Development and optimization of technology for the extraction and conversion of micro algal lipids to biodiesel

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Faculty of Applied Sciences at the Durban University of Technology.

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DECLARATION BY STUDENT

I declare that this thesis, submitted for Doctor of Philosophy in Chemistry at the Durban University of Technology, is the original work of the author and has not been submitted for a degree at any other University. Where use is made of any author's work, it has been duly acknowledged.

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ABSTRACT

Fossil fuel reserves have been diminishing worldwide thus making them very scarce in the long term. These fuel sources and their by-products which are used commercially tend to produce large quantities of emissions. Some of them are believed to be toxic to flora and fauna. It is primarily for this reason that researchers worldwide have begun to seek out alternative sources of environmentally safe fuel. Biodiesel from algae is one of these sources that have been examined over the last few decades. Biodiesel has been produced from other plant-based material and waste oils in countries like America and Japan. However, the use of food based crops for biodiesel production has been challenged as it has an impact on food production on an international scale. Algae have only recently been investigated for their feasibility for biodiesel production on a large scale.

The aim of this study was to investigate and develop technologies for biodiesel production from algae. The species of algae chosen were chlorella *sp* and scenedesmus *sp*., since they are indigeneous to Kwazulu Natal in South Africa. Samples were obtained from a local raceway pond and prepared for analysis. Drying protocols used freeze, oven and sun drying for initial preparation of the samples for analysis. Sun drying was the least energy intensive but most time consuming. At laboratory scale, oven drying was chosen as the best alternative. Lipid extraction methods investigated were the separating funnel method, the soxhlet method, microwave assisted extraction (MAE) and the expeller press. Thirteen solvents covering a range of polarities were used with the extraction methods to determine the efficiency of the solvent with these methods. Optimization of the MAE method was conducted using both the one factor at a time (OFAT) method and a design of experiment (DOE) statistical method. The shelf life of algal biomass was determined by ageing the samples for approximately three months. Direct and in-situ transesterification of lipid extracts to produce biodiesel was investigated using both acid and base catalysis. Qualitative and quantitative analyses were conducted using Fourier transform infra-red (FTIR) and gas chromatography (GC). Chemical and

physical characterization of the biodiesel produced from the algal lipid extracts were compared to both local and international standard specifications for biodiesel.

In terms of extraction efficiency, it was found that soxhlet and microwave assisted extraction methods were almost equally good. This was proved by the MAE method yielding an average of 10.0% lipids for chloroform, ethanol and hexane after 30 mL of solvent was used in an extraction time of 10 minutes, while the soxhlet method yielded 10.36% lipids using an extraction volume of 100 mL of solvent with an extraction time of 3 hours. Chloroform, ethanol and hexane were more efficient than the other ten solvents used. This was shown by these three solvents producing lipid quantities between 10% to 11% while all the other solvents produced lipid quantities between 2 and 10 %. The best extraction efficiency was achieved by the binary solvent mixture made up of chloroform and ethanol in a 1:1 ratio. Under the conditions optimized, this solvent ratio yielded a lipid content of 11.76%.

The methods chosen and optimized for extraction are very efficient, but the actual cost of production of biodiesel need to be determined. Physical methods like the expeller press are not feasible for extraction of the type of biomass produced unless algae are pelletized to improve extraction. This will impact on the cost of producing biodiesel. The transesterification protocols investigated show that the base catalysis produced biodiesel with a ratio of saturates to unsaturates conducive to a good fuel product. The direct esterification method in this study proved to be better than the in-situ method for biodiesel production. The in-situ method was also more labour intensive. Chromatography was found to be a fast and efficient method for qualitative and quantitative determination of biodiesel. Characterization tests showed that the quality of biodiesel produced was satisfactory. It also showed that the methods used in this study were feasible for the satisfactory production of biodiesel which meets local and international specifications.

DEDICATION

Dedicated to my beloved parents Ramluckan and Deomathie Unnur who would have been proud of this achievement.

