and can be avoided if the catalyst is neutralized with an acid after the derivatization has taken place (Glass, 1971).

The solvent system used in the *in situ* derivatization must be miscible with the water present in the sample as well as the generated FAME. Suter et al. (1997) suggested the use of dioxane, with the addition of dimethylformamide (DMF), to fully solubilize fat, especially PUFA, and bring all elements of the reaction into a single solution. Other solvents were suggested, including benzene, dichloromethane,

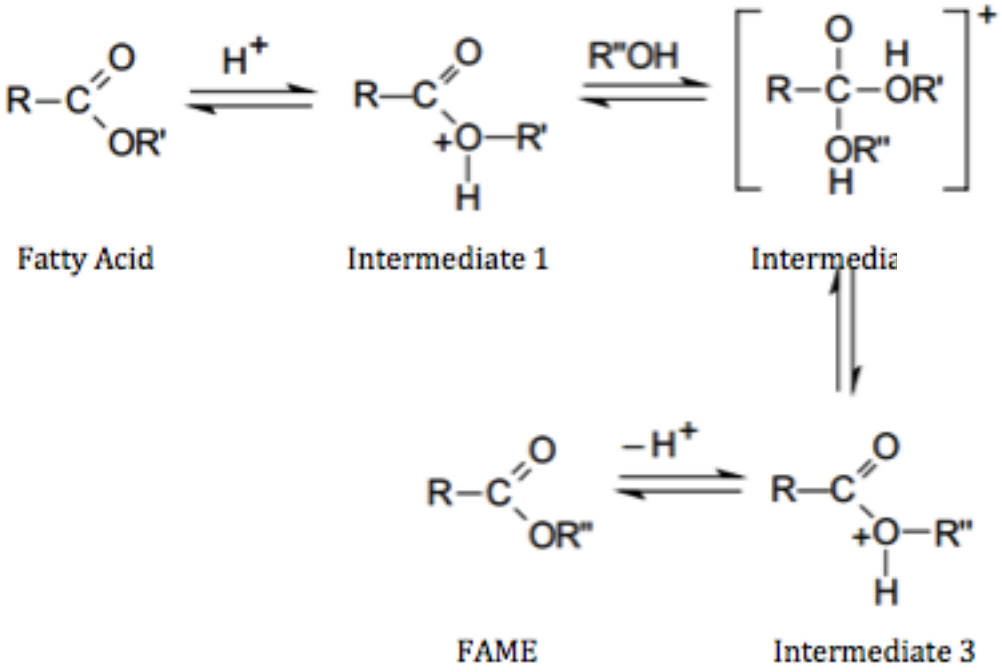

Figure 3.4. Acid-catalysed transesterification reaction. Source: Christie (2011).

toluene, or tetrahydrofuran, but were all judged inferior to dioxane when derivatizing samples which had a high water content, such as milk or ice cream (Suter et al., 1997). Samples were treated with dioxane and 5 % sodium methoxide in methanol and were left to react before the reaction was stopped and FAME were extracted with heptane. The results of the study indicated good reproducibility of the results and no effect of the water content on the transesterification reaction (~5 % water contributed by the sample). Compared to other *in situ* derivatization reactions, such as the Park & Goins (1994) method, this method required larger aliquots of several reagents and solvents, and required several steps.

Applying the strengths of both basic and acidic catalysts, one *in situ* method first transesterified the sample with a fast, base-catalyzed reaction, and then applied

an acidic catalyst to the solution to esterify any remaining FAs and neutralize the basic catalyst before saponification occurred (AOCS, 1998). This technique was popularized by the American Oil Chemists Society (AOCS) whose official methods have been implemented in numerous *in situ* derivatization studies (Ichihara et al., 1996; Jalali-Heravi et al., 2004; Ehimen et al., 2010).

Park & Goins (1994) described a method where food-based samples (e.g. meat, egg yolk, infant formula, etc.) were treated with methylene chloride and 0.5 N NaOH in methanol, then heated at 90 °C for 10 min. After cooling, the solutions were mixed with 14 % BF_3 in methanol and reheated at 90 °C for another 10 min. The addition, incorporation, and extraction of hexane to the sample successfully recovered all FAME. Comparison to a traditional method (i.e. Folch et al. (1957) extraction with AOCS derivatization) yielded nearly identical results for all of the samples. Specific samples tested were egg yolk (high in phospholipid), liquid infant formula (high water content), and meats (tissue samples), all of which had their FA accurately profiled (Park et al., 1994).

O'Fallon et al. (2007) performed a similar reaction to Park & Goins (1994); however, they deliberately incorporated water into the reagents for their two step FAME synthesis. Fresh samples were mixed with KOH in water and MeOH. The solutions were incubated at 55 °C for 90 min, cooled, acidified with 24 N H_2SO_4, and heated again at 55 °C for 90 min. Hexane was used to extract the generated FAME. The method deliberately saponified the samples to create FFA with a basic catalyst and then esterified the FFA with an acidic catalyst. This method represented a 22 and 14 % improvement over just base- or acid-catalysed reactions, respectively. The

direct method's proficiency was especially evident with PUFA, as this method out-performed acidic and basic catalysts in the extraction of EPA by 10.3 and 17.5 % and DHA by 15.7 and 26.8 %, respectively. O'Fallon et al. (2007) suggested that water contents up to 33 % by volume did not hinder reaction performance.

Focusing more strongly on a single catalytic reaction, a third *in situ* method, proposed by Lepage & Roy (1986), used 2:1 methanol:benzene as solvent with 5:100 acetyl chloride:methanol. The mixture was held at 100 □C for 1 hr to generate FAME. This method is considered the standard for *in situ* FAME synthesis of biological samples, though criticism has been drawn to the use of benzene, as it is a known carcinogen (Barreto et al., 2009). Benzene's role within the reaction is to solubilize nonpolar lipids, and can thus be replaced with other reagents, such as hexane, methylene chloride, toluene, and tetrahydrofuran (Park et al., 1994). Lepage & Roy (1986) recovered up to 20 % more lipids from samples of milk and adipose tissue when compared to traditional extraction methods, such as Folch et al. (1957) with acetyle chloride in methanol derivatization. Water levels >5 % inhibited the reaction and there was no benefit in using reaction times >1 hr.

A base-catalysed *in situ* reaction was proposed by Zeng et al. (2009) for the derivatization of lipids in milled sunflower seeds. The initial *in situ* screening tested KOH and NaOH in methanol, the presence of a co-solvent, diethoxymethane (DEM), and reaction times and temperatures ranging from 10 – 60 min and 20 – 65 °C, respectively. This initial screening produced FFA contents of 0.67 – 9.61 % lipids, indicated a slight preference for NaOH over KOH, determined a need for DEM to bring the moisture from the sample into solution, and demonstrated that the lowest

reaction time and temperature, 10 min and 20 °C, respectively, were the preferential reaction conditions.

Further adjustment to *in situ* methods could include investigation of their ideal reaction temperature and length of reaction. Investigation in reducing the reaction temperature to better preserve the lipids is of great interest, as is reducing the length of reaction to make the *in situ* method more efficient. Standard derivatization often occurs at a high temperature for an extended period of time, such as 100 °C for 1 hr associated with acid catalyzed derivatization, while some methods that use basic catalysts are performed at 45 °C or as low as ambient temperature (Ichihara et al., 2010). Basic reactions can proceed at ambient temperature proficiently, potentially making the heating of the basic reagent in the Park & Goins (1994) method needless. Shortening the length of reaction may prevent the lipids in the sample from being overexposed to a catalyst, which can produce artifacts and unwanted reactions, such as saponification with a basic reagent (Bautista et al., 2009).

3.7 Internal Standard

To accurately quantify the FAME produced by a specific method, the use of an internal standard is imperative. The standard must be of similar material to that which is being extracted and must not co-elute with any components present in the sample. Pure standards of FA are a popular choice. However, it may be difficult when working with marine samples to select an appropriate FA to use as internal

standard; the FA profile of marine-based samples are diverse, so it can be difficult to find a FA to serve as internal standard that is not already present.

In order to avoid interference between the sample and the internal standard, the standard is selected to not co-elute with FAME. Typical lipids selected as standards are usually straight chain FAs with an odd number of carbon atoms, as these species are uncommon in nature (Ackman, 1965). For instance, a typical internal standard is tridecanoic acid (C13:0), used by both the Lepage & Roy (1986) and O'Fallon et al. (2007). Meier et al. (2006) and Abdulkadir & Tsuchiya (2008) both suggest nonadecanoic acid (19:0) and both use marine samples for their studies. Suter et al. (1997) used several internal standards, including triundecanin (a TAG consisting of 11:0 FA), 1-tetradecene (14:1 alkene), and methyl nonanoate (19:0 FAME). It is not uncommon to incorporate several internal standards into a sample to act as a source of reference for all of the internal standards and their unique response factors.

The timing of the addition of the internal standard can have a significant effect on the determination of FA concentration (Han, 2010). For the most consistent results, it is best to incorporate the internal standard as soon as possible in sample workup. Any potential losses of the sample through transfers or incomplete derivatization will be reflected in the internal standard amount.

Another internal standard approach is to incorporate a known amount of a standard whose solubility is the same as the anticipated FAs found in the sample, but does not participate in the derivatization reaction. This kind of internal standard is useful when testing the proficiency of a derivatization reaction, as the amount of

internal standard in the sample will be consistent regardless of how thoroughly the derivatization reaction proceeded. The same effect is not observed with FAs as internal standards because they are subject to the same limitation of derivatization as the lipids in the sample, although methyl esters of FA would work well as they are already a derivatized FA. An example of an internal standard which does not participate in the derivatization reaction is 5-α-cholestane, a saturated 27-carbon hydrocarbon that elutes by GC after the typical range of FA structures in marine lipids, whose latest eluting FA is usually 24:1 (Padre et al., 2007, Fig. 3.5). As a saturated hydrocarbon, there is no functional group that can be methylated, which ensures consistency in the amount of 5-α-cholestane present in each sample (Alonso et al., 1995). The consistency in the content of cholestane in the sample accounts for any sample losses throughout the derivatization as an equivalent proportion of internal standard to sample would be present in the reagents.

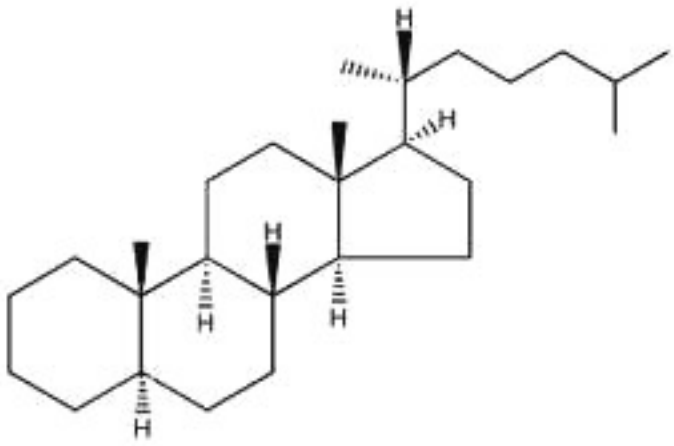

Figure 3.5. Structure of 5-α-cholestane. Source: Leray (2011).

3.8 Lipid Class Analysis

To assess the success of the derivatization reaction, the lipid classes of the hexane-extracted phase of the sample can be analyzed with instruments suited to separating lipid classes. Thin layer chromatography with flame ionization detector (TLC-FID) is based on the affinity principle between the sample and the stationary (silica on chromarod) and mobile (solvent system) phases. The separated sample is then analyzed in the FID by measuring the change in voltage applied across the flame as it interacts with each separated band of the sample, producing a chromatogram of the peak responses. GC works on a similar principle, where vaporized FAME is retained on a liquid stationary phase and elutes into the FID as it passes the length of the column, generating a chromatogram.

Quantifying the peak areas of TLC-FID chromatograms can be done by comparison of peak areas to that of the internal standard, the quantity of which is known. To produce a more accurate calculation of the contents of the components in TLC-FID, standard curves are generated for each anticipated lipid class based on area response. These calibration curves can be used to determine an absolute quantity of the individual component, as the intensity of the FID signal varies with the structure it is encountering (Parrish, 1987). The internal standard can then be used to relate the calibrated peak areas back to the original sample content. This same process is used to determine FA content in GC chromatograms; however, FA responses are of a generally known quantity and part of the analytical software associated with the GC.

CHAPTER 4: EXPERIMENTAL DESIGN

The goal of this project was to develop or adapt a rapid *in situ* transesterification method to quantify the FAs in microalgal samples by GC. Current methodologies require long extractions and multiple changes of glassware, both of which contribute to sample loss and inefficient use of lab time. The initial stage of the project was to narrow down the numerous candidates of *in situ* derivatization methods and apply them to microalgal cultures with comparison to the traditionally used method, Folch et. al. (1957) extraction followed by Hilditch et al. (1964) derivatization (F&H). The Folch et al. (1957) method repeatedly extracts lipids from biological samples using a 2:1 ratio of chloroform:methanol with mechanical grinding to promote extraction. The Hilditch et al. (1964) derivatization method transesterifies acyl lipids with a H_2SO_4 catalyst at 100 °C for 1 hr. Of the candidate *in situ* techniques reviewed, two were selected for comparison, based on methods by Park & Goins (P&G; 1994) and Lepage & Roy (L&R, 1986). The P&G method directly derivatizes the acyl lipids within a biological sample by transesterifying with two catalysts, first using NaOH (basic), followed by BCl_3 (acidic; originally BF_3 from the P&G method; see Section 3.5 above), each reacted at 90 °C for 10 min. The L&R method directly derivatizes acyl lipids within a biological sample by transesterifying with an acid catalyst, acetyl chloride, at 100 °C for 1 hr. These two methods were anticipated to adequately manage the high water contents of microalgal samples, had relatively rapid speeds of reaction, and each represented a different *in situ* technique; Park & Goins (1994) incorporated the dual catalyst technique which

utilizes the strengths of base- and acid-catalyzed derivatization, while Lepage & Roy (1986) solely utilized an acidic catalyst.

Due to the availability of instrumentation, TLC-FID was used in this research for lipid class determination, while GC was used to identify and quantify FAs.

The two selected *in situ* methods, P&G and L&R, were first applied to small aliquots of dense microalgal culture and FAME production was compared to the traditional method, F&H. The methods were then applied to filtered microalgal culture to better replicate the type of microalgae sample more likely used in a lab setting. The comparisons led to the selection of the P&G method for further study.

The P&G method was optimized for sample load and water content through several experiments as the original method did not suggest an absolute lipid limit, though it did suggest a 0.10 mL homogenized aqueous aliquot of biological sample, indicating a water load of <0.1 mL. Due to the oleaginous nature of microalgal samples, it was necessary to establish a lipid load to ensure efficient derivatization. It was also necessary to establish a water tolerance for the method as water may interfere with derivatization reactions and typical microalgal sample preparation includes filtration onto filter paper, which may retain between 0.2 to 0.5 mL of water. The maximum lipid loading experiments used a range of menhaden oil contents to represent high sample loading, while low sample loading was tested with filtered microalgal culture. The sample to solvent ratio was verified by maintaining a standard sample application of filtered microalgal culture and using one, two, and three times the volume of reagents. The maximum water tolerance of the method was tested with a range of menhaden oil contents to represent high

sample loading, while low sample loading was tested with small quantities of extracted microalgal lipid and filtered microalgal culture. To investigate water tolerance with whole microalgal cells, samples were dewatered onto filter paper and further dried by increasing the filtration time by 0, 30, and 60 s after visible dryness was observed at the surface of the culture. The results of the loading and water tolerance experiments confirmed the P&G method as an appropriate method for derivatizing microalgal cultures dewatered onto filter paper.

The method was then optimized for reaction temperature and length of reaction. The base- and acid-catalyzed steps of the reaction were treated as two different variables and were optimized with a full factorial design, changing their reaction temperature to 20 (ambient temperature), 55, and 90 °C (original reaction temperature of the method), while maintaining the length of reaction at 10 min for both catalysts. The literature concerning optimal reaction times for basic and acidic derivatization catalysts suggested the use of higher temperatures for acidic catalysts and lower temperatures for basic catalysts. Thus, the basic reaction temperature of the P&G method was maintained at ambient temperature while the acid-catalysed reaction was held at 80, 90, and 100 °C. The length of reaction for the basic and acidic catalysts were then varied from the original 10 min with a full factorial design using 1, 5, and 10 min at the optimum reaction temperatures.

The results of the experiments established an appropriate working range of lipid load and water tolerance, and the optimal reaction temperatures and times for the basic and acidic catalysts.

CHAPTER 5: MATERIALS AND METHODS

5.1 Materials

5.1.1 Laboratory Equipment

5.1.1.1 Glassware

All glassware used directly in the extraction or derivatization procedures was cleaned thoroughly to remove residual lipids and to avoid contamination. The glassware, primarily 10 mL glass centrifuge tubes with Teflon-lined caps, were washed, dried, and triple rinsed with methanol and chloroform, and once with dicholoromethane, then left to dry. Rinsing with a solvent was done by adding approximately 1 mL of the solvent to the centrifuge tube, capping it, shaking it well, and discarding the solvent.

5.1.1.2 Preparing the Filter Paper

The filter papers used were Whatman GF/C 47 mm glass fiber filter papers and were handled with dicholomethane-rinsed tweezers and spatulas. Filter papers used to determine dry masses of microalgal culture were placed in tared and labeled fluted aluminum weigh boats and their mass was measured with a Mettler Toledo balance (maximum weight 210 g, mininum weight 0.01 g, precision to 0.1 mg).

5.1.1.3 Solvents and Chemicals

All Optima grade solvents and all chemicals were purchased from Sigma.

5.1.2 Preparation of Supplemental Materials for Reactions

5.1.2.1 Internal Standard Preparations for Derivatization Reactions

For all three methods, an internal standard was prepared with a precisely measured amount of 5-α-cholestane dissolved in the organic solvent(s) used in the respective method (Table 5.1). All internal standards were precisely measured with a syringe of appropriate capacity (2.0, 1.0, and 0.25 mL capacity for F&H, L&R, and P&G, respectively). Between uses, the internal standard preparations were stored in a -20 °C freezer and brought to ambient temperature and vortexed before use.

Table 5.1. Internal standard preparations for three methods.

Derivatization method	5-α-cholestane concentration (mg/mL)	Solvent	Amount Solvent per reaction (mL)
F&H	0.067	Dichloromethane	1.5
L&R	0.1	3:2 MeOH:Hexane	1.0
P&G	1.0	Dichloromethane	0.1

5.1.2.2 Microalgal Cultures Used in Filtration Experiments

The Aquatron facility at Dalhousie University supplied three microalgal cultures, *Tetraselmis sp., Isochrysis sp*., and *Ankistrodesmus falcatus*. The dry mass of microalgae used for each experiment is listed in Table 5.2 (see APPENDIX A for detailed measurements). All of the cultures had been cultured photoautotrophically in filtered seawater for 2-3 weeks in 200 L columns lit with 4x 40 W fluorescent tubes and bubbled with sterile air for constant mixing and aeration. Different cultures were used in different experiments due to availability and access, as well as to provide insight into the method's ability to directly transesterify acyl lipids of varying microalgal cultures.

Table 5.2. Summary of microalgal cultures used in various experiments.

Experiment (results section)	Species	Dry mass (mg)
Slurry culture to select method (6.1.1)	*Tetraselmis sp.*	11.9 ± 0.6
Filtered culture to select method (6.1.2)	*Tetraselmis sp.*	4.9 ± 0.2
Lipid load with microalgae (6.2.2)	*Isochrysis sp.*	4.7 ± 0.2, 7.9 ± 0.5, 11.0 ± 1.3
Lipid load with increasing reagents (6.2.3)	*Isochrysis sp.*	13.8 ± 0.5
Water content with microalgae (6.3.3)	*Isochrysis sp.*	12.6 ± 3.1
Temperature variation (6.4.1)	*A. falcatus*	6.4 ± 0.1
Temperature refinement (6.4.2)	*A. falcatus*	6.4 ± 0.1
Reaction time variation (6.4.3)	*A. falcatus*	6.4 ± 0.1

The three cultures used in this research each had common lipids, primarily PL with some TAG (see Table A1. for trace amounts of TAG and PL identified by TLC-FID for each culture). All of the cultures are unicellular, mononucleated, and have cell walls (NCMA, 2012). *Tetraselmis sp*. is ovoid-shaped approximately 8-14 μm in length, *Isochrysis sp*. is round-shaped approximately 5-7 μm in length, and *A. falcatus* is spindle-shaped approximately 1-6 μm in length (AlgaeBase, 2012; NCMA, 2012). *Tetraselmis sp*. and *A. falcatus* are both chlorophytes, making their cells bright green colours (Chu et al., 1995; Day et al., 1996), while *Isochrysis sp*. is a haptophyte, which has a brown colouration (Andersen, 2004).

Cultures were transferred from the Aquatron to the lipids lab in 500 mL plastic Nalgene bottles, which were frequently rinsed with 10 % HCl solutions to prevent contamination. Cultures were occasionally stored for a short period (i.e. overnight) on lab benches with their caps loosely fitting.

5.2 Methods

5.2.1 Microalgal Culture Preparations

5.2.1.1 Preparing Microalgal Slurries Through Centrifugation

Microalgal cultures were condensed into a slurry through centrifugation and used in the original screening of the two *in situ* methods. Lipids were also extracted from these slurries and used in the water tolerance experiment with microalgal

lipid. Microalgae samples were placed in 40 mL glass centrifuge tubes and centrifuged in an International Equipment Company HN-SII centrifuge at 750 RPM for 10 min, or until the media in the upper portion of the test tube was clear with a dense slurry of microalgae at the bottom. The clear supernatant was decanted and discarded, while the slurries of microalgae were pooled into a single container. This process was repeated until a desired content of microalgae was achieved. This method produced microalgae slurries consisting of 5-10 % solids.

5.2.1.2 Filtering Microalgal Culture

5.2.1.2.1 Preparing Filtered Microalgal Cultures

Microalgal cultures were dewatered onto glass fiber "C" filter paper, Whatman GFC, using vacuum filtration with a 300 mL filtration apparatus. A water trap was attached to a water aspirator and fit with a rubber collar. A 1 L filter flask was attached with thick rubber hosing to the rubber collar of the water trap. The funnel of the filter apparatus was inserted into the mouth of the filter flask with a rubber collar, the 47 mm mesh filter was laid on the funnel with a rubber gasket, and filter paper was laid on top of the mesh filter. The filter paper was wetted with UV sterilized and filtered seawater (UVSFS) from a squeeze bottle before filtering. The graduated top of the filter apparatus was clamped to the top of the unit. A vacuum was applied from the water aspirator. Microalgal cultures were thoroughly mixed to prevent culture settling and volumes of culture were measured with a glass pipette and rubber bulb. Glass pipettes were cleaned and triple rinsed with cholorform and

methanol before use and their volumes were calibrated for use at ambient temperature. A specific volume of microalgal culture was applied to the wetted filter paper and filtered to visible dryness. The glass pipette was then rinsed with ~10 mL UVSFS by removing the rubber bulb and rinsing the interior of the pipette from the top down directly onto the filter paper. The graduated cup of the apparatus and the microalgal culture on the filter paper was also rinsed with ~10 mL UVSFS, filtered to visible dryness on top, and vacuum was continued to be applied for ~10 s, or until no substantial amounts of water were seen dripping from the funnel. The graduated cup was removed and the interior was wiped down with a Kimwipe. A spatula was inserted at the edge of the filter paper to break the vacuum seal and the filter paper was removed from the mesh filter. If the sample was to be derivatized, the filter paper was rolled and folded with a spatula and tweezers and inserted into a chloroform and methanol triple-rinsed centrifuge tube. If the sample was to be dried for mass determination (see Section 5.2.1.2.2 below), the filter paper was simply peeled from the mesh filter and returned to its labeled aluminum weigh boat. The mesh filter was wiped with a Kimwipe and the next sample was processed following the above procedure.

5.2.1.2.2 Dry Mass Determination

Masses of filters and aluminum weigh boats were recorded prior to microalgae filtration. Microalgal cultures were filtered onto weighed filter paper and weighed again to establish wet filter mass. The weigh boats were placed on glass trays and dried in an oven held at 100 °C for at least 12 and up to 24 hr. After drying,

the filters were placed in a desiccator to cool to ambient temperature. Masses of the filters plus dried cultures were then recorded. To assure full dryness of the samples, cultures were returned to the 100 °C oven for an hour, cooled to ambient temperature in the desiccator, and re-weighed. If the two dry masses agreed within 0.0001 g, the samples were assumed to be fully dried. Triplicate dry mass determinations per culture were carried out to ensure consistency.

5.2.2 Lipid Extractions

5.2.2.1 Traditional Extraction: Modified Folch et al. (1957) for Microalgal Samples Dewatered Through Centrifugation or Filtering

Samples used in the traditional Folch et al. (1957) extraction were concentrated aliquots of *Tetraselmis sp.* slurry (0.10 mL) that were either placed directly into a 10 mL centrifuge tube or first filtered onto filter paper, contributing approximately 11 and 5 mg dry microalgal mass, respectively. These two masses were derived from two independently prepared microalgal slurries of *Tetraselmis sp.* (see Section 5.2.1.1). Chloroform (2.0 mL) and ice-cold methanol (1.0 mL) were added and a triple-rinsed glass rod was used to grind the sample. The rod was rinsed with 1.0 mL solution of 2:1 chloroform to methanol, followed by 0.50 mL chloroform-extracted distilled water. The tube was nitrogen purged to avoid oxidation of the lipids, vortexed, and sonicated in an ice bath for 4 min. The tube was centrifuged at 750 RPM for 10 min or until the organic and aqueous phases separated. The bottom (organic) layer was removed from the centrifuge tube with

the double pipetting technique where a long Pasteur pipette was inserted into a short pipette, air was gently expelled from the tip of the pipettes as they passes through the organic/aqueous interface to avoid pipetting any aqueous phase, and the short pipette was used as a bridge through the aqueous phase. Without removing any of the interface or top phase, the bottom phase was pooled into another solvent-rinsed and labeled centrifuge tube. Both pipettes were rinsed into the original centrifuge tube containing the sample with 3 x 1.0 mL ice-cold chloroform and reserved. The sample was vortexed, sonicated, and centrifuged in the same manner as before. The bottom layer was again double-pipetted and pooled with the organic phase and the pipettes were again rinsed with 3 x 1.0 mL ice-cold chloroform. This process was repeated at least once more, or until no colour remained in the organic phase of the original centrifuge tube with the sample. The organic phase was then blown down under a stream of nitrogen, followed by application of vacuum to ensure all solvent had been removed. Samples were then immediately derivatized or stored in 2.0 mL chloroform in a nitrogen-purged tube, sealed with DuraSeal, and stored in a 4 °C refrigerator.

5.2.2.2 Bligh and Dyer (1959) Method for Bulk Lipid Extraction

A concentrated slurry of microalgal culture (1.0 mL) comprised of 2:1 ratio of *Tetraselmis sp*. and *Isochrysis sp*. was dispensed directly into a pre-rinsed 10 mL centrifuge tube for bulk microalgal lipid extraction (extracted lipid used in Section 5.2.4.2.2). An aliquot of 3.75 mL 1:2 chloroform:methanol solution was added and the tube was nitrogen purged. The sample was sonicated for 10-15 min, 1.25 mL

chloroform was added, and the sample was mixed by vortex for 1 min. Distilled water (1.25 mL) was added and the sample was again mixed by vortex for 1 min. The tube was centrifuged at 750 RPM for 10 min or until the phases were separated by a thin interface. The bottom phase of the sample was collected with three repetitions of the double pipetting technique (see 5.2.2.1 above) and pooled into a labeled pre-rinsed centrifuge tube. The extracted lipid was diluted in chloroform to 0.5 % lipid by weight in a nitrogen-purged test tube, sealed with DuraSeal, and stored at 4 °C.

5.2.3 Methods for FAME Synthesis

5.2.3.1 Traditional FAME Method: Hilditch et al. (1964) Procedure for Samples Less than 100 mg Lipid

Methylene chloride (1.5 mL) with internal standard 5-α-cholestane (Table 5.1) and 3.0 mL Hilditch reagent (0.5 N H_2SO_4 in anhydrous Na_2SO_4-dried methanol, freshly prepared daily) was added to a 10 mL centrifuge tube containing the lipid extract produced with the Folch et al. (1957) extraction (see Section 5.2.2.1 above). The tube was nitrogen-purged, vortexed, and placed in a 100 °C heating block for 1 hr. The original volume was marked on the outside of the tube and periodically checked to ensure that solvent did not evaporate during heating. The tube was occasionally shaken during heating to promote mixing. The sample was cooled to ambient temperature, 3.0 mL hexane and 1.0 mL distilled water were added and the sample was vortexed. The sample was centrifuged at 750 RPM for 2-

5 min, or until a clean interface was achieved. The top layer, containing hexane and FAME, was pipetted into a labeled, pre-rinsed centrifuge tube. The sample was rinsed at least once more with hexane, or until the hexane extract was clear, and extracts were pooled into the corresponding centrifuge tube. Distilled water (2.0 mL) was added to the hexane extracts and the sample was vortexed and centrifuged at 750 RPM until a clean interface was achieved (2-5 min). The top layer was pipetted into a labeled, pre-rinsed centrifuge tube, without carrying over any of the interface or bottom layer. A ~1 g scoop of anhydrous Na_2SO_4 was added to the sample and it was vortexed. The hexane extracts were finally transferred to a labeled, pre-rinsed, and weighed vial. Hexane was evaporated under a stream of nitrogen and any remaining traces of hexane were removed by vacuum evaporation. Once fully dried, the pre-weighed vial was again weighed to determine the mass of the FAME produced. Samples were reconstituted with hexane to a concentration of approximately 0.5 mg/mL. Samples were nitrogen-purged, sealed with DuraSeal, and stored in a 4 °C refrigerator.

5.2.3.2 Direct FAME Synthesis Methods

5.2.3.2.1 Modified Lepage & Roy (1986) Method: Acetyl Chloride Catalyzed *in situ* Derivatization

A concentrated aliquot of *Tetraselmis sp.* slurry (0.10 mL) was placed directly into a pre-rinsed 10 mL centrifuge tube or dewatered onto filter paper, and contributed ~11 and 5 mg of dried culture, respectively. These two masses were

derived from two independently prepared microalgal slurries of *Tetraselmis sp.* (see Section 5.2.1.1). Then, 1.0 mL of a 3:2 methanol:hexane solution containing 5-α-cholestane internal standard (Table 5.1) was added. The original method used methanol:benzene which was exchanged here for methanol:hexane due to availability and safety. Freshly prepared acetyl chloride solution (1.0 mL prepared as a 5:100 acetyl chloride:methanol volume ratio by gradually dripping the acetyl chloride into cool, anhydrous Na_2SO_4-dried methanol in a fume hood) was added, the solution was nitrogen-purged, vortexed, and the solution level marked. The sample was placed in a 100 °C heating block for 1 hr and was periodically checked for leaks and shaken to promote mixing. When cool, 1.0 mL distilled water and 1.0 mL hexane were added to the centrifuge tube and vortexed. The top layer was removed and placed in a clean vial. Another 1.0 mL hexane was added to the original centrifuge tube, vortexed, and pipetted and pooled in the vial with the first 1.0 mL of hexane. The hexane-extraction was repeated once more if the previous extraction was significantly pigmented. Distilled water (1.0 mL) was added to the pooled hexane extracts and vortexed for a water rinse. The tube was centrifuged and the top layer of hexane was removed, placed in another clean tube, and dried over ~1 g anhydrous Na_2SO_4. The hexane extracts were moved to a last pre-weighed, clean tube and blown to dryness under a stream of nitrogen, followed by an application of vacuum to ensure all traces of hexane were removed. The samples were weighed and reconstituted to a concentration of approximately 0.5 mg/mL with hexane. Samples were nitrogen-purged, sealed with DuraSeal, and stored at 4 °C.

5.2.3.2.2 Modified Park & Goins (1994) Method: NaOH (Basic) Followed by BCl_3 (Acidic) Catalysis *in situ* Derivatization

The preparation of specific samples and modifications to standard procedure outside of the initial screening tests are described in Section 5.2.4, while the standard P&G method used in this study is described below.

Samples of *Tetraselmis sp*. were condensed into a slurry (0.10 mL) or dewatered onto glass filter papers, contributing ~11 and 5 mg dried culture, respectively, and were inserted into triple-rinsed 10 mL centrifuge tubes with Teflon-lined caps. These two masses were derived from two independently prepared microalgal slurries of *Tetraselmis sp.* (see Section 5.2.1.1). Dichloromethane (0.10 mL) with internal standard was added (Table 5.1), followed by 1.0 mL of the basic reagent (0.5 N NaOH in methanol). The tube was nitrogen-purged, capped, and sonicated for ~5 min. The samples were then heated for 10 min at 90 °C on a heating block. The tubes were taken off the block and cooled to ambient temperature. Then, 1.0 mL of the acidic catalyst (14 % BCl_3 in methanol, purchased prepared and stored in 4 °C refrigerator) was added to the tubes. The original method used BF_3 which has been reported to cause artifacts in chromatograms and thus was exchanged for BCl_3 (Ackman, 1998). The tubes were nitrogen-purged, capped, vortexed, and heated for 10 min at 90 °C on a heating block, and were then cooled to ambient temperature. When cool, 1.0 mL distilled water and 1.0 mL hexane were added to the centrifuge tube and vortexed. The top layer was removed and placed in a cleaned and triple-rinsed vial. Another 1 mL hexane was added to the original tube and the mixture was vortexed. The hexane

layer was removed and added to the first 1.0 mL of hexane. The hexane extraction was repeated if the previous extraction was heavily pigmented. The hexane extracts were washed with 1.0 mL distilled water to remove any aqueous carry-over. The top layer of hexane was then pipetted to another clean vial containing a ~1 g scoop of anhydrous Na_2SO_4 to dry the hexane of any remaining water. The extracts were moved to a pre-weighed, clean vial and blown to dryness under a stream of nitrogen, followed by an application of vacuum to ensure all traces of hexane were removed. The samples were weighed and reconstituted with hexane to a concentration of ~0.5 mg/mL. Samples were stored nitrogen purged, sealed with Duraseal, in a 4 °C refrigerator.

5.2.4 Optimization of the P&G Method

5.2.4.1 Sample Loading Optimization

5.2.4.1.1 High Sample Loading with Menhaden Oil

To determine an appropriate lipid load for the P&G method, 0.1, 0.3, 0.5, 1.0, 3.0, and 5.0 mg menhaden oil was applied to dry filter papers from hexane-diluted stock solutions (Table 5.3). The range of menhaden oil added was selected to represent two different loading classes of lipid: 0.1 – 0.5 mg for low sample loading and 1.0 – 5.0 mg for high sample loading. Bulk solutions of menhaden oil were prepared by weighing samples of menhaden oil into triple-rinsed 40 mL capacity

glass centrifuge tubes and diluting with hexane measured by pipette. Volumes of hexane-diluted menhaden oil were added to a 10 mL glass centrifuge tube containing a GFC filter paper using a 2.0 mL capacity syringe that had previously been cleaned and triple-rinsed. The menhaden oil solutions were then blown to dryness with a stream of nitrogen, followed by application of vacuum to ensure all hexane had been removed. To replicate the water content of a homogenized aqueous aliquot, 0.1 mL distilled water was added to each sample, followed by derivatization with the P&G method (see Section 5.2.3.2.2 above).

5.2.4.1.2 Low Sample Loading with Filtered *Isochrysis sp.*

In conjunction with the lipid load tolerance, the method's cellular load was also tested to get an approximate range of accuracy with lipid quantity from filtered

Table 5.3. Contribution of menhaden oil to low and high loading sample sizes.

Loading Class	Concentration of bulk solution (mg/mL)	Volume solution added (mL)	Amount menhaden oil (mg)
Low	0.250	0.40	0.10
	0.250	1.2	0.30
	0.250	2.0	0.50
High	2.50	0.40	1.0
	2.50	1.2	3.0
	2.50	2.0	5.0

cultures. Amounts of 50, 75, and 100 mL of *Isochrysis sp*. culture, contributing approximately 4.7, 7.9, and 11 mg of dry culture, respectively, were dewatered onto glass filters, vacuum filtered until visible surface dryness with an additional ~10 s of vacuum filtration, and transesterified with the P&G method (5.2.3.2.2).

5.2.4.1.3 Increasing Ratio of Reagents to Sample with Filtered *Isochrysis sp.*

Samples (100 mL) of mature *Isochrysis sp*. culture contributing ~14 mg dry culture were dewatered onto GFC filter paper until surface dryness was observed with an additional ~10 s of vacuum filtration. The samples were transesterified with the P&G method (see Section 5.2.3.2.2 above) using one, two, and three times the standard quantities of reagents (Table 5.4). Each sample was initially mixed with 0.10 mL dichloromethane containing the internal standard, 5-α-cholestane. The volumes of dichloromethane included in Table 5.4 represent the volume added in addition to the dichloromethane that was added containing the internal standard.

Table 5.4. Volumes of reagents used in the increasing ratio of reagents to sample experiment.

Sample	Volume dichloromethane (mL)	Volume 0.5 N NaOH in MeOH (mL)	Volume 14% BCl_3 in MeOH (mL)
Single	0.00	1.0	1.0
Double	0.10	2.0	2.0
Triple	0.20	3.0	3.0

5.2.4.2 Water Tolerance Optimization

5.2.4.2.1 Water Tolerance with High Amounts of Menhaden Oil

The functional water content of the sample was investigated to ensure the water trapped on the filter was not suppressing the transesterification reaction. Large amounts of menhaden oil (10 mg) were added to 10 mL centrifuge tubes by placing 1.0 mL of a 10 mg/mL solution of menhaden oil in hexane onto a GFC filter. The hexane was then evaporated to dryness with a stream of nitrogen, followed by an application of vacuum. Increasing quantities of distilled water, 0.0 to 0.50 mL in 0.10 mL increments, were added to the sample with a 1.0 mL capacity micropipetter. The samples were then derivatized with the P&G method (see Section 5.2.3.2.2 above).

5.2.4.2.2 Water Tolerance with Low Amounts of Extracted Microalgal Lipid

The water tolerance of the P&G method was investigated with extracted microalgal lipid and increasing quantities of distilled water. A solution of 5.0 mg/mL extracted microalgal lipid in chloroform was applied in 0.10 mL aliquots with a syringe onto glass fiber filter papers in triple-rinsed 10 mL glass centrifuge tubes and blown to dryness with a stream of nitrogen, followed by an application of vacuum to evaporate any remaining hexane. Distilled water quantities ranged from 0.0 to 0.50 mL by increments of 0.10 mL measured with a micropipette equipped with a 1.0 mL tip. The samples were then derivatized with the P&G method (see Section 5.2.3.2.2 above).

5.2.4.2.3 Water Tolerance with Filtered *Isochrysis sp*.

Water tolerance was tested using whole microalgal cells by filtering 100 mL *Isochrysis sp.* culture, contributing ~13 mg dried culture. The cultures were dewatered through filtration by applying vacuum to the filtered cultures until visible dryness was apparent. Cultures were then rinsed and vacuum was again applied until visible dryness; additional vacuum filtration was applied for 0, 30 and 60 s, contributing 0.55, 0.36, and 0.28 mL of water to the sample, respectively. The samples were then derivatized with the P&G method (see Section 5.2.3.2.2 above).

5.2.4.3 Reaction Temperature Optimization

The reaction temperatures of the base- and acid-catalysed reactions were optimized with a full factorial design with two variables, changing the basic and acidic reaction temperature independently between three reaction temperatures: 20 (ambient temperature; selected to represent "no heating"), 55 (a common derivatization reaction temperature; O'Fallon et al, 2007), and 90 °C (the original temperature of the P&G method), while maintaining the reaction time at 10 min for each reaction step (Table 5.5). The samples were then derivatized with the P&G method (see Section 5.2.3.2.2 above).

Table 5.5. Basic and acidic catalysis reaction temperatures for temperature optimization experiment (base and acid reactions both at 10 min).

Sample Number	Base Temperature (°C)	Acid Temperature (°C)
1	20	20
2	20	55
3	20	90
4	55	20
5	55	55
6	55	90
7	90	20
8	90	55
9	90	90

5.2.4.4 Optimization of the Length of Reaction

A full factorial design with two variables was also used to optimize the length of reaction for the base- and acid-catalyzed reactions. The reaction times were independently varied for basic and acidic catalysis, using reaction times of 1, 5 and 10 min at 20 and 90 °C for basic and acidic catalysts, respectively (Table 5.6). The samples were then derivatized with the P&G method (see Section 5.2.3.2.2 above).

Table 5.6. Basic and acidic catalysis reaction time for length of reaction optimization experiment (20 and 90 °C reaction temperatures for basic and acidic catalysts, respectively).

Sample Number	Base Reaction Time (min)	Acid Reaction Time (min)
1	1	1
2	1	5
3	1	10
4	5	1
5	5	5
6	5	10
7	10	1
8	10	5
9	10	10

5.2.5 Lipid Profiling

5.2.5.1 TLC-FID

The hexane-extracted phase of a sample was measured into a clean vial, blown to dryness with nitrogen, and reconstituted to 10 times the original hexane concentration with chloroform to achieve a concentration of ~5 mg/mL. A capillary tube was used to apply 1.0 µL of sample to the TLC-FID chromarods. The sample was focused on the chromarods with acetone by lowering the rod rack into a small development tank of 80 mL acetone (Optima grade) until the spotted samples were saturated with acetone and focused into a narrow band. The rack was removed from the tank and air-dried for a few seconds, then focused again until the tight bands were saturated with acetone. The rack was set for 5 min in a constant humidity chamber created using a supersaturated solution of NaCl in distilled water in a beaker inside of the chamber. Rods were then placed in a small development tank containing 80 mL of the hexane (Hex):petroleum ether (PE):ethyl ether (EE):formic acid (FoA) solvent system (made freshly from volumetric ratio of 48:48:4:0.25 Hex:PE:EE:FoA measured in mL) and the chromarods were developed for 25 min. After a brief air-drying, the rod rack was put into a 100 °C oven for 3 min to vaporize any remaining solvent clinging to the rack before being analyzed by the TLC-FID (Iatroscan MK V/6, Spotsylvania, Virginia).

FAME, FFA, TAG, and PL were identified by comparison with the retention times of standards applied to a separate chromarod and measured by TLC-FID for each sample batch. Peak areas were calculated with manual integration software

Peak Simple, using a 0.1 peak area count rejection threshold. The individual lipid class and internal standard peak areas were calibrated with standard curves prior to analysis to assure their response measured by the FID was standardized for each individual lipid class. The lipid class proportions were reported as a percent of total lipid classes identified in a sample, where total lipids included FAME, FFA, TAG, and PL, if present. The lipid classes were also reported as a concentration (mg lipid/mg dry sample mass) using the peak area of the internal standard, 5-α-cholestane, as the reference quantity.

The FAME peak areas from the TLC-FID chromatograms were selected to calculate lipid content of the sample, disregarding FFA, TAG, and PL, if present, as this reflects the lipid content which would traditionally be calculated from chomatograms generated by GC (i.e. only FAME are determined by GC, making the FAME peak the source of lipid data for a sample derivatized and analyzed by GC). The FAME peak area count was compared to the area count of the known quantity of internal standard, 5-α-cholestane, and the lipid content for the sample was calculated.

Total lipid peak areas were the combined peak area counts of FAME, FFA, TAG, and PL, where applicable for each sample. This value was once again compared to the peak area of 5-α-cholestane to quantify the total solubilized lipids of a sample, regardless of whether or not they were derivatized fully. This value was used to help determine if an experiment was fully solubilizing the lipids of a sample as the varying conditions and solvent systems may have been insufficient.

5.2.5.2 GC Analyses

FAME in hexane (0.5 mg/mL) were quantified using a Perkin Elmer Autosystem gas chromatograph equipped with an autosampler and a flame ionization detector (FID). FAME were separated using a flexible fused silica column (30 x 0.25 mm ID) coated with 50 % cyanopropyl polysiloxane (0.25 µm film thickness; J&W DB-23, Agilent Technologies, Folsom, California) and helium (He) was used as the carrier gas, flowing at 1 mL/min. Samples were injected in 1 µL volumes into the injector, which was held at 250 °C, and samples were run with splitless injection, with a split-flow rate of 30 mL/min He. The initial oven temperature was 50 °C and was held for 1 min before ramping at 45 °C/min until reaching 153 °C. This temperature remained for 2 min before ramping again at 2.3 °C/min to 174 °C. This was held for 0.2 min before ramping at 2.5 °C/min to 210 °C, which was held for 2 min and is the final oven temperature. The runtime for this program lasted approximately 35 min. Hydrogen and air flowed to the detector at 45 and 450 mL/min and the FID was held at 280 °C. The chomatograms produced by Star software were compared to a standard chromatogram of menhaden oil FAME and FAs were identified by retention times. Peak areas were manually integrated and adjusted using empirical response factors. FAME were reported as percentages of total FAME identified and as concentrations (mg lipid/mg dry culture) relative to the internal standard.