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PREFACE

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An evaluation of the efficacy of using selected solvents for the extraction of lipids from algal biomass by the soxhlet extraction method

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HIGHLIGHTS

- Thirteen solvents with varying characteristics were used in the extraction of algal biomass.
- Ethanol, chloroform and hexane produced average of >10% lipid extracts.
- Time-based trials showed optimum extraction efficiency at 3 h.
- Binary mixtures gave greatest extraction efficiency with 1:1 chloroform:ethanol.

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ABSTRACT

The use of solvents for the extraction of lipids from algal biomass has been a method of choice for many years. The soxhlet extraction method was chosen because of its simplicity in operation, relative safety and potential for upscaling to industrial plant level. The source of algal biomass was a raceway pond. *Chlorella* sp. which is known to produce larger amounts of oil than other indigenous species was used for this investigation. Thirteen solvents spanning a range of polarities and solubilities were selected for this study. Extraction methodology involved the use of single solvents, selected binary solvent mixtures and time-based extractions which were varied from 1 to 5 h. Ultraviolet (UV) spectroscopy was used to determine chlorophyll content of the lipid extracts and gas chromatography was used for the identification and quantitation of the lipids. Analysis showed that ethanol, chloroform and hexane were generally more efficient in the extraction of lipids than the other solvents studied, producing lipid contents in excess of 10%. The time-based trials indicated that the optimum extraction time was 3 h for the solvents selected. The binary solvent mixture with the greatest extraction efficiency (i.e. >10% lipid extract) was obtained with the 1:1 mixture of chloroform:ethanol. Chlorophyll quantities varied for each solvent extract with chloroform and methanol producing the highest values at >1%. Chromatography was effective in identifying lipids used in the production of biodiesel.

1. Introduction

The gradual global depletion of fossil reserves has made it imperative for most countries to seek viable alternative sources of energy [1]. The continued use of petroleum based fuels is becoming unsustainable because of the diminishing fuel reserves worldwide. This, compounded by the environmental impact of carbon dioxide emissions, has prompted the search for more environmentally friendly and renewable fuel sources [2,3]. For these reasons, renewable and carbon neutral biofuels have grown in importance as environmental and economically sustainable fuels. First generation biofuels sources from edible oil such as soybean, palm and canola have a negative impact on food supplies, while second generation non-edible sources, exemplified by jatropha, require vast amounts of arable land. In the light of the above observations, algae based biofuels are considered to be a viable alternative since they do not impact on food supply. Furthermore, they can also be grown on any available land, water or saline [4,5]. Microalgae, like plants, use sunlight and the photosynthetic process to produce lipids, but they do so more efficiently [2]. Microalgae have therefore been regarded as a promising and potentially renewable fuel source that could replace fossil fuels [5].

Since the amount of lipids in microalgae is relatively small (on average between 15% B 30% depending on the algal species), it is crucial that the selected extraction procedure is efficient enough to extract the maximum quantity of lipids possible [6,7]. Lipids are made up of a diverse group of biological substances, some of

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Efficacy of drying and cell disruption techniques on lipid recovery from microalgae for biodiesel production

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HIGHLIGHTS

- Microwave and sonication techniques are compared for efficient lipid extraction.
- Qualitative lipid variation caused by cell disruption and drying methods is studied.
- Energy consumption calculated for drying and lipid extraction processes.
- Lipid quality has been assessed for its suitability for biodiesel production.
- Sun drying with efficient microwave extraction could be possible processing step.

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ABSTRACT

Downstream processing of microalgal biomass presents a significant challenge to large scale biodiesel production. *Scenedesmus* sp. which is known to be a potential feedstock for biodiesel production was cultivated in an open circular pond. The biomass productivity peaked at day 21 with a yield of 1.16 g L^{-1}. Biomass was harvested by gravitational settling followed by centrifugation. Harvested biomass was dried using the freeze drying, oven drying and sun drying processes followed by lipid extraction which yielded 29.65%, 28.63% and 28.33% lipid g^{-1} DCW (dry cell weight) respectively. Lipids were extracted from microalgal biomass dried by selected drying techniques using microwave and sonication for cell disruption in the presence of mixture of chloroform and ethanol (1:1). Microwave assisted extraction of sun dried biomass yielded 28.33% lipid g^{-1} DCW, as compared to 18.9% lipid g^{-1} DCW achieved by sonication assisted extraction. The saponification and acid values of the lipid obtained from *Scenedesmus* sp. dried by selected drying techniques showed high saponification and acid value indicating presence of high free fatty acid content. Effect of different drying and cell disruption technique on fatty acid profile of lipids extracted from *Scenedesmus* sp. biomass was also studied. These values indicate promising potential of the oil produced for conversion into biodiesel.