5.2.6 Statistics

All of the peak areas generated by TLC-FID were analyzed with SPSS software 11.0 to assess normality of data and statistical equivalence. The data were tested with ANOVA or MANOVA to determine statistical difference between means. Bonferonni and Tukey's post-hoc tests were used with a p value for rejection of 0.05, adjusted for multiple comparisons.

CHATPER 6: Results

6.1 Comparing *in situ* and Extraction/Derivatization Methods

6.1.1 Comparison of Three Derivatization Methods with Slurry Culture of *Tetraselmis sp.*

The only lipid group identified by TLC-FID for the three methods was FAME, making its relative peak area 100% of all lipid groups found (Fig. 6.1). The standard deviation associated with the results of the three methods was zero as FAME was the only lipid group found. The samples were all statistically equivalent to each other. No residual microalgal lipids (TAG and PL) were detected, nor was any FFA detected.

Using the internal standard to correct for losses during sample preparation, the lipid class content of the derivatized samples was determined from the lipid class peak areas for each method for FAME and total lipids and expressed as lipid mass/dry mass of culture (Fig. 6.2). These independent values represented the total FAME concentration formed through transesterification, while total lipids indicated the amount of FAME formed through transesterification plus unreacted, but solubilized, lipids.

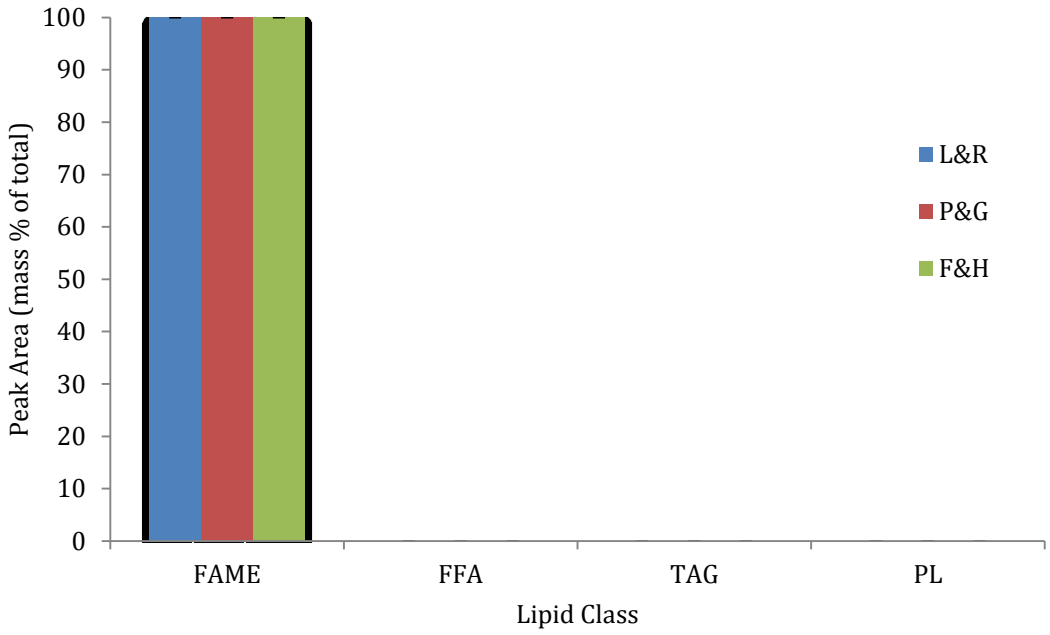

Figure 6.1. Lipid classes expressed as peak area proportions for three extraction/derivatization methods with 0.10 mL slurry culture of *Tetraselmis sp.* (mean ± SD, n = 3).

Despite dispensing identical microalgal aliquots and the use of identical internal standard masses, ANOVA indicated that the lipid content for the three methods varied significantly (Fig. 6.2; $F(2,6) = 20.6$, $p < 0.017$) based on the FAME and total lipid peak areas (note: MANOVA was not used for two variables as FAME and total lipid were equivalent in value). Results were identical for FAME and total lipids as all solubilized lipids were converted to FAME (Fig. 6.1). Post-hoc tests indicated that P&G and F&H were not statistically different, suggesting that P&G produced results equivalent to the F&H method. Though L&R and F&H were

statistically equivalent as well, L&R generated a significantly lower lipid content compared to P&G.

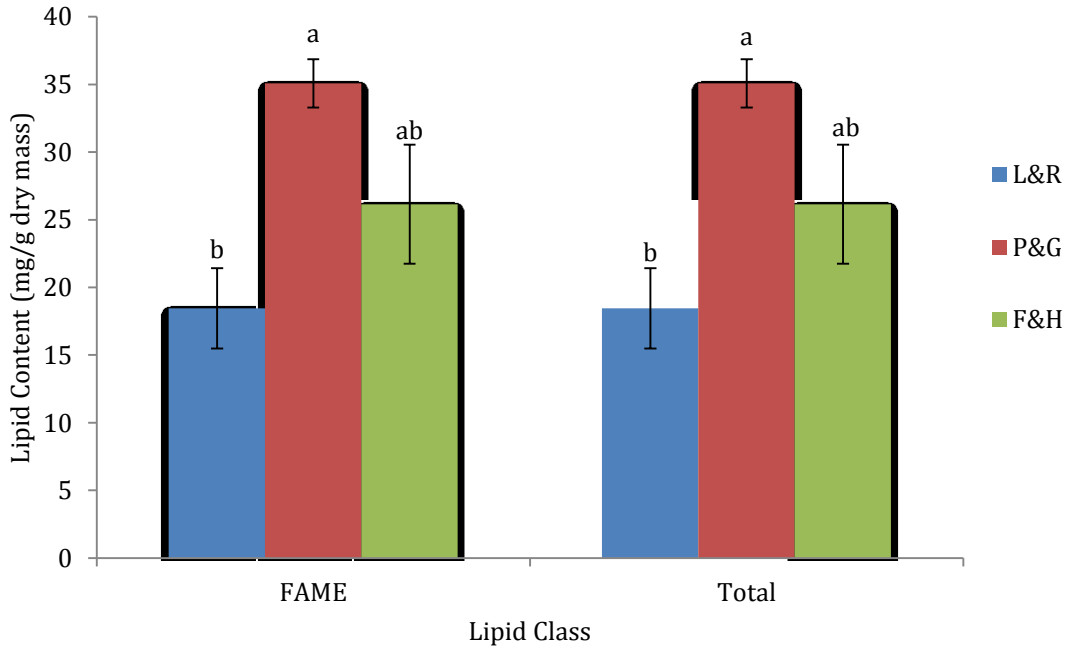

Figure 6.2. Lipid content for three extraction/derivatization methods with 0.10 mL slurry culture of *Tetraselmis sp.* (mean ± SD, n = 3). Results with different letters are significantly different.

To ensure that derivatization was equivalent for all FA, the FA profile of the microalgae was determined through GC for the three different methods (Fig 6.3). The same FAs were identified for all three methods and the individual FA contents had some agreement between all methods (L&R to F&H, L&R to P&G, and P&G to F&H; Fig. 6.3). The ten FAs present in the greatest proportions, 14:0, 16:0, 16:1n-7, 18:1n-9, 18:1n-7, 18:2n-6, 18:3n-3, 18:4n-3, 22:5n-6, and 22:6n-3, varied in their

content among the three methods. Expressed as mass proportional data, there was no significant effect on the same ten FAs due to the method used on individual FA proportions (Table. 6.1; MANOVA: Wilks Lambda = 2.40×10^{-8}, $p \leq 0.05$).

Table 6.1. Ten highest proportion FA's from *Tetraselmis sp.* slurry culture analyzed by GC (mean ± SD, n = 6). Different letters per FA denote significant differences.

FA	Proportion (mass % of total)		
	L&R	P&G	F&H
14:0	21.01 ± 0.75^{a}	19.67 ± 1.26^{a}	21.98 ± 0.23^{a}
16:0	16.36 ± 0.31^{b}	16.59 ± 0.34^{b}	16.79 ± 0.07^{b}
16:1n-7	2.06 ± 0.09^{c}	2.17 ± 0.08^{c}	1.99 ± 0.02^{c}
18:1n-9	26.53 ± 0.67^{d}	25.78 ± 0.96^{d}	26.50 ± 0.19^{d}
18:1n-7	1.34 ± 0.02^{e}	1.28 ± 0.04^{e}	1.30 ± 0.02^{e}
18:2n-6	3.71 ± 0.08^{f}	4.16 ± 0.07^{g}	3.83 ± 0.03^{fg}
18:3n-3	2.74 ± 0.11^{hi}	2.99 ± 0.16^{h}	2.56 ± 0.02^{i}
18:4n-3	8.95 ± 0.38^{j}	10.85 ± 0.86^{k}	9.08 ± 0.08^{j}
22:5n-6	1.97 ± 0.08^{l}	1.68 ± 0.11^{l}	1.75 ± 0.02^{l}
22:6n-3	8.17 ± 0.28^{m}	8.76 ± 0.17^{m}	7.90 ± 0.09^{m}

There was very little difference in the FA portions between methods, with 7 of the 10 FAs producing equivalent proportions for all three methods.

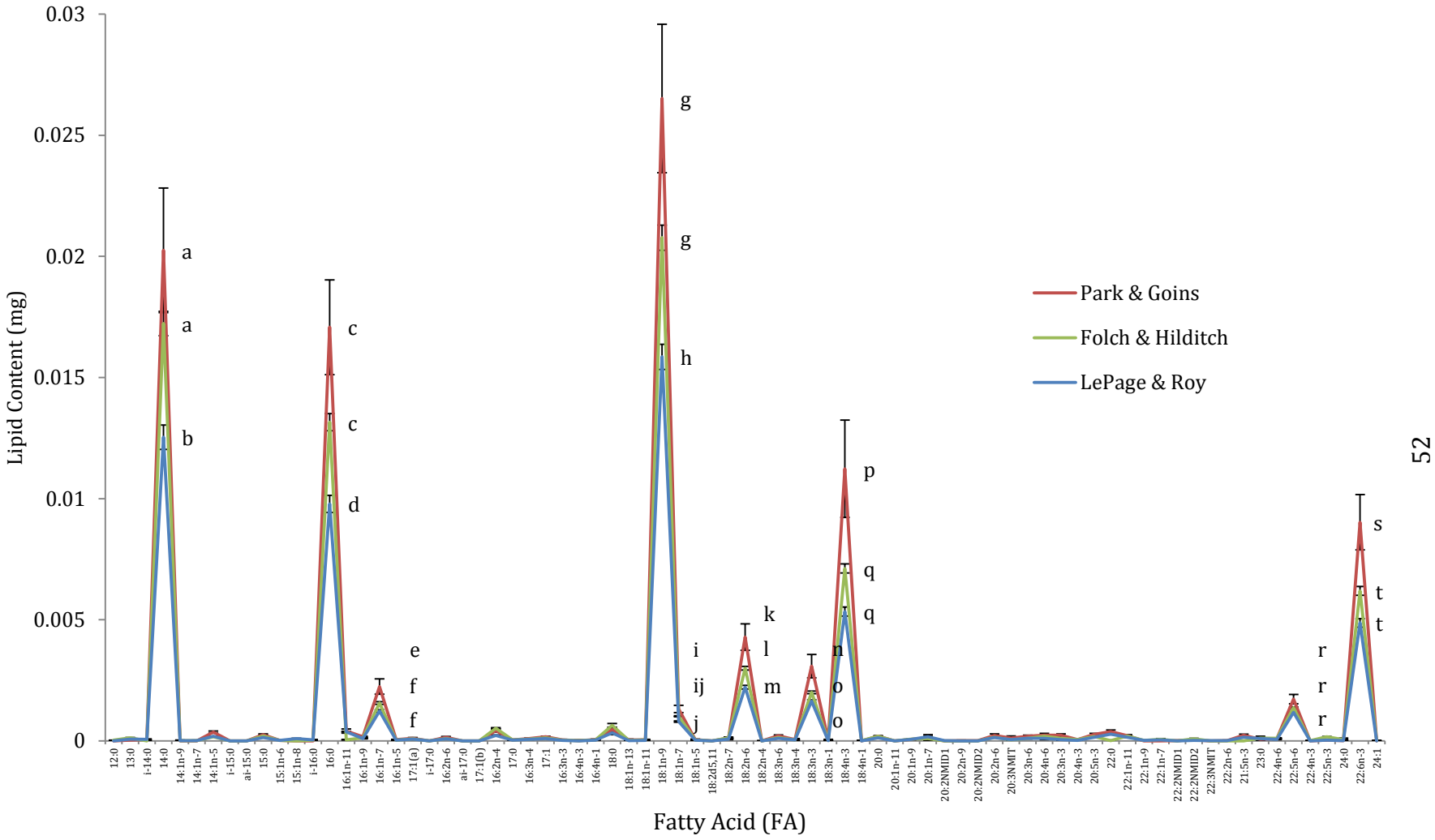

Figure 6.3. Chromatogram of fatty acids transesterified by L&R, P&G, and F&H for 0.10 mL slurry culture of *Tetraselmis sp.* (mean ± SD, n = 6). Results with different letters are significantly different.

Total FA content from the GC data was calculated with the internal standard for all three methods and compared to the values calculated with TLC-FID (Fig 6.4). For each method, FAME content determined by TLC-FID and GC were identical.

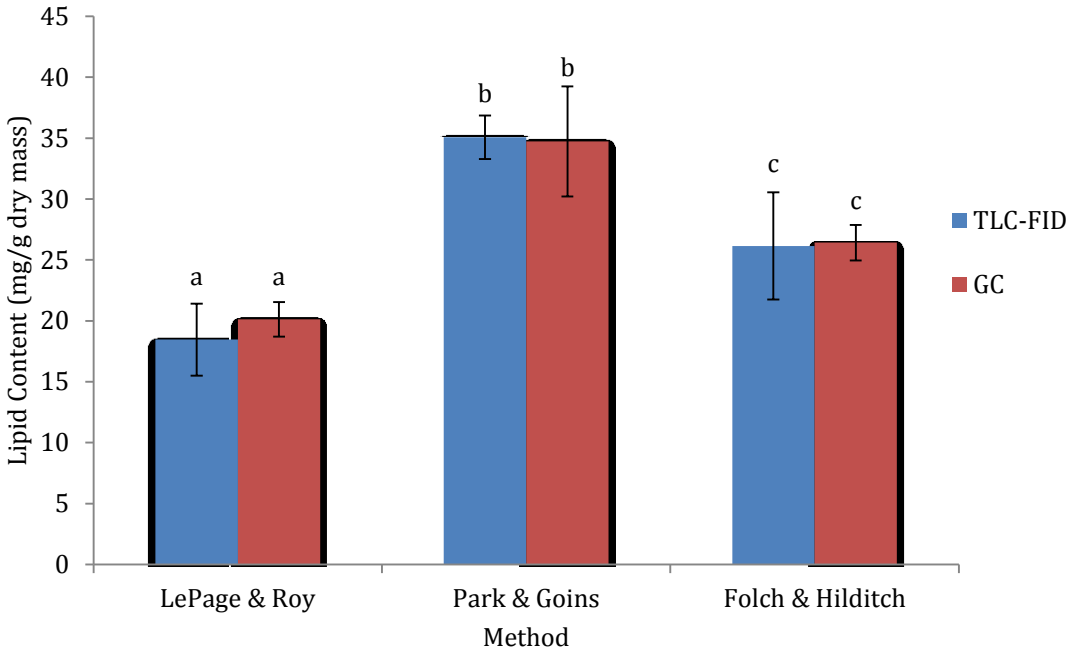

Figure 6.4. Comparison between FAME contents determined by TLC-FID and GC for three derivatization methods with a 0.10 mL slurry culture of *Tetraselmis sp.* (mean ± SD, n = 3). Results with similar letters are not significantly different.

6.1.2 Comparing Three Derivatization Methods with Filtered Culture of *Tetraselmis sp.*

Samples of *Tetraselmis sp*. were filtered with glass filter paper and FAME was produced with the three methods (Fig. 6.5). MANOVA indicated differences in the data (MANOVA: Wilks Lambda = 0.2043, $p \leq 0.005$) and that derivatization method

had a significant effect on FAME ($F(2,14) = 7.65$, $p < 0.017$) and TAG ($F(2,14) = 12.2$, $p < 0.017$) proportions, but not PL ($F(2,14) = 0.15$, $p > 0.017$). Bonferroni post-hoc tests indicated that results of the P&G and F&H methods were statistically equivalent for FAME and TAG. There was not a significant quantity of unreacted TAG or PL for both the P&G and F&H methods. The L&R method, however, generated statistically less FAME than the P&G method and more TAG than the other two methods.

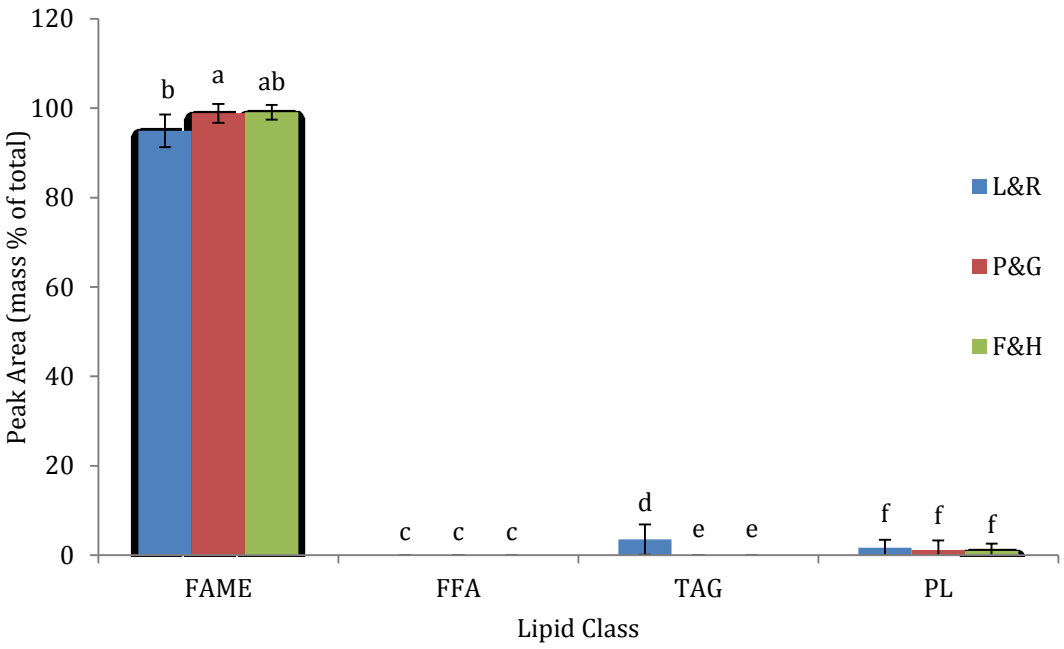

Figure 6.5. Lipid classes expressed as peak area proportions for three extraction/derivatization methods with ~5 mg filtered culture of *Tetraselmis sp.* (mean ± SD, n = 3). Results with different letters are significantly different.

With filtered cultures, there were no significant differences for FAME or total lipid content on a dry weight basis (Fig. 6.6; MANOVA: Wilk's Lambda = 0.4619, $p \leq 0.05$). There was a trend towards higher lipid content for the P&G method; similarly the L&R method showed a trend of lower lipid content. This lower level was expected due to the presence of unreacted TAG reported in Fig 6.5. The large standard deviations observed here were due to the small amounts of lipid that were used in the experiments.

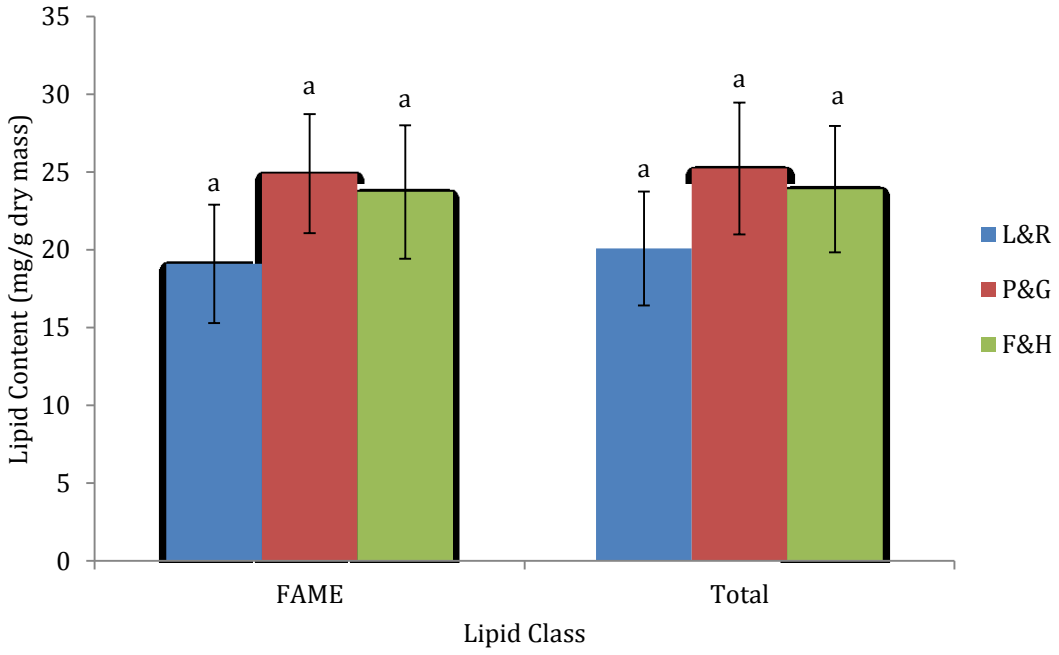

Figure 6.6. Lipid content for three extraction/derivatization methods with ~5 mg filtered culture of *Tetraselmis sp.* (mean ± SD, n = 3). Results with similar letters are not significantly different.

6.1.3 Result of Preliminary Testing of Three Methods

From the results of the three methods, the P&G method was judged to be consistently as proficient as the F&H while taking approximately one sixth of the time to react within one vessel. The trends for lipid content and conversion of FAME from TAG and PL indicated that the P&G method was better suited to microalgal samples than L&R; therefore, P&G was selected for further study. Because they were not present in any of the preliminary results, FFA will only be included in the following lipid class summaries when found.

6.2 Lipid Load with P&G *in situ* Method

6.2.1 High Sample Loading with Menhaden Oil

The derivatization of varying quantities of menhaden oil (0.10, 0.30, 0.50, 1.0, 3.0, and 5.0 mg) with the P&G method identified FAME as the major lipid class with traces of TAG also present (Fig. 6.7). PL was not detected in any sample, which was to be expected as menhaden oil is comprised entirely of TAG. MANOVA indicated differences in the data (MANOVA: Wilks Lambda = 0.000317, $p \leq 0.05$) and that sample loading had an effect on both FAME ($F(5,12) = 244$, $p < 0.008$) and TAG ($F(5,12) = 287$, $p < 0.008$). Post-hoc tests indicated that FAME and TAG results derived from oil amounts between 0.3 and 3.0 mg were all equivalent (Fig. 6.7) with discrepancies arising for the lower and higher values. Nearly 100% conversion of sample to FAME was observed, although small amounts of residual TAG remained.

The 5.0 mg load gave the highest unreacted TAG yield. There was a clear trend in the data indicating that lipid in a lower sample load was more fully converted to FAME.

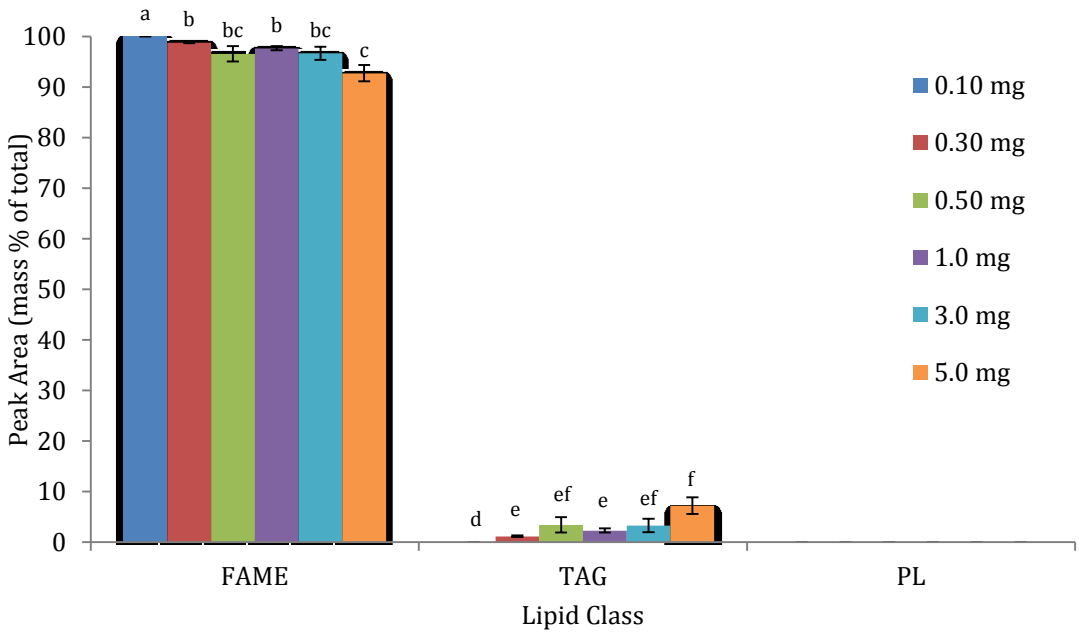

Figure 6.7. Lipid classes expressed as peak area proportions for high sample loading with increasing amounts of menhaden oil (mean ± SD, n = 3). Results with different letters are significantly different.

To compare lipid content as FAME and total peak area (Fig. 6.8), all of the results were reported as mg lipid yield per mg original lipid content. MANOVA indicated some differences in the data (MANOVA: Wilks Lambda = 0.009, $p \leq 0.05$) and again showed an effect from sample load on FAME ($F(5,12)=100$, $p < 0.008$) and total lipids ($F(5,12)=118$, $p < 0.008$). As above, the lipid content for both FAME and total peak areas demonstrated agreement with the amount of lipid applied for

sample loads from 0.10 to 1.0 mg. However, application of loads of both 3.0 and 5.0 mg produced yields significantly greater than the amount of lipid originally applied.

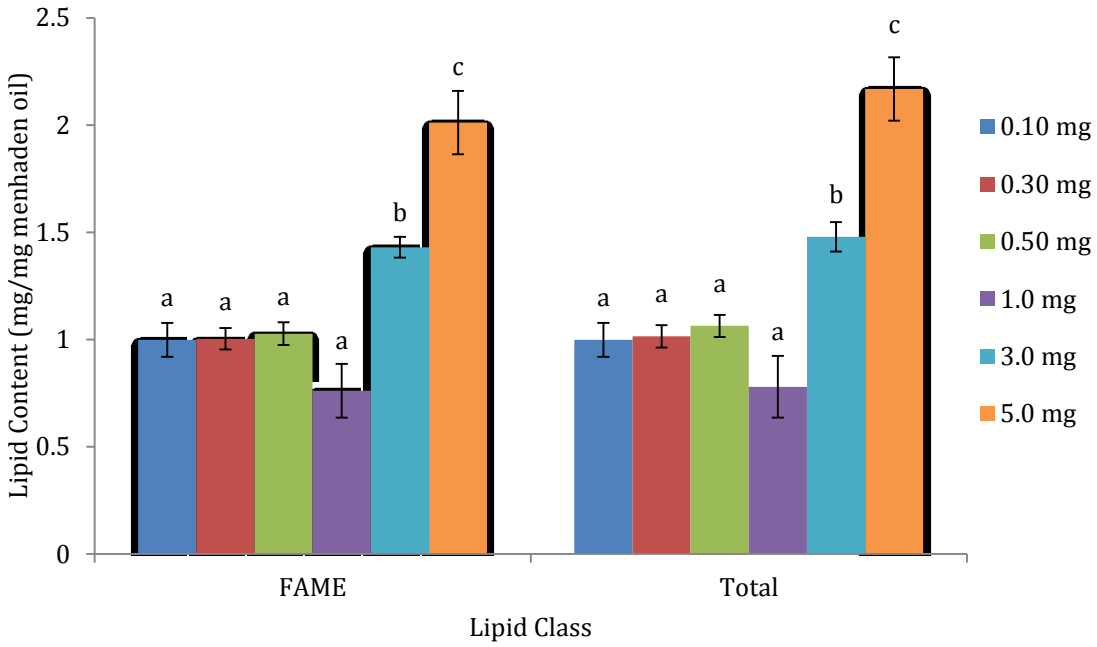

Figure 6.8. Lipid quantity for high sample loading with increasing quantities of menhaden oil expressed per quantity of menhaden oil originally applied (mean ± SD, n = 3). Results with different letters are significantly different.

6.2.2 Low Sample Loading with Filtered Culture of *Isochrysis sp.*

For the filtered samples, MANOVA did not show an overall difference (Fig. 6.9; MANOVA: Wilks Lambda = 0.3098, $p \geq 0.05$), indicating that, at low levels, sample loading did not have a significant effect on lipid class composition. There was an observed trend of decreasing FAME conversion as the sample load increased, indicating a more complete transesterification reaction with smaller sample loading.

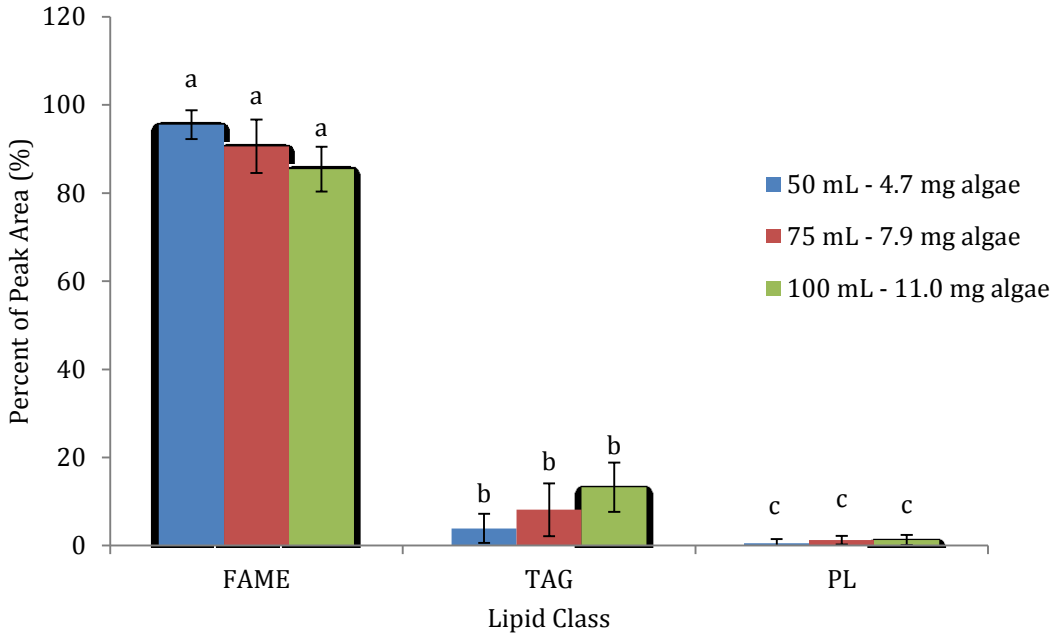

Figure 6.9. Lipid classes expressed as peak area proportions for sample loading with 50, 75, and 100 mL filtered microalgal culture, *Isochrysis sp*. (mean ± SD, n = 3). Results with similar letters are not significantly different.

MANOVA also did not show a significant difference for lipid content when evaluated on a dry mass basis (Fig 6.10; MANOVA: Wilks Lambda = 0.4250, $p \geq 0.05$). However, another decreasing trend was observed as the sample load increased, indicating a more complete transesterification reaction with smaller sample loading.

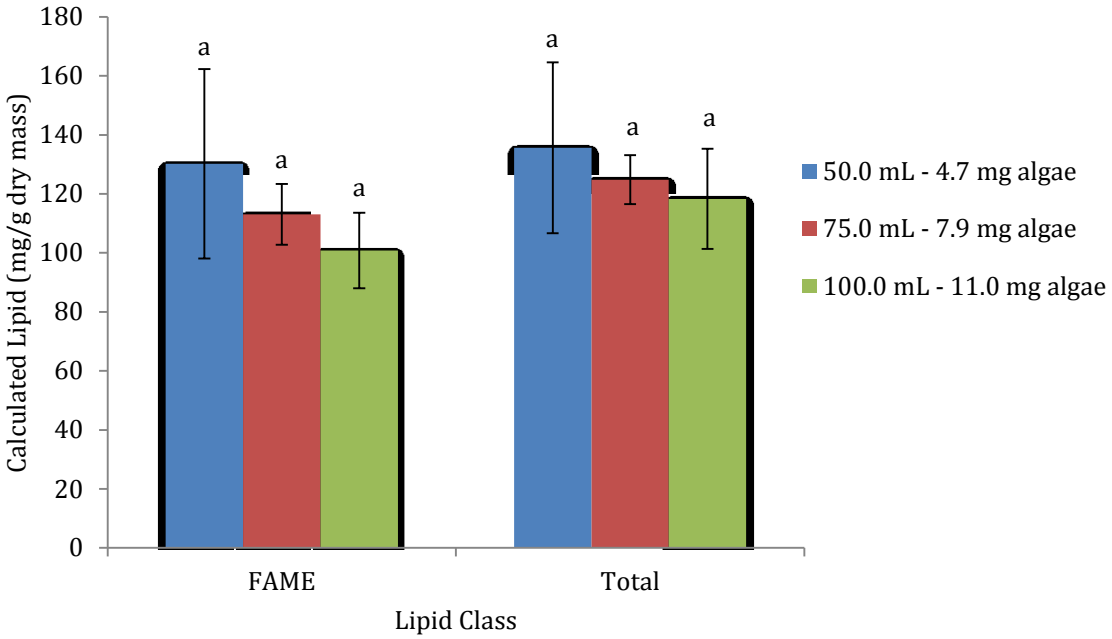

Figure 6.10. Lipid content for sample loading with 50, 75, and 100 mL filtered microalgal culture of *Isochrysis sp.* (mean ± SD, n = 3). Results with similar letters are not significantly different.

6.2.3 Loading Test with Increasing Ratio of Reagents to Sample Using Filtered *Isochrysis sp*.

MANOVA indicated no differences in the conversion of sample lipids to FAME with an increasing ratio of reagents to sample (Fig. 6.11; MANOVA: Wilks Lambda = 0.5311, $p \geq 0.05$).

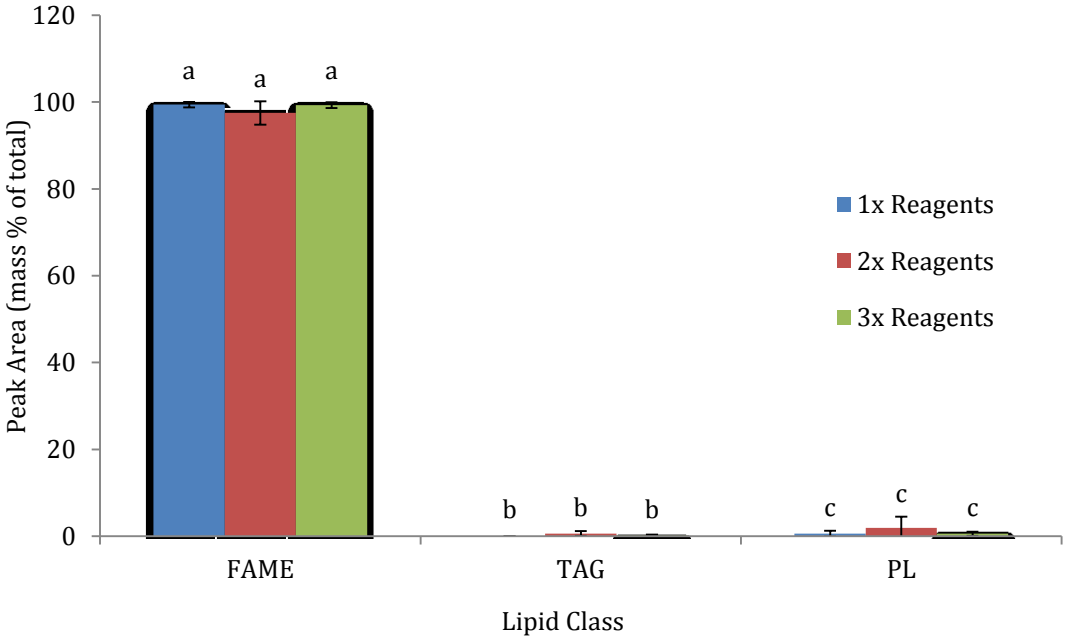

Figure 6.11. Lipid classes expressed as peak area proportions for increasing ratio of reagents to sample (regular amount [1x], double [2x], and triple [3x] the regular amount) with 100 mL filtered culture of *Isochrysis sp*. (mean ± SD, n = 3). Results with similar letters are not significantly different.

The ratio of reagents to sample had no effect on lipid content expressed on a dry weight basis (Fig. 6.12; MANOVA: Wilks Lambda = 0.3939, $p \geq 0.05$). The large standard deviations associated with the results of testing 1 and 3x reagents cannot be directly linked to any known shortcoming of the method, sample, or measurement procedures, thought may be attributed to the small sample size of microalgae being analyzed.

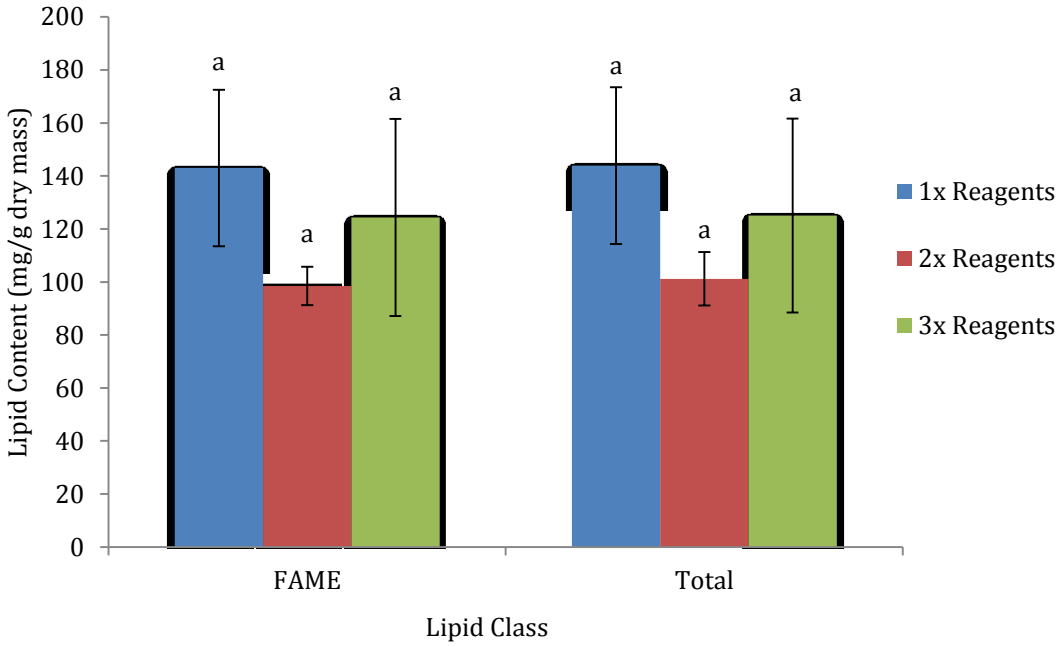

Figure 6.12. Lipid content for increasing ratio of reagents to sample with 100 mL filtered culture of *Isochrysis sp.* (mean ± SD, n = 3). Results with similar letters are not significantly different.

6.3 Water Tolerance

6.3.1 Effect of Water Content with High Quantities of Menhaden Oil

There were significant differences with water content on lipid proportions when tested with high contents (10 mg) of menhaden oil (Fig. 6.13; MANOVA: Wilks Lambda = 0.000035, $p \leq 0.008$). There was a significant effect from water content for both FAME ($F(5,12)=248$, $p < 0.008$) and TAG ($F(5,12)=2032$, $p < 0.008$) with a sharp decrease in FAME proportion as the water content increased, demonstrating a

strong trend between water content and conversion of lipids to FAME for higher contents of lipid. FAME and TAG were the only lipid classes identified by TLC-FID.

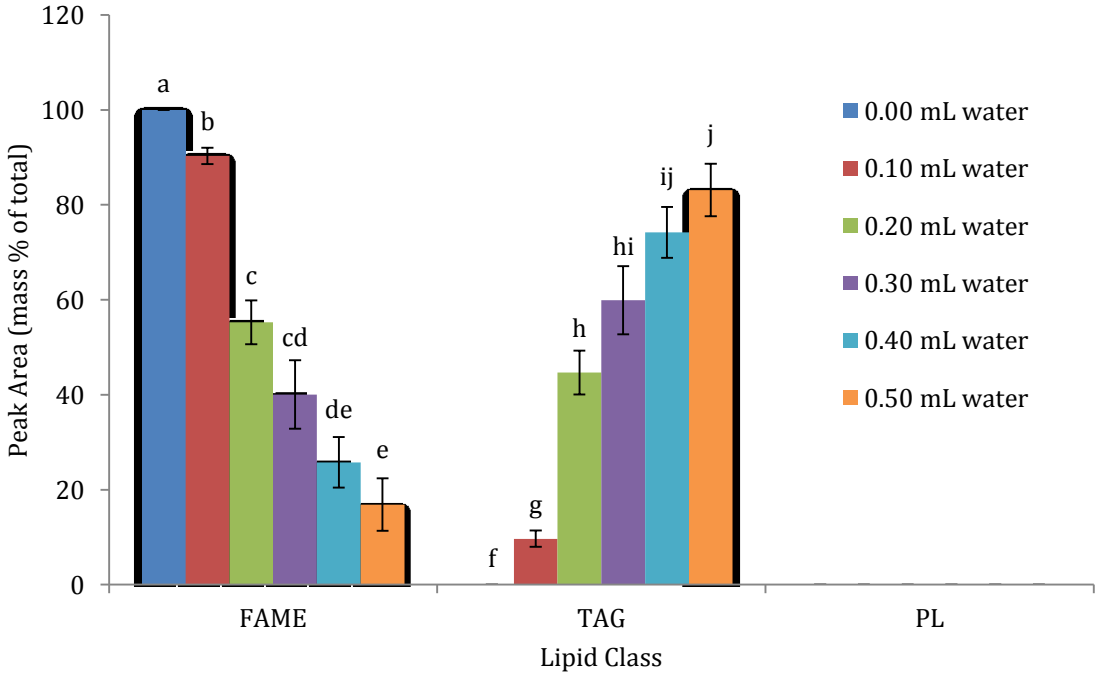

Figure 6.13. Lipid classes expressed as peak area proportions for increasing water content with high lipid loading (10 mg) of menhaden oil (mean ± SD, n = 3). Results with different letters are significantly different.

Lipid contents could not be evaluated as concentrations because there was a relatively high proportion of lipid to internal standard, 5-α-cholestane, producing an unreliable result.

6.3.2 Effect of Water Content with Low Quantities of Microalgal Lipid

MANOVA reported no effect from increasing water content with microalgal lipid samples on the FAME proportions of the data (Fig. 6.14; MANOVA: Wilks Lambda = 0.4177, $p \geq 0.05$). There was no TAG and very little PL detected for the samples, indicating good solubility and complete reaction of all lipids.

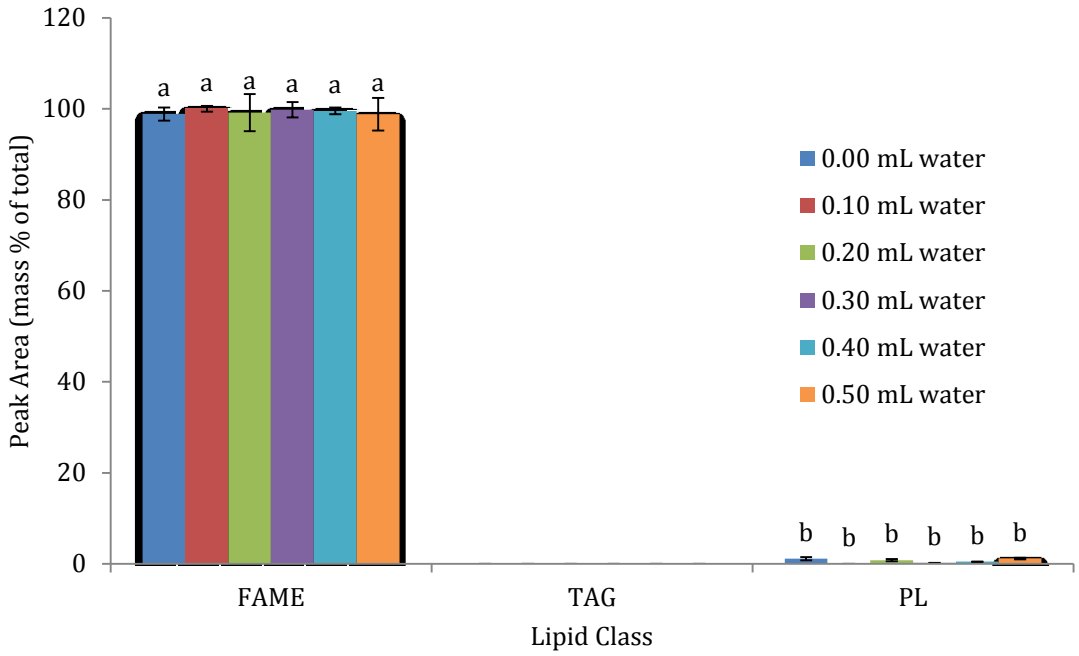

Figure 6.14. Lipid classes expressed as peak area proportions for increased water content with low lipid loading (0.5 mg) of microalgal lipid (mean ± SD, n = 3). Results with similar letters are not significantly different.