1. Introduction

Microalgae have emerged as promising feedstocks for biodiesel production. Microalgal biodiesel has several advantages over crop based biodiesel such as high potential for biomass production with minimal land and freshwater requirement; no arable land requirement, no food security concerns; a higher carbon-dioxide sequestration potential; wastewater utilization during cultivation; and the production of value-added products from de-oiled microalgal biomass. The production of a sustainable and economically viable biodiesel from microalgae is however still a challenge [1–6].

Biodiesel production from microalgae is a multi-step process including cultivation, harvesting and dewatering of microalgal biomass, extraction of lipids from biomass, and conversion of lipids to biodiesel. Photoautotrophic cultivation of microalgae is widely accepted as a more economically viable method for large scale microalgal biomass production. Microalgae essentially require light, carbon dioxide, inorganic nutrients, and water for their growth [7]. Microalgae are either cultivated in open raceway ponds or in photo-bioreactors for a high biomass and lipid production. The choice of technique for cultivation and production of microalgal biomass with high quantity of lipids depends upon microalgal strain selection, culture conditions, land area availability, natural light and production scale [2].

Harvesting and dewatering of microalgal biomass are crucial steps in commercial production of microalgae. The harvesting

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The evaluation and optimization of parameters for the Microwave assisted extraction (MAE) method for the extraction of lipids from algal biomass.

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LIST OF ABBREVIATIONS

µL	microlitre
µV	microvolt
Ala	Alpha linolenic acid
ALE	Algal lipid extract
ANOVA	Analysis of variance
AOCS	American oil chemists' society
AR	Analytical Reagent (grade)
ASE	Accelerated solvent extraction
ASTM	American standards for testing materials
ATR	Attenuated total reflectance
AV	Acid value
BDH	British Drug House
CCD	Central Composite Design
CN	Cetane number
CO	Carbon monoxide
DCW	Dry cell weight.
DHA	Docosahexanoic acid
DOE	Design of Experiment
DU	Degree of unsaturation
dwt	Dry weight
EPA	Eicosapentaenoic acid
FA	Fatty acid/s
FAAE	Fatty acid alkyl esters
FAEE	Fatty acid methyl ester/s
FAME	Fatty acid methyl ester/s
FFA	Fatty acid ethyl ester/s
FID	Flame ionization detector
FMAE	Focussed microwave assisted extraction
FTIR	Fourier transform infra-red

GC	Gas Chromatography or Gas chromatograph
GC-MS	Gas Chromatography - Mass spectrometry
HPLC	High performance liquid chromatography
HTL	Hydrothermal liquefaction
HTT	Hydrothermal methods
IV	Iodine value
LA	Linoleic acid
MAE	Microwave Assisted Extraction
MARS5	Microwave Accelerated Reaction System 5
ME	Methyl ester
min.	minute
mL	millilitre
MUFA	Monounsaturated fatty acid (methyl esters)
NLEA	Nutrition labelling and education act (US)
NMR	Nuclear magnetic resonance
OS	Oxidation stability
PBR	Photobioreactor
PFE	Pressurized fluid extraction
PHWE	Pressurised hot water extraction
PLE	Pressurized liquid extraction
PM	Particulate matter
PMAE	Pressurized microwave assisted extraction
PSE	Pressurized solvent extraction
PUFA	Polyunsaturated fatty acid (methyl esters)
RSM	Response Surface Methodology
RTP	Resonance Temperature Probe
SFA	Saturated fatty acid (methyl esters)
SO	Sunflower oil
SOP	Standard operating procedure
SOX	Soxhlet extraction
SV	Saponification value