MANOVA again indicated no effect on the lipid content due to the varying water content (Fig. 6.15; MANOVA: Wilks Lambda = 0.2550, $p \geq 0.05$). Consistency in the lipid content is observed for all of the samples for both FAME and total lipids,

with the data converging near 1 mg/mg extracted lipid. The equivalence between FAME and total lipids corroborated the lipid class data (Fig. 6.14) that showed a nearly 100% conversion of lipids to FAME for all samples. The mean and standard deviation of the 0.50 mL water sample are much larger than the other samples, though there is no significant difference between it and the rest of the samples. These characteristics indicate less consistency for samples with higher water content.

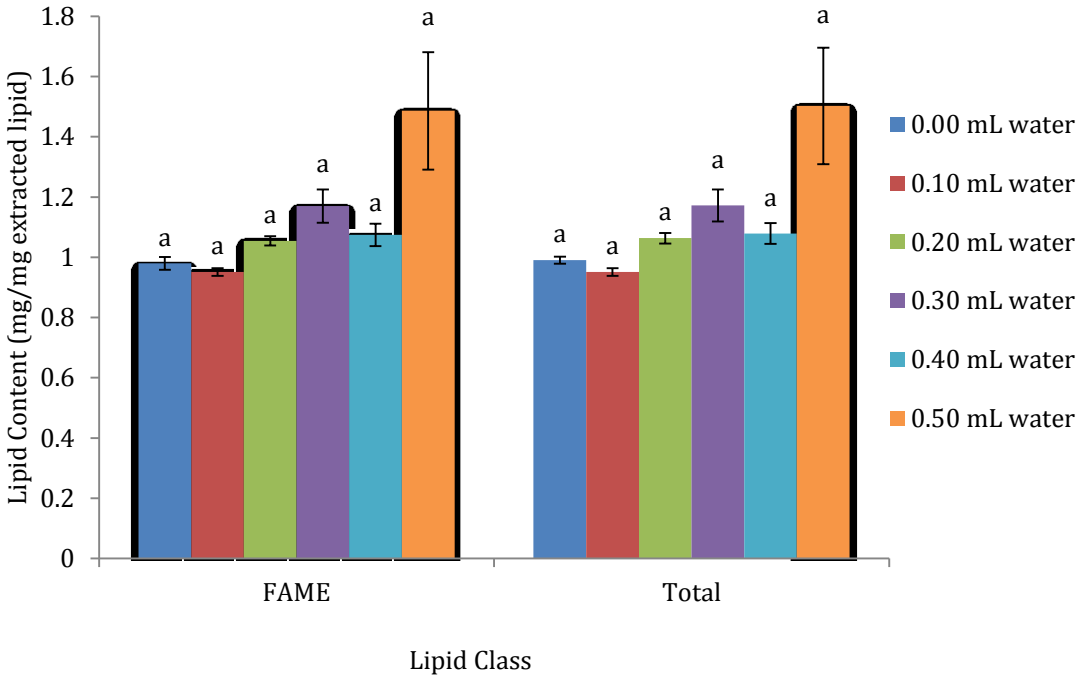

Figure 6.15. Calculated lipid content for increasing water content with low loading (0.5 mg) of microalgal lipid (mean ± SD, n = 3). Results with similar letters are significantly equivalent.

6.3.3 Effect of Water Content with Filtered Culture of *Isochrysis sp.*

MANOVA reported no statistical difference in the data due to water content variation (Fig. 6.16; MANOVA: Wilks Lambda = 0.03652, $p \leq 0.05$). The influence of the water content on the method's ability to transesterify the microalgal lipids was negligible as the content ranged from 0.28 to 0.55 mL of water and the peak area percentage of FAME for all of the samples was high and no statistical differences were determined between samples.

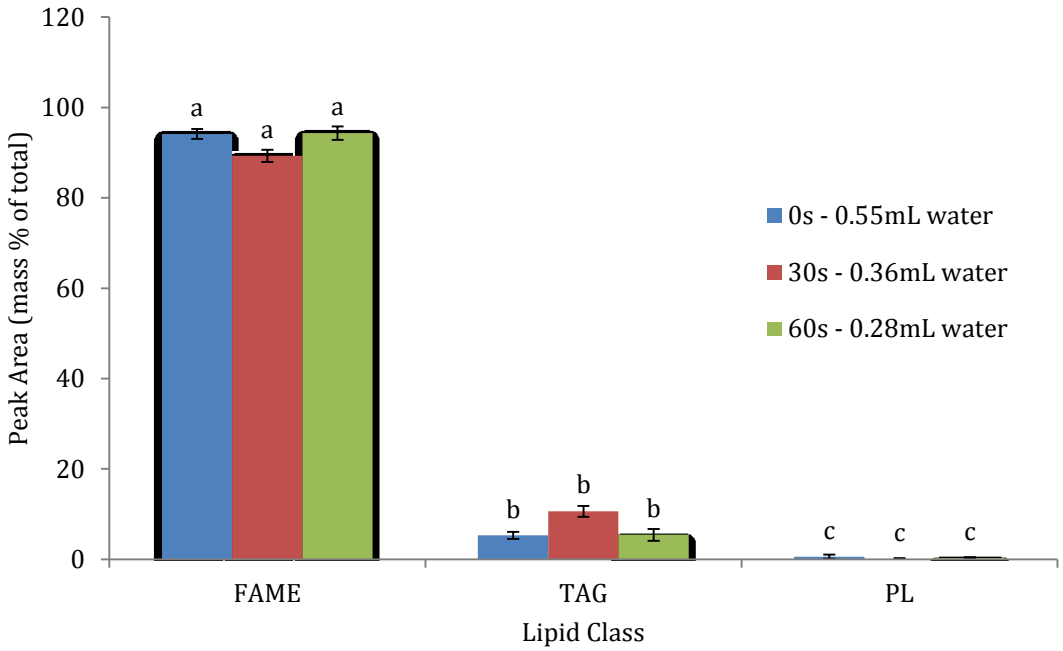

Figure 6.16. Lipid classes expressed as peak areas proportions for increased water content with 100 mL filtered culture of *Isochrysis sp*. (mean ± SD, n = 3). Results with similar letters are not significantly different.

There was also no effect on the lipid content calculation of filtered microalgal culture due to water content derived from variation in length of filtration (Fig. 6.17; MANOVA: Wilks Lambda = 0.04074, $p \leq 0.05$). No trends implicating the benefits of shorter or longer filtering times were observed.

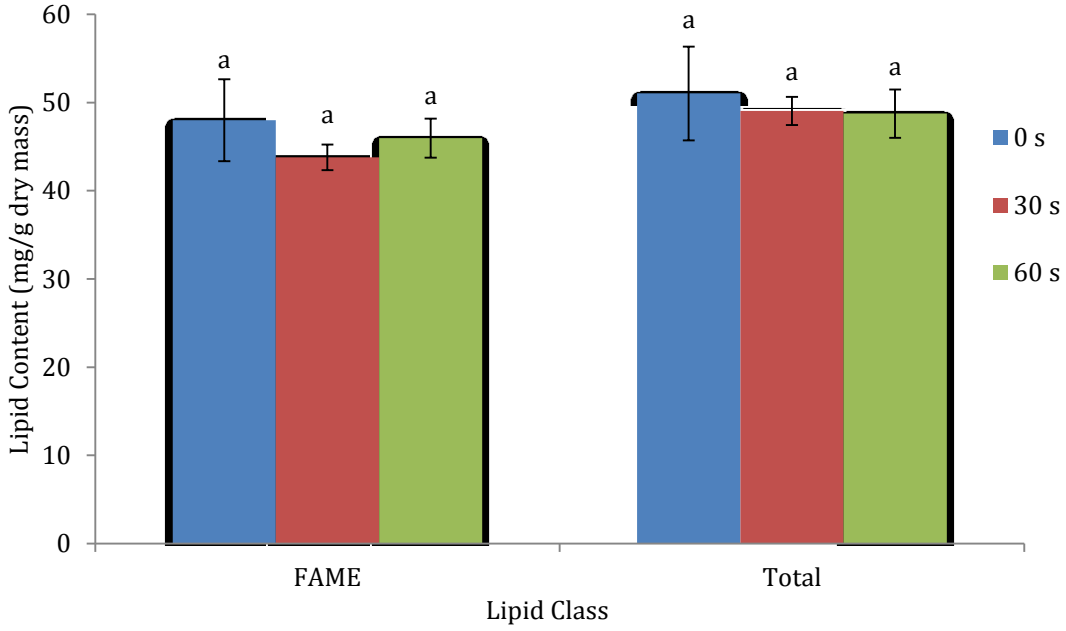

Figure 6.17. Lipid content for increased water content with 100 mL filtered culture of *Isochrysis sp*. (mean ± SD, n = 3). Results with similar letters are not significantly different.

6.4 Optimization of the P&G Method

6.4.1 Reaction Temperature Optimization with Full Factorial Design Using Filtered *Ankistrodesmus falcatus*

MANOVA indicated that temperature had a significant effect on FAME proportions (Fig. 6.18; MANOVA: Wilks Lambda = 0.000804, $p \leq 0.05$; $F(8,18) = 27.3$, $p < 0.006$). FAME proportions at the acid reaction temperature of 20 and 55 °C were significantly lower than those at 90 °C (Fig. 6.18). Within a single acid reaction temperature, base reaction temperature had no effect on FAME proportions. The amount of FAME produced varied for each acid reaction temperature, with a constant upwards trend as the acid reaction temperature increased.

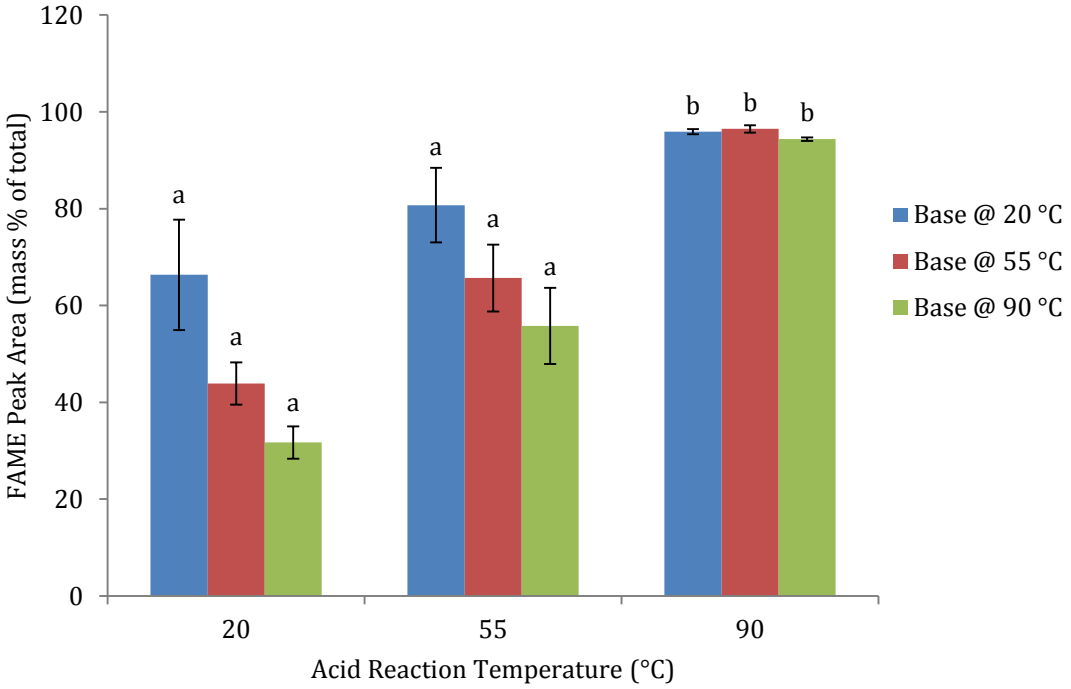

Figure 6.18. FAME peak area proportions for optimization of reaction temperatures with full factorial design of 50 mL filtered culture *A. falcatus* (mean ± SD, n = 3). Results with different letters are significantly different.

A relatively small amount of TAG and PL were detected in the samples (see APPENDIX B), while FFA was a significant peak (Fig. 6.19). MANOVA indicated differences (MANOVA: Wilks Lambda = 0.000804, $p \leq 0.05$) in the FFA content with changes in the acid reaction temperature ($F(8,18) = 61.9$, $p < 0.006$). FFA results for acid catalyzed reactions performed at 90 °C were significantly less than those carried out at 20 and 55 °C, with trends of higher base reaction temperatures having produced more FFA. Within a single base reaction temperature, there was a consistent decrease in FFA proportions with increasing acid reaction temperature.

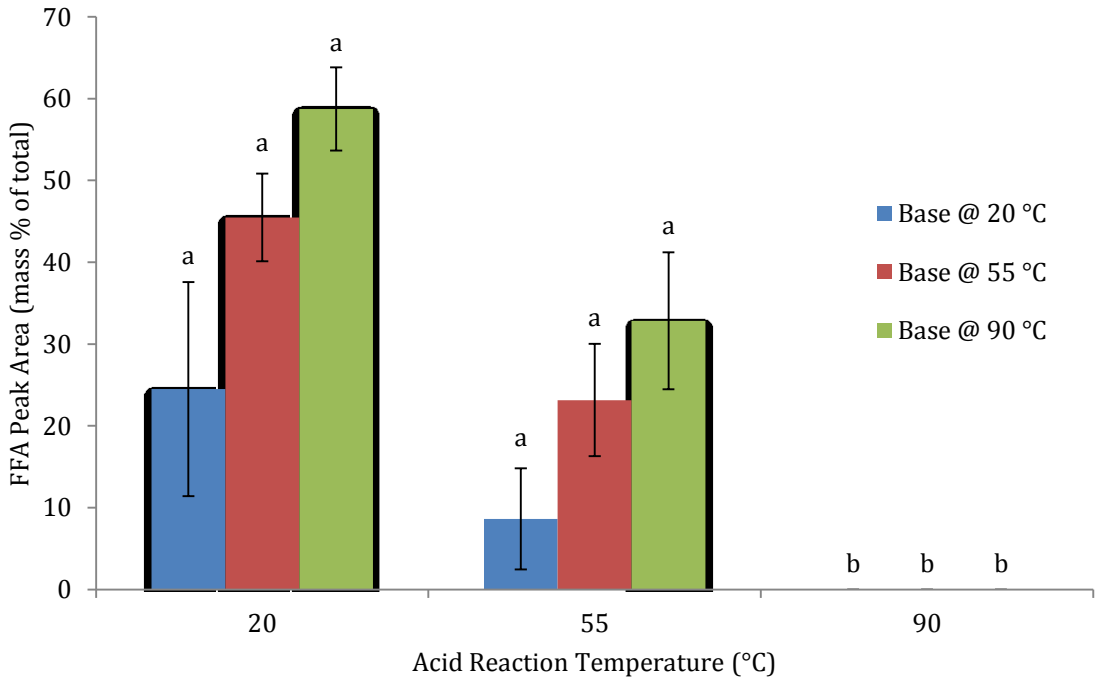

Figure 6.19. Residual FFA peak area proportions for temperature optimization with full factorial design of 50 mL filtered culture *A. falcatus* (mean ± SD, n = 3). Results with different letters are significantly different.

MANOVA indicated a significant effect of temperature (Fig. 6.20; Wilks Lambda = 0.001587, $p \leq 0.05$) on FAME content ($F(8,18) = 13.5$, $p < 0.006$). The basic temperature reaction of 90 °C generated the same FAME content regardless of the acidic catalysis temperature. Higher yields were consistently achieved with base-catalyzed reaction temperatures of 20 and 55 °C, which were consistent with the FAME proportions observed in the previous figure (Fig. 6.19).

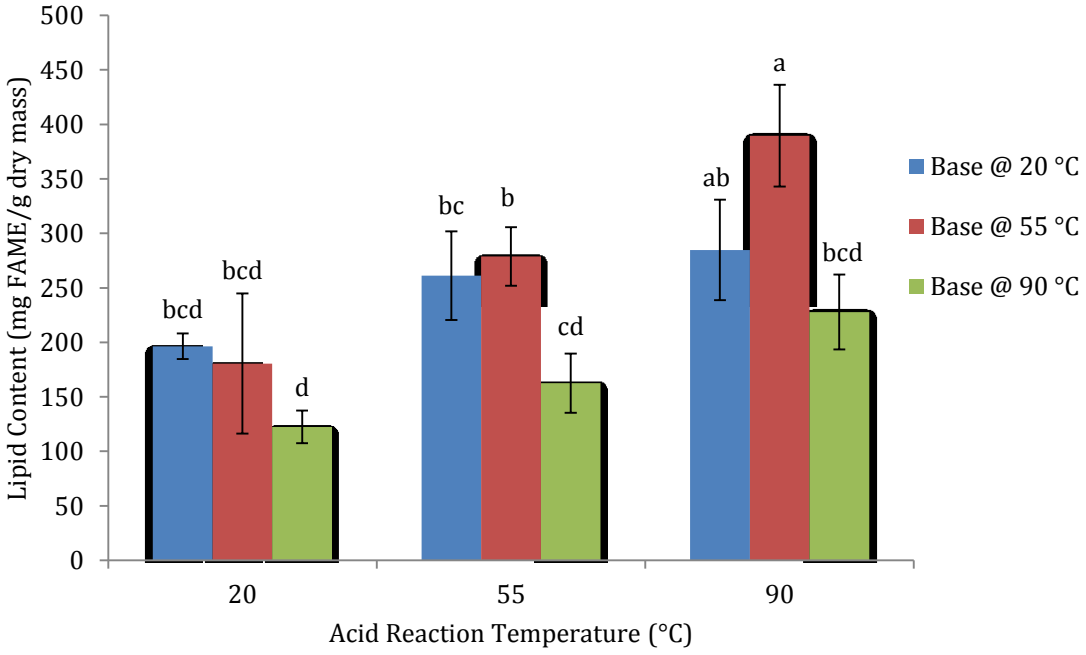

Figure 6.20. Lipid content of FAME for temperature optimization with full factorial design of 50 mL filtered culture *A. falcatus* (mean ± SD, n = 3). Results with different letters are significantly different.

MANOVA did not suggest a significant effect of temperature (Fig. 6.21; Wilks Lambda = 0.001587, $p \leq 0.05$) on the lipid content from total lipids ($F(8,18) = 3.82$,

$p < 0.006$). Trends demonstrate a higher reported mean for samples with base reaction temperature at 55 °C, though no significant differences were reported.

From these results, it was concluded that a lower base catalyzed reaction temperature may benefit the conversion of lipid to FAME, while the acid catalysis temperature should remain at 90 °C for ideal transesterification.

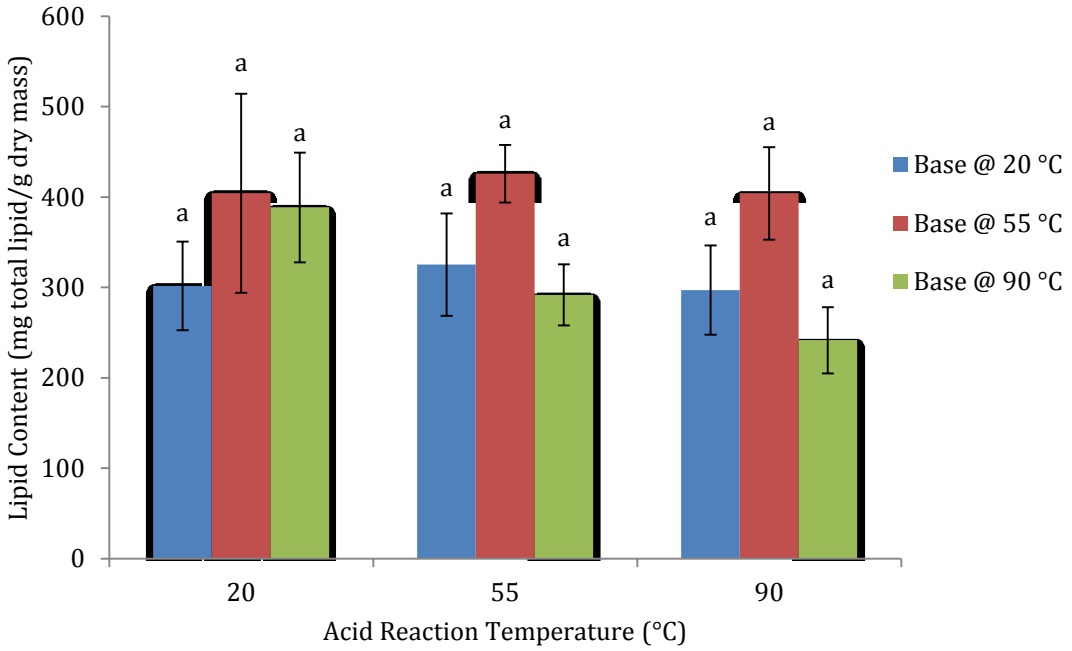

Figure 6.21. Total lipid content from total lipids for temperature optimization with full factorial design of 50 mL filtered culture *A. falcatus* (mean ± SD, n = 3). Results with similar letters are not significantly different.

6.4.2 Ensuring Proper Reaction Temperature for Acid Catalysis when Base Catalysis Occurred at Ambient Temperature

MANOVA did not show an effect of acid catalysis temperature when 70, 80, and 90 °C were considered with a basic catalyst temperature of 20 °C (Fig. 6.22; MANOVA: Wilks Lambda = 0.1546, $p \geq 0.05$). The FAME peak area percentage was also very high, with small trace amounts of TAG and PL remaining, indicating good conversion of lipids to FAME for all conditions.

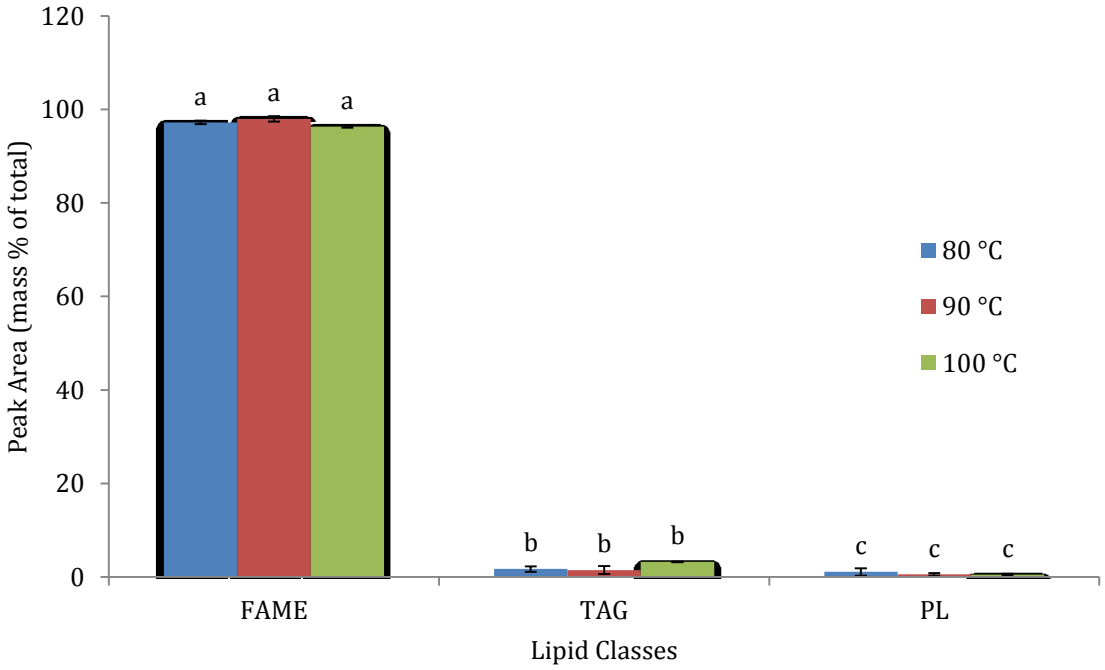

Figure 6.22. Lipid classes expressed as peak area proportions for acid catalyzed reaction at 80, 90, and 100 °C with base catalyst reacted at 20 °C with 50 mL filtered culture of *A. falcatus* (mean ±SD, n = 3). Results with similar letters are not significantly different.

Lipid contents on a dry weight basis did not demonstrate any significant differences regardless of reaction temperature (Fig. 6.23; MANOVA: Wilks Lambda = 0.08613, $p \geq 0.05$).

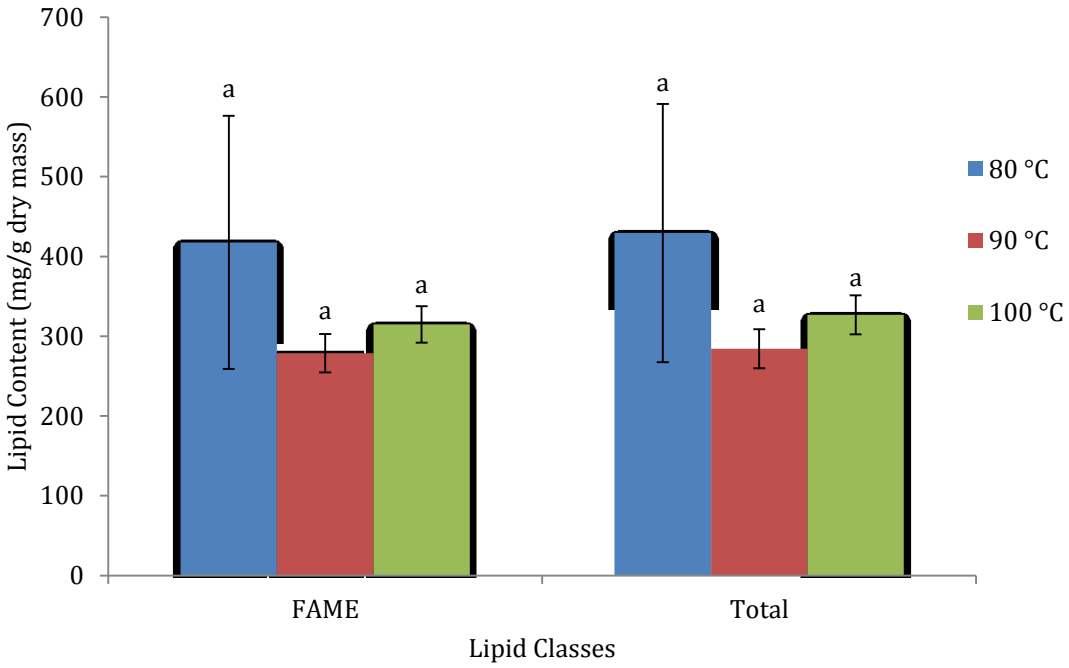

Figure 6.23. Lipid content relative to dry mass for acid-catalysed reaction at 80, 90, and 100 °C with base-catalysed reaction at 20 °C with 50 mL filtered culture, *A. falcatus* (mean ± SD, n = 3). Results with similar letters are not significantly different.

6.4.3 Optimization of Reaction Time with Full Factorial Design Using Filtered *Ankistrodesmus falcatus*

Reaction time had a significant effect on FAME proportions (Fig. 6.24; MANOVA: Wilks Lambda = 0.01203, $p \leq 0.05$), as there was a significantly greater

FAME yield with a 10 min acidic catalyst length of reaction compared to shorter reaction times ($F(8,18) = 17.3$, $p < 0.006$). There was also an upward trend as the acidic catalyst length of reaction increased, regardless of the basic catalyst reaction time, demonstrating a dependence of the extent of the transesterification reaction on the acidic catalyst reaction time. Only the 10 min acidic catalyst reaction time gave an acceptably high percentage of FAME. There was a slight trend of higher FAME content when the basic catalyst reaction used a shorter reaction time compared to the associated acidic catalyst reaction time, which may indicate less saponification and fewer byproducts of the reaction when run for a shorter period.

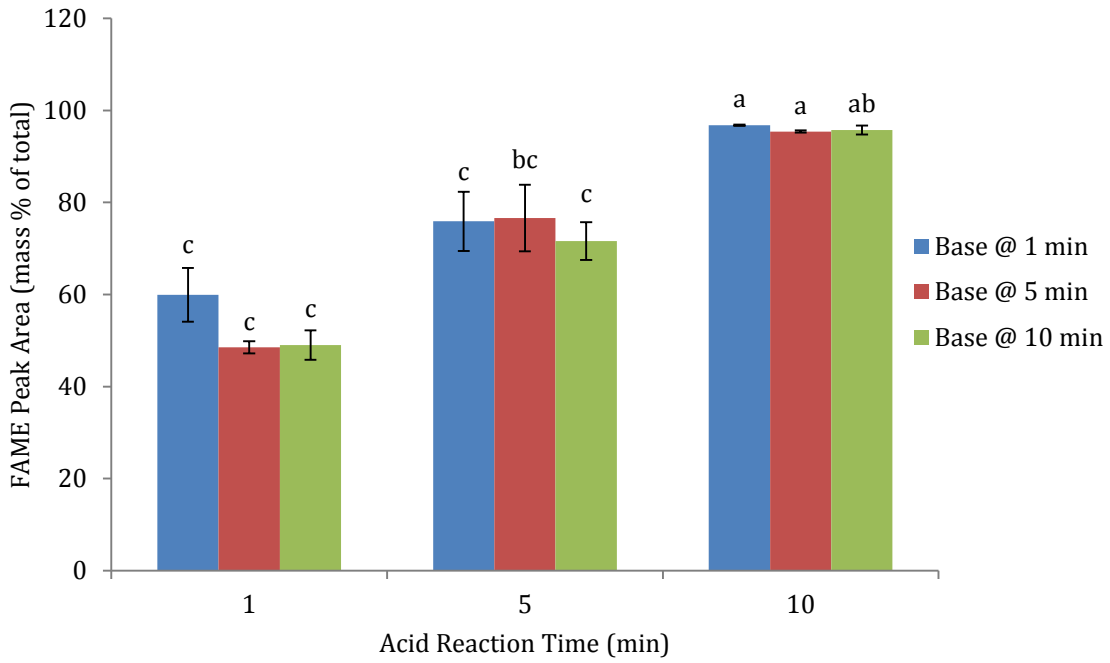

Figure 6.24. FAME peak area proportions of 50 mL filtered culture *A. falcatus* for reaction time optimization with full factorial design (mean ± SD, n = 3). Results with different letters are significantly different.

Only trace amounts of TAG and PL were present in all replicates; however, substantial FFA peak areas were evident in some trials (Fig 6.25). MANOVA reported significant differences (MANOVA: Wilks Lambda = 0.01203, $p \leq 0.05$) in the FFA content due to the varying lengths of reaction ($F(8,18) = 10.6$, $p < 0.006$). FFA were not present in the 10 min acidic catalyst samples; a trend was apparent that longer acidic reaction times produced less FFA, while basic catalysis time had no strong trends in FFA production (Fig. 6.25). This again confirms the dependence between the length of acidic catalyst and the progression of the reaction, as the incomplete reaction may be measured by the production of FFA.

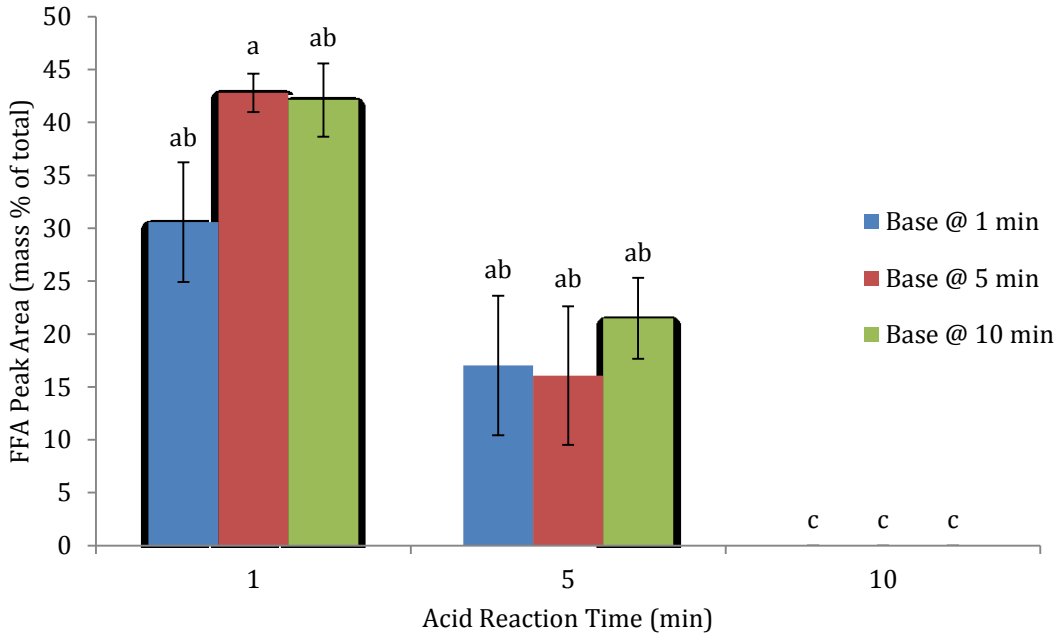

Figure 6.25. FFA peak area proportions of 50 mL filtered culture *A. falcatus* for temperature optimization with full factorial design (mean ± SD, n = 3). Results with different letters are significantly different.

MANOVA demonstrated significant differences (Fig. 6.26; MANOVA: Wilks Lambda = 0.004932, $p \leq 0.05$) with a significant effect of reaction time on FAME content expressed on a dry weight basis ($F(8,17) = 4.54$, $p < 0.006$). This is not consistent with the post-hoc comparisons, which indicated statistical equivalence for all of the calculated lipid content from FAME (Fig. 6.26). The p-value was reported as 0.00423, which was somewhat close to the cut-off adjusted value of 0.00556. This equality indicated a subtle effect on the varying catalysis reaction times in determining lipid content from FAME. There is an observable increasing trend in the lipid content with longer acid catalysis reaction times.

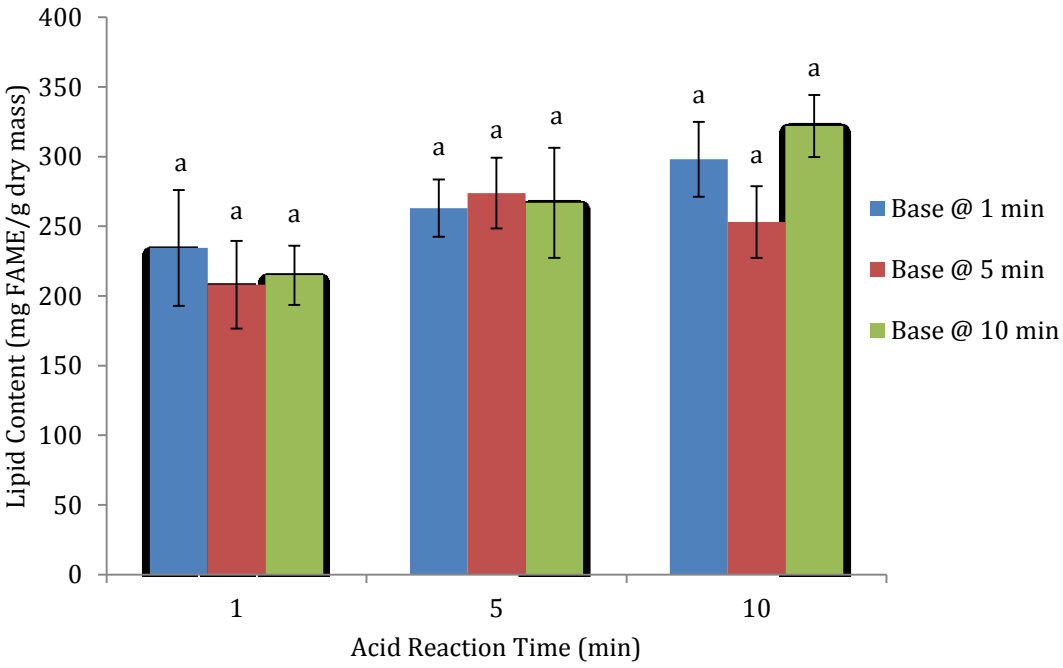

Figure 6.26. FAME content for reaction time optimization with full factorial design of 50 mL filtered culture of *A. falcatus* (mean ± SD, n = 3). Results with similar letters are not significantly different.

Reaction time had no significant effect on the lipid content (Fig. 6.27; MANOVA: Wilks Lambda = 0.004932, $p \leq 0.05$). The results of the lipid calculations from the total lipid peak areas were statistically equivalent, suggesting equivalent solubilization of the lipid into the solution.

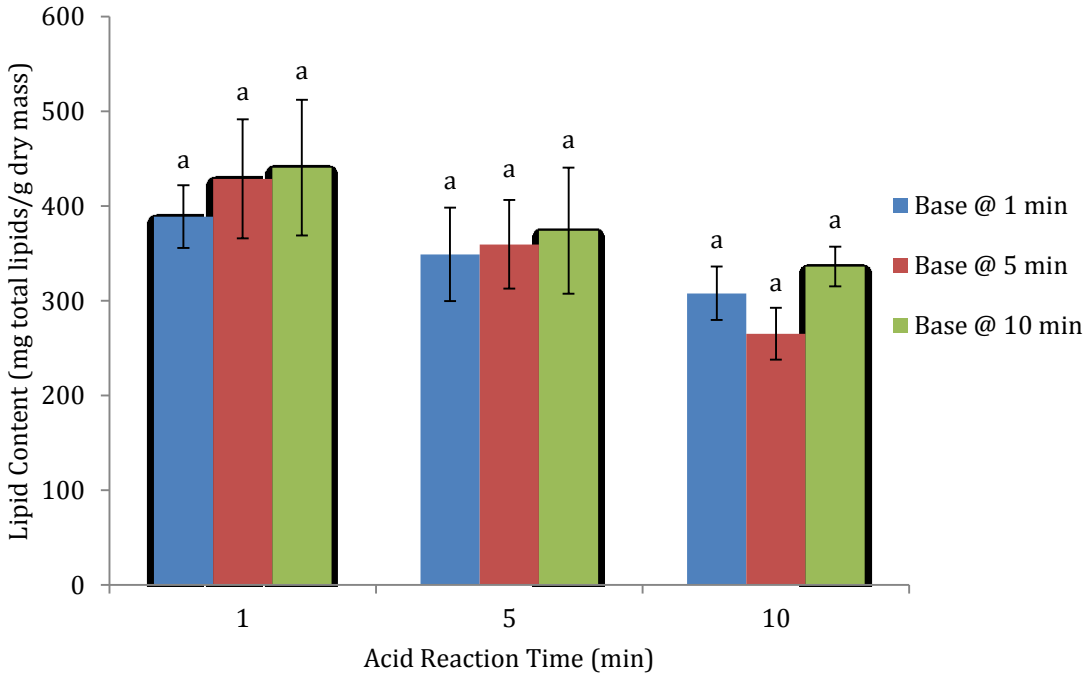

Figure 6.27. Total lipid content for reaction time optimization with full factorial design of 50 mL filtered culture *A. falcatus* (mean ± SD, n = 3). Results with similar letters are not significantly different.

From this experiment, a trend was observed between shorter reaction times with the basic catalyst and longer reaction times with the acidic catalyst in fully converting sample lipids to FAME with the minimal amount of FFA production.

CHAPTER 7: Discussion

7.1 Comparison of Three Derivatization Methods: P&G, L&R, and F&H

7.1.1 Slurry Culture

7.1.1.1 Lipid Classes – FAME

The results of the derivatization of microalgal slurry lipids to FAME for all three methods demonstrated successful derivatization all of the accessible or solubilized lipids within the sample (Fig. 6.1). The sample size (0.10 mL of ~12 % by weight slurry of microalgal cells) suggests an appropriate lipid and water loading of the microalgae sample and complies with the most stringent restriction of the *in situ* methods, namely the suggested maximum of 0.10 mL for water tolerance of the P&G method. However, this was not critical for the F&H method, where the lipid and water tolerance levels are higher due to the initial lipid extraction step, prior to derivatization (Folch et al., 1957). The extracted lipid is then dried, assuring that water content is negligible for the Hilditch derivatization reaction.

7.1.1.2 Lipid Content

There are inconsistencies in the lipid content of the microalgae samples for the three methods, ranging from less than 20 for L&R to nearly 35 mg lipid/g of dry microalgal mass for P&G (Fig. 6.2). Although there was not a statistically significant difference between the F&H method and the two *in situ* methods, the lipid contents

for L&R and P&G were significantly different; F&H was intermediate in its lipid content while P&G was the highest and L&R was the lowest. This difference was surprising considering that FAME was the only lipid class identified in the samples, indicating no residual unreacted TAG or PL from the original sample, nor the production of FFA from incomplete transesterification. It is also unlikely that there would be losses of these lipid classes with the discarded liquid of the supernatant as it was comprised mostly of distilled water, residual methanol, and other aqueous byproducts (Christie, 2011). All samples were rinsed thoroughly with hexane to extract FAME, which would also be the preferential solvent, compared to the aqueous phase, in which TAG, PL, and FFA would solubilize. The lipid contents were calculated based on the presence of the internal standard, of which each sample was supplied with a measured, consistent amount, making this an unlikely source of the discrepancy. However, the three methods did use different solvent systems, which may have led to differences in each method's miscibility with the water present in the sample, as well as altered the methods' ability to solubilize the lipids in the sample and permeate the microalgal cells. These ideas will be expanded in combination with the following discussion of experimental results.

7.1.2 Filtered Culture

7.1.2.1 Lipid Classes

The lipid class results of the comparison of the three methods with filtered microalgal culture were similar to those of the slurry culture, although there was a

poorer conversion of lipid to FAME. Figure 6.5 shows a large proportion of FAME for all of the methods, although also present are small amounts of TAG for the L&R method and PL for all three methods. The amount of microalgae applied to the filter was 0.10 mL of a condensed slurry equivalent to ~5.0 mg dry microalgae, which was then rinsed, contributing ~0.20 mL of trapped water on the filter. The cause of the incomplete derivatization was most likely the increased amount of water associated with the filter. An increase in water content is often detrimental to the extent of derivatization reactions (Lepage et al., 1986).

Another source of discrepancy in the derivatization could have been the presence of the filter, which may act as a matrix within which lipids may be retained. It is difficult to relate this hypothesis to other *in situ* derivatization methods, as most existing *in situ* methods were not developed for cultures deposited on filter paper. As well, most methods for determining lipid classes in filtered cultures grind their filters prior to any reactions to facilitate access of solvent to lipid (Pernet et al., 2003). Grinding was omitted for the two *in situ* derivatization methods to ensure a rapid reaction and to avoid sample loss. This omission did not seem to contribute to the overall derivatization as greater lipid yields were not found with F&H, which does grind samples. Examination of the filters after transesterification revealed pure white filters that were thoroughly soaked in the primarily methanol solvent system, implying both full contact between the sample, the catalyst(s), and the organic solvents.

7.1.2.2 Lipid Content

There was no significant difference in the lipid contents for the three methods using filtered culture, though a visible trend in the mean lipid content again demonstrated L&R as producing the lowest lipid quantity (Fig. 6.6). This discrepancy can be explained in part by the residual TAG in the L&R samples (Fig. 6.5), as well as likely suffered from the same solvent system hindrance as the slurry culture experiment, (above, Section 7.1.1.2).

7.1.3 Solvent Systems of Respective Methods

The organic solvents used in each method (methylene chloride and methanol, methanol and benzene, and chloroform and methanol for the P&G, L&R, and F&H methods, respectively) all had similar polarities to both solubilize lipids and combine with the water of the aqueous samples. However, the polarity of the L&R method may have been altered from its original design with the replacement of benzene with less toxic hexane (Han, 2008). However, in terms of polarity and lipid solubility, a more suitable replacement for benzene may have been toluene as opposed to hexane (Carvalho et al., 2005; Lane et al., 1989; Lepage et al., 1986), though the replacement of the equally toxic toluene does little to improve the exposure risk that benzene carries (Miyagi et al., 1999). This alteration of replacing benzene for hexane may have created a more strongly non-polar solution compared to the other methods as hexane is more non-polar than benzene (Reichardt, 2003). The more non-polar solvent may have been repelled by the high water content of the whole microalgal cell, limiting the contact of the acid catalyst with the *in situ*

lipids. The lipids of microalgae are largely PL, primarily phosphatidylethanolamine (Sanina et al., 2004), which are soluble in alcohols, with some TAG, which is soluble in less polar organic solvents, like chloroform, though neither require an extremely non-polar solvent to solubilize. Solvent systems for the extraction of lipids from samples with membranes (i.e. phospholipid bilayer of cells) are thus intentionally developed with more polar solvents to adequately permeate the membrane (Leray, 2011).

7.1.4 Investigations into Lipid Classes and Content Variation

The frequent transfers of the extracted lipid of the F&H method could contribute significant errors to the final lipid determination as the internal standard was added to the sample after extraction for the slurry culture comparison (Fig. 6.2). This procedure was corrected in the filtered culture experiment where the internal standard for the F&H method was added during the extraction portion of the method, thus carrying through the losses of any residual lipid throughout the experiment (Fig. 6.6). The lipid content for slurry culture generated by the F&H method was not drastically different from the result generated for the filtered culture, indicating the extra sample handling without internal standard did not seem to have a significant effect.

With the high quantity of water within the filtered cultures, a spontaneous creation of liberated PL into liposomal structures could have occurred, causing discrepancies in the lipid yield (Fig. 6.2, Fig. 6.6). The structural integrity of the liposome could have restricted the permeability of the solvents and stopped the

lipids from reacting with the catalysts. The creation of liposomes comprised of PL encased between a solid substrate and water has been documented as occurring as soon as one minute after mixing (Yamada et al., 2007). This phenomenon may have occurred between the aqueous phase of the reagents and the glass fibers of the filter paper containing the microalgae; the multifaceted surface of the filter and the abundance of water clinging to the filter makes this phenomenon more likely.

It is unlikely that variation in the quantity of microalgae applied to the filter could be the cause of the variation in FAME yield, as samples were well mixed and measured precisely for all methods, for both slurry and filtered microalgal cells (Fig. 6.2, Fig. 6.6). The similarities in the results of the two experiments demonstrated the robustness of the F&H and P&G methods; water content on the filters was difficult to control, yet did not seem to have a significant effect on FAME yield.

7.1.5 Effect of Water Content on Basic and Acidic Catalysts

The success of the independent methods did not agree with a number of literature reports (Sukhija et al., 1988; Griffiths et al., 2010; Yang et al., 2011), which suggested that water present in amounts of 10-20 % by volume would be detrimental to the basic and acidic catalysts, with as little as 6 % by volume contributing to saponification reactions with a basic catalyst (Suter et al., 1997). However, Lepage & Roy (1986) reported no influence of up to 30 % water by volume on the esterification of FA standards, although 20 and 30 % water by volume water had increasingly detrimental effects on the derivatization of TAG

standards, with an exacerbated effect demonstrated as FA chain length increased. Their experiments comparing their *in situ* method to the traditional Folch et al. (1957) method demonstrated a higher conversion of lipid to FAME for the *in situ* method for samples of human milk and adipose tissue where water content of the samples was maintained at 10 % by volume. This suggested that *in situ* methods could be used with biological tissues with water contents <10 % by volume.

The individual rates of derivatization and saponification reactions may offer some insight to the unexpected proficiency of the primarily base-catalysed reaction (P&G) over the primarily acid-catalyzed reaction (L&R) in derivatizing microalgal samples with excessive water contents. Literature suggests that higher water contents with a basic catalyst may lead to saponification and can create FA salts, which react and compete with the catalyst (Ma et al., 1998), although the production of FA salts may proceed at a fraction of the speed of the transesterification reaction (Suter et al., 1997). When the acidic catalyst was then applied after the initial base reaction with the P&G method, the basic catalyst was neutralized and the acidic catalysis reaction proceeded, effectively stopping any saponification reactions caused by the basic catalyst. The acidic catalysis reaction then acts as a clean-up step for residual FFA, any generated FA salts, and other traces of lipid which were not derivatized by the base catalyst, fully esterifying and transesterifying all of the lipids present in the sample. Though the presence of water was high in the sample, the miscibility between water and methanol is good, allowing both catalysts to react readily with the *in situ* lipids.

7.1.6 Comparison to GC Data

The high water content of the filtered culture compared to the slurry contributed to a lower total FAME conversion, with traces of TAG and PL in all trials, especially L&R (Fig. 6.5). Incomplete transesterification is capable of producing undesirable compounds, such as monoacylglycerol (MAG), diacylglycerol (DAG), and FFA (Xie et al., 2006). These compounds have low volatility and do not elute properly on GC columns, often condensing on glass injection liners and the stationary phase of the GC column. Though not always requiring the replacement of a GC column, the presence of these undesirable compounds can create errant peaks on future GC chromatograms should the compounds pass through the column (J&W, 1998). With the formation of undesirable compounds, the FAME concentration determined by GC will not be accurate. The acyl lipids lost to byproducts of FFA, MAG, and DAG would not be quantified by GC, skewing the mass of lipid for the sample (Levine et al., 2010).

FAME concentrations determined by TLC-FID and GC were equivalent for their respective methods (Fig. 6.4). Due to the sensitivity of the GC analysis, the equivalence between these two quantification methods lends support to the accuracy and precision of the TLC-FID quantification method.

The mass proportions of the ten highest FA contributors to the total FAME were not statistically different for the three methods (Table 6.1); however, in the lipid contents (Fig. 6.3) there was only one instance in which P&G agreed with L&R, where the mass of 22:5n-6 was equivalent for all three methods. This result corroborates what had been observed from the TLD-FID data: FA masses for the

individual *in situ* methods and F&H were often similar but the two *in situ* methods rarely produced equivalent results; however, the mass proportions of individual FA demonstrated consistency between the methods, indicating no significant preference of a method for derivatizing a particular type of FA.

7.1.7 Summary of the Comparison of the Three Methods

The purpose of comparing the methods was to determine if the F&H method could be improved upon with both shorter reaction and sample workup times. Both of the *in situ* methods applied directly to the sample without prior extraction of lipids reduced potential losses and improved the speed of sample processing. The original derivatization method (Hilditch et al., 1964) required an hour of reaction time, similar to the L&R method. Considering the lack of statistical differences in FAME and lipid yield between P&G and F&H, and the improved speed of reaction, P&G was determined to be the best candidate with which to proceed.

Alternate *in situ* methods considered for study included and Abdulkadir & Tsuchiya (2008), O'Fallon et al. (2007), Suter et al. (1997), and many others. The consideration for these methods was not extended beyond initial literature review as they required greater sample preparation or reaction time or they required expensive or proprietary equipment.

7.2 Lipid Load with P&G *in situ* Method

7.2.1 Selection of Menhaden Oil for High Lipid Loading

The amounts of menhaden oil were selected to approximate the typical mass of lipid found in filtered microalgae, which can contain anywhere from 5-70 % lipid per mass of dry biomass. It was not feasible to use microalgal lipids for this experiment due to the difficulty in isolating large quantities of lipid from microalgae. Menhaden oil was selected for its availability, purity, and similarity to microalgal FA profiles, as it is a marine lipid source. It is comprised entirely of TAG, which would be readily transesterified by the basic catalyst (Carrapiso et al., 2000). A marine lipid sample composed of TAG better represents a stressed microalgal sample, which has a preference for accumulating large quantities of storage lipids as TAG, rather than PL (Lombardi et al., 1995). TAG also has low solubility in the solvent system of the method, primarily methanol, which further helps to demonstrate the robustness of the method for non-ideal reaction conditions.

No differences in the derivatization reaction results were attributed to the lipid class of the lipid samples used in the loading experiments as the P&G method showed proficient derivatization of moderate amounts of both TAG and PL, typical lipids in an microalgal sample (above, Section 7.1.1.1). Although PL has a higher preference for solubilization in methanol than TAG, the nature of the *in situ* reaction does not entirely depend on full solubilization of lipids, so long as the solvent system can adequately permeate the biological matrix of the sample and achieve sufficient contact between the catalysts and the lipids. However, it is logical to

assume that, with limited reaction time (i.e. 10 min per catalyst) and a varying content of water, there may be a slight preference for the derivatization of PL over TAG due to the primarily methanol solvent system employed by this method.

7.2.2 Lipid Class Composition and Concentration for High Lipid Load with Menhaden Oil

The lipid class results for the experiments with high lipid loading with menhaden oil and low loading with filtered microalgal culture demonstrated a relationship between higher lipid loads and a lower conversion of lipid to FAME. For instance, there was a significant decrease in FAME yield as the lipid load increased from 0.10 to 5.0 mg in the high lipid loading experiment with menhaden oil (Fig. 6.7). There are few differences between the lipid loads, although there was a trend to decreasing conversion of lipid to FAME as the loading increased, with 5.0 mg lipid dropping to nearly 90 % conversion while 0.10 mg was at 100 %. Water content of the samples was kept constant as the Park & Goins (1994) method suggested an aqueous sample load of 0.10 mL. Although the method does not specify a lipid limit, the aqueous aliquot of 0.10 mL suggested by P&G was for homogenized samples in water, such as a 20 % solution of egg yolk (the yolk having ~25 % lipid by weight, contributing ~5 mg lipid to the 0.10 mL aliquot). P&G reported full derivatization of their biological and food samples. Menhaden oil, though readily transesterified by the base catalyst (above, Section 7.2.1), may be present at too great of an amount with the higher lipid loadings relative to the amount of the basic catalyst. However, the molar amount of methanol is several orders of magnitude

larger than that of FA in a 5 mg quantity of lipid, making it very unlikely that methanol is a limiting reagent, even with high sample loading.

Both FAME and total lipids showed unusual patterns as lipid load increased (Fig 6.8). Lipid loads of 0.10 to 1.0 mg produced relatively consistent FAME and total lipid yields, indicating a total solubilization of the lipids and full conversion of lipids to FAME. The 3.0 and 5.0 mg applications, however, produced 50 and 100 % higher lipid quantities than the other samples. These impossibly high yields were likely related to the size of the internal standard peak relative to the other lipids. As lipid load increased, so did the peak areas of FAME and TAG (the combination of the two being "total" lipids). To quantify, these peaks were compared on the same measurement scale to the peak area of the constant amount of 5-α-cholestane added. Thus, as FAME and TAG peaks increased, a relatively smaller peak for 5-α-cholestane was produced, diminishing the precision of the 5-α-cholestane peak area.

The agreement in FAME and total lipid results with samples of 0.10 to 1.0 mg indicated that the method was sensitive enough to handle small sample sizes with good accuracy and precision. From these results, the highest advisable lipid content of pure lipid was set conservatively at 1.0 mg for filtered microalgae samples.

7.2.3 Solubility of Menhaden Oil in the Reaction Solvent System

In this experiment, where the only variable was the quantity of lipid applied, one of the sources of incomplete transesterification could be the reduced solubilization of menhaden oil in the solvent system, reducing the exposure of the lipid to the catalysts present in the primarily methanol-based system. This

hypothesis is made more likely by the state of the sample, in which menhaden oil is directly applied to the reaction vessel as a pure sample of TAG. This makes it very accessible to be solubilized in contrast to whole cell microalgal samples which contain lipids within a biological matrix. This, however, was not observed with high quantities of lipid. TAG has limited solubility in methanol and solubilizes more readily in non-polar solvents (Suter et al., 1997). The presence of water, which is miscible with methanol, may also have been a contributing factor, increasing the polarity of methanol and further reducing the solvent system's ability to solubilize large quantities of TAG.

7.2.4 Lipid Class Composition and Concentration for Low Lipid Loads with Filtered *Isochrysis sp*.

There was not a significant difference in the lipid class results for the low lipid loading experiment with the filtered microalgal cultures; however, there was a clear trend of decreasing conversion of lipid to FAME as the amount of filtered culture increased (Fig. 6.9). The experiment was designed to demonstrate a real-world scenario of filtering microalgal cultures in varying quantities and observing their lipid to FAME conversion. The trends clearly indicated a preference for low microalgal sample loading for the best FAME conversion. There were two possible explanations for this shift in conversion: 1) with higher sample loads, there was more microalgae with higher quantities of lipid that must be extracted into solution; and 2) higher culture loads also have increased water content. Water content on the filter was controlled as much as possible by ensuring consistency in the period of

time that vacuum was applied after the bulk water was removed from the culture sample. The increase of lipid in the sample, though relatively small in comparison to the high lipid loading with menhaden oil, was likely exacerbated by the increasing presence of water, shifting the solvent system to a higher polarity and likely limiting its effectiveness in extracting lipids from the microalgae.

FAME and total lipid content of the samples did not show a significant variation with increasing lipid load (Fig. 6.10), partially due to the large standard deviations. The 50.0 mL cultures had exceptionally large variation in replicates for unknown reasons. The same decreasing trend in FAME content was observed here with increasing sample size. Coupled with an increase in water content as sample content increased, the trends indicated that full derivatization can more easily be attained with smaller culture volumes. However, because these trends are not statistically significant, their importance is diminished.

The highest sample loading, 100 mL, used ~11 mg of microalgal culture (Table 5.2), which contributed ~10 % lipid per dry mass (Fig. 6.10) and had the lowest FAME and total lipid content. This accounted for ~1 mg of lipid in the sample, which was the advisable lipid load as determined by menhaden oil, so greater FAME derivatization was anticipated (see Section 7.2.2 above). Although these two samples were not identical—a primarily TAG oil compared to a primarily PL microalgae culture—the moderate application of ~1 mg was within an advisable quantity of lipid applied to a filter. Compared to earlier experiments using filtered microalgal culture with varying contents of water, this trend is somewhat unique

and does not entirely represent typical outcomes of filtered microalgal culture with ~1 mg of lipid (see Section 7.1.1 above).

Further study into the ideal ratio of reagents to sample used 100 mL volumes of *Isochrysis sp*. with more favourable results on FAME production (see Section 7.2.5 below). These results of incomplete transesterification with a moderate ~1 mg lipid load are likely anomalous.

7.2.5 Increasing Ratio of Reagents to Sample with Filtered *Isochrysis sp.*

To ensure that the results of the experiments using low sample loads with filtered culture were not due to saturation of reagents, insufficient amount of reagents, or an overwhelming quantity of water associated with the sample, differing quantities of reagents with a constant volume of the same filtered culture (100 mL) were tested. This resulted in identical FAME and total lipid content for all three of the tests (Fig. 6.11). This was particularly significant as the volume of culture applied to the filter (100 mL contributing ~13 mg of microalgae) was a relatively large sample load. This confirmed that the quantity of reagents used is appropriate for the typical amount of filtered microalgal sample, even with high sample loads.

7.3 Water Tolerance with P&G *in situ* Method

7.3.1 Effects of Increasing Water Content on Derivatization of Lipids

High quantities (10 mg) of menhaden oil were applied to glass fiber filters and mixed with increasing quantities of water. A decreasing conversion of lipid to FAME as the water content increased was obvious (Fig. 6.13). This experiment was extreme in that the only result that produced 100 % conversion to FAME was the sample with 0.0 mL water, which is not feasible when working with wet biological samples. The sample with 0.10 mL water showed ~90 % conversion of lipids to FAME, with its unreacted TAG creating ~10 % residual lipids. The recommended aqueous sample size for the P&G method was a 0.10 mL homogenized aqueous aliquot (Park & Goins, 1994) contributing a lipid content of ~5 mg. In this case, the 10 mg lipid tested was twice the amount that Park & Goins (1994) would have encountered in their samples, and higher than normally encountered in small quantities of microalgae. As water content increased, the polarity of the solvent would have also increased, likely causing the TAG to experience diminished solubility. FFA, which would represent an incomplete reaction, were not identified here, while the presence of unreacted lipids (TAG) supports the idea that the lipids were not exposed to the catalysts at all. Dunn et al. (1994) reported the formation of microemulsions in a combined system of soybean oil, alkaloid, and methanol, with concentrations of water as little as 1.0-1.5 %. The P&G base catalyst, NaOH, has a higher solubility in water than in methanol so, should this phenomenon be occurring with increasing amounts of water in the experiment, the sample's lipids

would have decreased exposure to the catalyst as it is associated with the aqueous phase. Regardless of the specific cause, these results indicated that the higher the lipid concentration, the lower the water content must be to achieve proper derivatization.

With low applications (0.5 mg) of microalgal lipid, a high dependence on water content was not observed (Fig. 6.14). The conversion of lipid to FAME was nearly 100 % for all of the samples, despite the water content being increased up to 0.50 mL. The only other lipid class identified was PL, which was found in trace amounts. This implies good derivatization of solubilized lipids into solution within a reasonable range of water content for small applications of extracted lipid, extending the robustness of the method to include direct applications of lipid to be derivatized in small quantities. The proficiency of derivatization in this experiment may have also been due to the prevalence of PL in the extracted microalgal lipids, which preferentially solubilize in alcohols, like methanol.

There was no statistical difference in FAME and total lipid concentrations (mg/mg applied lipid), which were centered around 1 mg/mg (Fig. 6.15). The trial with the highest water content, 0.50 mL, had a very high mean, corresponding to approximately 50 % more lipid than the known applied quantity. Coupled with the largest standard deviation in the group, these results indicated a lack of reproducibility in peak areas for samples with higher water contents, despite the complete conversion of lipid to FAME. These results once again demonstrated a preference for lower sample loading.

7.3.2 Effects of Increasing Water Content on Derivatization of Filtered *Isochrysis sp*.

Although there seemed to be little influence of water on the efficiency of derivatization of microalgal oils, it was necessary to confirm this result with filtered cultures, since there may also be a matrix effect due to non-lipid components in the cells. The varying content of water trapped on the filter did not have a significant effect on lipid proportions remaining after derivatization of filtered cultures (Fig. 6.16). All samples showed a slightly higher residual TAG content than had been seen with the other filtered cultures, although the source of this remains unknown. The results indicate that, for small quantities of lipid in an microalgal sample, a large range of water associated with the sample will have very little effect on the derivatization reaction. These results agree with those summarized when microalgal oil was used as the sample (see Section 7.3.1 above).

Similarly, both FAME and total lipid content of the filtered cultures were consistent with varying water contents (Fig. 6.17). This consistency suggests that water content had little effect on the derivatization of the biological samples when applied in moderate amounts. This experiment was performed with a low and consistent amount of microalgae, removing the influence of microalgal load, and little change was observed as the water content increased. Because this experiment was designed to replicate typical filtering procedure in the lab, it can be concluded that the water contributed by normal filtration will not limit the method's derivatization capability, despite the water content ranging from 0.28-0.55 mL.

7.4 Optimization of the P&G *in situ* Method

7.4.1 Dependence of Basic and Acidic Catalysts on Reaction Temperature

The conversion of lipid to FAME showed a strong dependence on the acidic reaction temperature (Fig. 6.18). As the temperature decreased, conversion dropped significantly. The effect was best observed with the basic reaction temperature at 90 °C and the acidic reaction temperature at 20 °C; here, the conversion to FAME was only around 30 %. FAME conversion was also higher for lower temperature basic reactions, which may be an effect of the creation of FFA or FA salts with basic catalysis at higher temperatures. This was in agreement with literature, which indicates excessive time or heat will contribute to a higher production of FA salts and FFA in the presence of a basic catalyst (Ackman, 1998). The creation of FFA was confirmed by TLC-FID and their proportion of total lipids increased dramatically when the temperature of the acidic reaction was lowered, especially when paired with higher basic reaction temperatures (Fig. 6.19). FFA were not detected in the reactions where the acidic reaction temperature was 90 °C, confirming that the higher temperature for the acidic reaction was necessary to esterify the FFA to FAME (Lepage et al., 1986). The FAME conversion and absence of FFA at 90 °C acidic reaction temperature were the same for all of the basic reaction temperatures, demonstrating that there was not a significant relationship between basic reaction temperature and conversion to FAME. However, the trends do indicate that a lower basic reaction temperature will be generally more beneficial to the final acidic reaction with a reduced production of FFA. These results indicated

that excessive, unnecessary heating of the basic reagent may actually promote saponification and the formation of byproducts, such as FA salts and FFA, which are only poorly esterified at low acid reaction temperatures.

FAME and FFA were the major lipid classes identified in the temperature optimization experiment, with very little TAG and PL detected. The absence of large quantities of the original lipids, TAG and PL, demonstrated good contact between the lipids and the basic reagent; however the presence of FFA indicated incomplete transesterification. Any FA salts or FFA generated by the base-catalysed reaction should have been derivatized by the acid-catalyzed reaction; however, this was not observed here at acidic reaction temperatures <90 °C. The highest quantities of FFA can be associated with lower acidic reaction temperatures, confirming the acidic catalyst's need for a high reaction temperature.

There was an increasing trend in the mass of FAME produced as the acidic reaction temperature increased, while the basic reaction temperatures of 20 and 55 °C generally gave higher FAME yields (Fig. 6.20), supporting the results found for lipid class proportions (Fig. 6.19). The variation in the lipid content was large, especially when the acidic reaction temperature was 90 °C; however, the standard deviations were also rather large. Results for total lipid produced were more equivalent across all acidic reaction temperatures, indicating a full and equivalent solubilization of lipids from the samples (Fig. 6.21). The basic reaction at 55 °C showed a trend of higher lipid content, while a decreasing trend of lipid content was apparent as the acidic reaction temperature increased while the basic reaction was held at 90 °C. These are, however, only trends; the large standard deviations led to

results that were not statistically different for reaction temperatures for the acid and base catalyzed reactions. This leads to the inference that the large variation in FAME content exists because the acidic catalyst required a higher reaction temperature (or longer reaction time) to properly derivatize the FFA generated by the basic catalysis (AOCS, 1998; Christie, 1993).

7.4.2 Minimum Temperature for Acid-Catalyzed Reaction

The acid reaction temperature was further analyzed to ensure that another, relatively high, reaction temperature would not be as proficient. The three temperatures investigated, 80, 90, and 100 °C, all produced identical lipid class proportions (Fig. 6.22). Results were also equivalent among temperatures for FAME and total lipid contents, indicating good solubilization of the lipids and appropriate reaction conditions for both basic and acidic reactions (Fig. 6.23). The agreement between 90 and 100 °C was expected because of the prevalence of the latter temperature in acid catalysed derivatization reactions (Lepage et al., 1986). The agreement between all of the reaction temperatures demonstrates that 80 °C is also a sufficient reaction temperature; however, the large standard deviation associated with the 80 °C sample, in addition to its high mean value, may indicate less consistency with this reaction temperature, making 90 °C a more reasonable selection for the acid reaction temperature.

7.4.3 Dependence of Basic and Acidic Catalysts on Reaction Time

The conversion of lipid to FAME showed a strong dependence on reaction time of the acidic catalyst (Fig. 6.24), with higher proportions of lipid converted to FAME as reaction time increased. The same dependency was not observed with the basic reaction time, which produced a mostly consistent result across all acidic reaction times. These results agreed with literature, which summarized acidic catalysts as requiring a relatively long reaction time (i.e. 1 hr) (Lepage et al., 1986) while basic reactions happen almost immediately (Suter et al., 1997). The reliability of the results was supported by the low standard deviations around the means.

The two main lipid classes identified by TLC-FID were FAME and FFA, which indicated an incomplete acidic reaction to convert the FFA and FA salts to FAME, likely generated from the base-catalyzed reaction (Fig. 6.25). As described above for the temperature optimization results (see Section 7.4.1.), the relative absence of TAG and PL indicated the basic reaction was consistently hydrolyzing those lipids, while the residual FFA points more to an incomplete acidic reaction with the short reaction time (Carrapiso et al., 2000).

Due to high variance, FAME masses were equivalent for all of the reactions with an increasing trend towards higher FAME content as acidic reaction time increased (Fig. 6.26). From the two earlier experiments, the lipid content was ~300 mg/g dry mass; the trial with the highest reaction time, 10 min, came closest to generating this amount of FAME. As expected, the opposite effect was observed for total lipids, where a decreasing trend was found with increasing acidic reaction

temperature (Fig. 6.27). For both FAME and lipid content, the basic reaction time had very little effect on lipid content.

7.4.4 Summarized Results of the Optimization Experiments

From the optimization reaction experiments, the conversion of lipids to FAME was influenced most strongly by the conditions of the acidic reaction, which required a temperature of 90 °C and a reaction time of 10 min, while the basic reaction generated a significantly higher content of FA salts and FFA at higher temperatures for longer reaction time. From these results, it is advisable to perform the basic reaction at ambient temperature and neutralize it quickly, in contrast to the basic reaction temperature of 90 °C for 10 min originally proposed by the P&G method (Park et al., 1994). However, the original reaction time and temperature of 10 min at 90 °C, as proposed by P&G, should be used for the acidic reaction.

CHAPTER 8: CONCLUSIONS

In this study, two *in situ* transesterification methods, Lepage and Roy (L&R; 1986) and Park and Goins (P&G; 1994), were assessed for their ability to transesterify microalgal samples. Their conversion of lipid to FAME was compared to a standard two-step method, Folch et al. (1957) lipid extraction followed by Hilditch et al. (1964) derivatization (F&H). For both slurry and GFC filtered cultures using *Tetraselmis sp.*, the L&R method produced the smallest FAME yield and left significant amounts of lipid unreacted. The P&G method converted lipid to FAME as well as the F&H method without requiring an extraction step or transferring sample between vials. The P&G method was therefore selected as the *in situ* method for further study.

The P&G method was tested for maximum lipid and water load incorporated by a sample. The maximum lipid load was assessed with samples of menhaden oil (0.1 – 5.0 mg) and GFC filtered *Isochrysis sp.* culture (4.7 - 11 mg), with a controlled quantity of water (0.1 mL) for menhaden oil and a range of water (0.27 – 0.35 mL) for the filtered microalgal culture. The maximum advisable lipid load was ~1 mg lipid for all experiments, with poorer conversion to FAME associated with ≥3.0 mg menhaden oil.

The maximum water load was tested with menhaden oil (10 mg), microalgal lipid (0.5 mg), and GFC filtered *Isochrysis sp.* culture (13 mg dry mass), with quantities of water ranging from 0.0 – 0.5 mL for menhaden oil and microalgal lipid, and 0.28 – 0.55 mL for the filtered culture. Water did not have a significant effect on

FAME yield for the microalgal lipid or filtered microalgal culture; however, there were reduced FAME yields with the menhaden oil experiment when water content was as little as 0.1 mL. From these experiments, it was determined that moderate sample loading with microalgal cultures worked well with the method, despite the large range of water that could be trapped in the filter paper. High sample loading was unadvised for both low and high water contents.

The P&G method was optimized with GFC filtered *A. falcatus* to establish ideal reaction temperature and length of reaction for each of its catalysts: basic and acidic. The reaction temperatures of both catalysts were varied in combination between three temperatures: 20 °C (ambient temperature), 55 °C, and 90 °C (the original reaction temperature). No significant effects were observed for FAME production with changing temperature for the basic catalyst; however, trends indicated reduced FAME yield and increased FFA production for the basic catalyst when temperatures >20 °C were used. Acid reaction temperature had a significant effect on FAME production, with a clear need for the highest reaction temperature, 90 °C, and the longest length of reaction, 10 min, for adequate FAME production.

The reaction temperature of the acidic catalyst was further analyzed to establish a minimum temperature and to reduce the potential for lipid degradation during the reaction. The acidic catalyst reaction temperature was varied at 80, 90, and 100 °C, while the basic reaction was held at ambient temperature. All three of the acidic catalyst reaction temperatures produced equivalent results; however, the variance in the replicates, along with a high mean, eliminated the 80 °C acidic

catalyst reaction temperature, and supported the conclusion of an ideal acidic catalyst reaction temperature of 90 °C.

For the length of reaction, both catalysts were varied in combination between three lengths of reaction: 1 min, 5 min, and 10 min (the original length of reaction). The basic reaction time had no significant effects on FAME production; however, trends indicated that longer reaction times with the basic catalyst resulted in increased FFA production. Length of reaction for the acidic catalyst did have a significant effect on FAME production, with a reduction in FAME and an increase in FFA production with decreasing reaction time.

From these results, it can be concluded that the P&G *in situ* transesterification method fully derivatizes microalgal lipid to FAME for GFC filtered microalgal cultures with ~1 mg maximum lipid loads, with no hindrance from the typical range of water associated with filtration. The basic catalyst was shown to perform best at a low reaction temperature (20 °C) for a short length of reaction (1 min), while the acidic catalyst required the original reaction temperature (90 °C) and length of reaction (10 min) to fully derivatize all remaining lipids. Utilizing this *in situ* method shortens the reaction time of microalgal FAME preparations from ~3 hr to ~20 min.

This research has successfully adapted an *in situ* transesterification method for applications with microalgae dewatered on GFC filter paper. Because the water and sample loading—0.1 mL homogenized aqueous aliquots of biological samples from the original Park & Goins (1994) method—was much lower than the tolerance found in this research, it would stand to reason that these sample preparations are

also appropriate for this adapted method. For verification, various biological samples (e.g. milk, egg yolk, meat, macroalgae, etc.) could be tested to demonstrate the robustness of this method.

As well, water tolerance established with high loading of menhaden oil (see Section 6.3.1) demonstrated that 100 % FAME conversion could be achieved for 10 mg of lipid with this method when no water content was present (Fig. 6.13). To adapt this method to pure lipid samples, a maximum lipid loading experiment with no water added to the sample could establish the maximum lipid tolerance of the method. For these larger lipid samples, it would be necessary to adjust the amount of internal standard used to more closely match the expected FAME or FA peak areas generated by TLC-FID and GC, respectively.

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APPENDIX A
MICROALGAL DRY MASSES

Table A1. Microalgal dry masses for various experiments.

Section	Sample		Mass Filter/Pan (g)	With Sample (g)	After Dry (g)	Water (g)	Dry Mass (g)
6.1.1		1	1.0021	1.1041	1.0133	0.0908	0.0112
		2	1.0132	1.1171	1.0253	0.0918	0.0121
		3	1.0092	1.1158	1.0215	0.0943	0.0123
6.1.2		1	0.9858	1.0897	0.9910	0.0987	0.0052
		2	1.0159	1.1125	1.0207	0.0918	0.0048
		3	1.0139	1.1191	1.0190	0.1001	0.0051
		4	1.0248	1.1273	1.0295	0.0978	0.0047
		5	1.0217	1.1248	1.0267	0.0981	0.0050
		6	0.9987	1.0987	1.0035	0.0952	0.0048
6.2.2	50 mL	1	1.0788	1.3536	1.0834	0.2702	0.0046
		2	1.0938	1.3589	1.0986	0.2603	0.0048
		3	1.0987	1.3900	1.1032	0.2868	0.0045
		4	1.0963	1.3994	1.1011	0.2983	0.0048
	75 mL	1	1.0958	1.3688	1.1037	0.2651	0.0079
		2	1.0868	1.3384	1.0940	0.2444	0.0072
		3	1.0808	1.3912	1.0890	0.3022	0.0082
		4	1.1045	1.3909	1.1129	0.2780	0.0084
	100 mL	1	1.1123	1.4539	1.1218	0.3321	0.0095
		2	1.1216	1.4655	1.1332	0.3323	0.0116
		3	1.0976	1.4899	1.1122	0.3777	0.0146
		4	1.0943	1.4720	1.1063	0.3657	0.0120
6.2.3		1	1.0897	1.3950	1.1029	0.2921	0.0132
		2	1.0828	1.3685	1.0970	0.2715	0.0142
		3	1.0903	1.3875	1.1042	0.2833	0.0139
6.3.3	0s	1	1.0948	1.6632	1.1117	0.5515	0.0169
		2	1.0929	1.6318	1.1077	0.5241	0.0148
		3	1.1170	1.6935	1.1349	0.5586	0.0179
		4	1.1164	1.6968	1.1327	0.5641	0.0163
	30 s	1	1.1383	1.4868	1.1494	0.3374	0.0111
		2	1.0928	1.4762	1.1048	0.3714	0.0120
		3	1.1046	1.4722	1.1156	0.3566	0.0110
		4	1.0860	1.4912	1.0975	0.3937	0.0115
	60 s	1	1.0990	1.4058	1.1097	0.2961	0.0107
		2	1.1155	1.3862	1.1248	0.2614	0.0093
		3	1.1032	1.3876	1.1125	0.2751	0.0093
		4	1.1029	1.4064	1.1128	0.2936	0.0099
6.4.1		1	1.0781	1.4567	1.0845	0.3722	0.0064
		2	1.1009	1.4565	1.1073	0.3492	0.0064
		3	1.0928	1.4311	1.0993	0.3318	0.0065

APPENDIX B
TLC-FID DATA

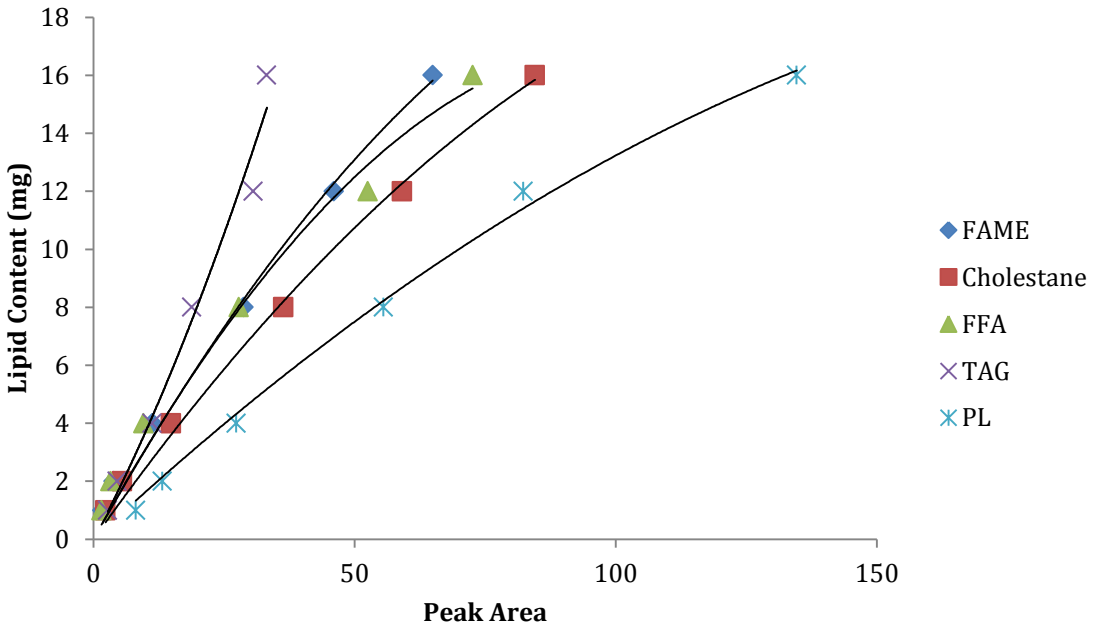

Figure B1. Calibration curve for five common lipids in TLC-FID data of microalgae.

Table B1. Lines of best fits for five common lipids in TLC-FID data of microalgae.

Lipid	a	b	R^2
Cholestane	-0.000451	0.254569	0.995058
FAME	-0.000561	0.323259	0.992999
FFA	-0.000812	0.328894	0.981896
TAG	0.004461	0.339775	0.978766
PL	-0.000457	0.167830	0.996210

For the equation $y = ax^2 + bx$ where y is lipid content (mg) and x is TLC-FID peak area.

Table B2. TLC-FID data for various experiments, reported as peak areas (PA).

Section	Sample	Number	Cholestane (PA)	FAME (PA)	FFA (PA)	TAG (PA)	PL (PA)
6.1.1	L&R	1	52.208	38.483	0	0	0
		2	54.631	29.325	0	0	0
		3	52.66	38.23	0	0	0
	P&G	1	21.301	26.849	0	0	0
		2	18.895	26.338	0	0	0
		3	26.148	34.713	0	0	0
	F&H	1	101.392	81.755	0	0	0
		2	127.832	115.087	0	0	0
		3	76.999	91.508	0	0	0

Section	Sample	Number	Cholestane (PA)	FAME (PA)	FFA (PA)	TAG (PA)	PL (PA)
6.1.2	L&R	1	46.637	11.955	0	0.343	0.548
		2	27.493	5.576	0	0.103	0.303
		3	42.603	11.036	0	0.478	0.985
		4	74.847	22.416	0	0.399	0
		5	82.131	28.107	0	0	0.162
		6	61.617	17.49	0	1.707	0
	P&G	1	42.136	15.552	0	0	0.448
		2	19.491	5.86	0	0	0
		3	15.351	6.865	0	0	0.735
		4	36.507	12.728	0	0	0
		5	32.315	11.007	0	0	0
		6	16.368	7.271	0	0	0
	F&H	1	unusable	unusable	unusable	unusable	unusable
		2	24.197	6.563	0	0	0.498
		3	66	21.253	0	0	0
		4	78.578	28.341	0	0	0.176
		5	97.284	39.461	0	0	0
		6	49.205	16.507	0	0	0.118
6.2.1	0.1 mg	1	17.198	12.174	0	0	0
		2	39.113	31.864	0	0	0
		3	30.625	24.717	0	0	0
	0.3 mg	1	20.616	48.698	0	0.42	0
		2	17.893	46.739	0	0.506	0
		3	20.76	52.58	0	0.587	0
	0.5 mg	1	14.57	62.791	0	1.134	0
		2	13.238	62.067	0	1.61	0
		3	13.297	56.273	0	2.509	0
	1.0 mg	1	13.048	86.976	0	1.357	0
		2	11.192	78.592	0	1.781	0
		3	12.146	83.639	0	1.54	0
	3.0 mg	1	2.214	84.121	0	1.669	0
		2	2.093	85.287	0	3.355	0
		3	1.92	74.576	0	1.642	0
	5.0 mg	1	0.795	70.591	0	5.49	0
		2	0.554	52.03	0	2.748	0
		3	0.867	73.473	0	4.133	0
6.2.2	50 mL	1	2.524	15.441	0	0.142	0
		2	3.957	19.367	0	0.6	0.634
		3	5.087	18.961	0	1.392	0

Section	Sample	Number	Cholestane (PA)	FAME (PA)	FFA (PA)	TAG (PA)	PL (PA)
6.2.2	75 mL	1	5.085	40.701	0	0.954	0.451
		2	3.919	28.863	0	2.06	1.382
		3	5.125	35.847	0	5.139	0.598
	100 mL	1	4.956	45.051	0	4.054	0.668
		2	4.33	42.059	0	4.206	2.265
		3	5.423	51.629	0	9.789	0.582
6.2.3	1 x	1	5.843	48.32	0	0	0.428
		2	8.427	49.502	0	0	0
		3	11.543	70.912	0	0	1.551
	2 x	1	9.221	39.623	0	0.371	0
		2	6.808	32.654	0	0.243	3.082
		3	6.817	29.581	0	0	0.45
	3 x	1	4.263	31.241	0	0	0
		2	9.395	37.41	0	0.131	0.609
		3	6.864	39.33	0	0	0.567
6.3.1	0.0 mL	1	0	55.425	0	0	0
		2	0	57.707	0	0	0
		3	0	58.063	0	0	0
	0.1 mL	1	0	62.727	0	5.9	0
		2	0	32.574	0	3.347	0
		3	0	64.21	0	4.29	0
	0.2 mL	1	0	75.152	0	30.321	0
		2	0	97.002	0	46.875	0
		3	0	110.391	0	42.641	0
	0.3 mL	1	0	20.584	0	16.986	0
		2	0	34.516	0	40.466	0
		3	0	19.714	0	21.705	0
	0.4 mL	1	0	9.774	0	18.514	0
		2	0	8.031	0	15.162	0
		3	0	10.15	0	28.309	0
	0.5 mL	1	0	12.94	0	28.936	0
		2	0	7.41	0	33.285	0
		3	0	20.581	0	60.652	0
6.3.2	0.0 mL	1	15.97	70.131	0	0	0
		2	14.863	59.078	0	0	3.65
		3	14.385	61.822	0	0	0
	0.1 mL	1	14.076	59.003	0	0	0
		2	13.655	54.171	0	0	0
		3	14.38	57.51	0	0	0

Section	Sample	Number	Cholestane (PA)	FAME (PA)	FFA (PA)	TAG (PA)	PL (PA)
6.3.2	0.2 mL	1	10.668	48.889	0	0	0.239
		2	9.046	38.464	0	0	0
		3	15.082	69.168	0	0	2.632
	0.3 mL	1	5.529	27.847	0	0	0
		2	7.155	36.749	0	0	0
		3	8.992	38.957	0	0	0.45
	0.4 mL	1	6.796	31.847	0	0	0
		2	8.004	32.66	0	0	0.62
		3	8.103	36.697	0	0	0.255
	0.5 mL	1	7.254	45.648	0	0	0.321
		2	3.672	28.305	0	0	0.521
		3	11.52	53.613	0	0	2.064
6.3.3	0 s	1	3.619	22.265	0	1.271	0.494
		2	4.621	24.519	0	1.022	0.219
		3	3.678	23.233	0	1.227	0.085
	30 s	1	6.121	30.634	0	2.756	0
		2	4.971	24.203	0	2.835	0.166
		3	5.473	27.121	0	2.856	0
	60 s	1	11.049	52.007	0	2.471	0.408
		2	8.882	44.086	0	2.722	0.284
		3	10.557	50.88	0	1.87	0
6.4.1	20 °C	1	4.849	51.44	7.447	5.552	0.143
	20 °C	2	3.82	43.43	13.96	5.24	0
		3	4.012	40.85	28.17	4.528	0
	20 °C	1	2.916	49.33	5.292	5.687	0
	55 °C	2	3.21	42.94	0.9310	3.507	0.189
		3	4.959	64.78	11.36	7.919	0
	20 °C	1	3.047	56.65	0	1.975	0.494
	90 °C	2	5.264	73.154	0	2.128	0
		3	4.279	68.302	0	2.513	0
	55 °C	1	2.231	28.15	23.70	5.341	0.964
	20 °C	2	3.01	30.93	31.24	5.891	0.668
		3	5.163	30.37	41.04	5.647	1.291
	55 °C	1	3.139	53.74	13.49	6.786	0.209
	55 °C	2	3.954	56.69	29.16	8.034	1.132
		3	2.864	42.32	11.04	5.531	0
	55 °C	1	3.2	61.023	0	1.507	0
	90 °C	2	2.266	53.917	0	1.616	0.976
		3	2.457	55.265	0	1.593	0

Section	Sample	Number	Cholestane (PA)	FAME (PA)	FFA (PA)	TAG (PA)	PL (PA)
6.4.1	90 °C	1	5.227	35.49	57.25	8.889	0
	20 °C	2	3.639	20.09	41.76	5.853	0.116
		3	3.547	25.07	59.89	5.238	0
	90 °C	1	5.517	54.69	18.71	7.916	0
	55 °C	2	5.338	37.63	30.48	6.872	0
		3	4.459	42.99	27.16	7.231	0
	90 °C	1	5.141	67.44	0	2.87	0.337
	90 °C	2	6.463	71.11	0	3.428	0
		3	3.908	56.80	0	2.966	0
6.4.2	80 °C	1	1.369	26.834	0	0.332	0.605
		2	3.098	34.477	0	0.739	0.164
		3	3.136	32.7	0	0.429	1.104
	90 °C	1	4.986	42.612	0	0.467	0.427
		2	4.443	41.663	0	0.901	0.194
		3	3.079	30.273	0	0.217	0.46
	100 °C	1	4.094	44.399	0	1.298	0.497
		2	3.519	33.837	0	0.984	0.152
		3	3.169	34.521	0	1.024	0.366
6.4.3	1 min	1	2.493	32.57	14.67	4.264	0.413
	1 min	2	3.665	37.29	25.21	5.27	0.593
		3	2.556	37.20	14.22	3.704	1.456
	1 min	1	2.731	38.78	11.79	3.14	0
	5 min	2	2.886	37.89	4.141	2.512	0.631
		3	2.582	39.58	9.292	3.326	0
	1 min	1	2.455	43.91	0	1.302	0
	10 min	2	4.196	66.14	0	1.722	0
		3	3.084	49.12	0	1.403	0
	5 min	1	2.533	30.81	27.73	5.186	0
	1 min	2	3.067	36.97	30.07	5.547	0
		3	3.148	28.72	26.73	3.837	0.114
	5 min	1	2.669	43.69	6.808	3.051	0
	5 min	2	3.86	53.74	6.688	3.925	0
		3	2.955	43.57	14.13	4.289	0.103
	5 min	1	3.752	54.754	0	2.253	0.113
	10 min	2	5.438	69.666	0	2.558	0
		3	3.818	58.146	0	2.338	0
	10 min	1	3.423	36.85	28.20	5.291	0.281
	1 min	2	2.213	26.89	25.64	4.098	0.399
		3	unusable	unsuable	unsuable	unusable	unusable

Section	Sample	Number	Cholestane (PA)	FAME (PA)	FFA (PA)	TAG (PA)	PL (PA)
6.4.3	10 min	1	2.308	31.49	6.737	2.254	0.132
	5 min	2	3.719	47.89	14.41	4.418	0.232
		3	2.068	34.45	11.78	2.754	0
	10 min	1	2.215	38.58	0	1.242	0
	10 min	2	3.19	53.20	0	2.545	0
		3	2.028	37.59	0	1.327	0