TAG	Triacylglycerol
TLS	Tri-laminar sheath
TUFA	Total unsaturated fatty acid (methyl esters)
UV	Ultraviolet
ρ	Density

CHAPTER 1

INTRODUCTION

Fossil fuel reserves worldwide are being gradually depleted and this has made it imperative for most countries to seek viable alternative sources of energy (Pinto *et al.*, 2005). The continued use of petroleum-based fuels is gradually becoming unsustainable. This is compounded by the environmental impact of carbon dioxide emissions. The continued demand for fossil fuels and the environmental impact of emissions resulting from the use of these fuels have prompted the search for more environmentally friendly and renewable fuel sources (Chisti, 2007; Vasudevan and Briggs, 2008). Renewable and carbon-neutral biofuels have grown in importance as environmental and economically sustainable fuels. The choice of sources for biofuel production is crucial. The use of edible oil extracted from, for example, soybean, palm and canola will have a negative impact on food supplies. On the other hand, non-edible sources such as jatropha, require vast amounts of arable land. Therefore, algae based biofuels are considered to be a viable alternative since they do not have an impact on food supply. Furthermore, they can also be grown on any available land, water or saline solution like sea water (Govindarajan *et al.*, 2009; Shen *et al.*, 2009). Microalgae, like plants, use sunlight and the photosynthetic process to produce lipids which can be converted to biodiesel (Chisti, 2007). Microalgae have therefore been regarded as having a promising potential as a renewable source of biofuels that could replace fossil fuels (Shen *et al.*, 2009).

Biodiesel is defined as a product obtained when vegetable oil or animal fat is chemically reacted with alcohol using a catalyst. Biodiesel can be used as an alternative fuel for diesel engines. Although it was first cited as a viable alternative in the early 70's, the high cost of producing it has prevented large scale production (Chisti, 2008). Recent developments, which have resulted in a fuel crisis world-wide,

have prompted new interest in biodiesel fuels as an alternative to fossil fuels. The quest for biodiesel arose out of a coarse prediction that fossil fuels may be depleted in the near future. Although the course of the future is unknown, the projections are based on the on-going scientific analysis of population growth and the demands placed on earthly resources by this population. Instability in the Middle Eastern countries, the primary suppliers of fossil fuels, has also encouraged the Western and European countries to forcefully seek out alternate sources of fuel. As a result of this, BRICS countries (Brazil, Russia, India, China and South Africa) followed suit in the hope of developing technologies for other sources of fuel.

The US Department of Energy (DOE) had revived its investment goals for algal biofuels as far back as 2008. This was in response to efforts for the lowering greenhouse gas emissions and producing affordable, reliable energy. This led to the investigation of new and renewable sources of fuel (Ferrell and Sarisky-Reed, 2010). The use of algae as a source of algal biofuels was investigated, but its downside is that the present cost of producing these fuels does not make it economically feasible. It is however envisaged that with improvement in technologies the costs will reduce sufficiently to make this type of fuel economically viable. The upside is that this fuel is not as harmful to the environment as fossil based fuels are. This has created an upsurge in research and development of biofuels.

Biodiesel from oil crops, waste cooking oil and animal fat is not expected to satisfy even a small fraction of the existing demand for transport fuels. Microalgae appear to be the only source of renewable biodiesel that may meet the global demand for transport fuels (Amigun *et al.*, 2006). Oil productivity of many microalgae greatly exceeds the oil productivity of the best oil producing crops. Estimated yields for corn are 15 gallons (US) of oil per acre per year, while microalgae produce more than 1850 gallons of oil per acre, based on actual biomass yields (Chisti, 2007).

In uses of microalgae as a source of oil, some form of extraction or conversion is required before the algae can be used as a biofuel (Meher *et al.*, 2004). Algal oils

have a variety of commercial and industrial uses, and can be extracted using a wide variety of methods. Generally algae need not be dried before oil extraction (Palligarnai and Briggs, 2008). The simplest method is mechanical crushing. Since different strains of algae vary widely in their physical attributes, various press configurations like the screw, expeller and piston are used for specific algae types (*Oilgae-Oil & Biodiesel from Algae,* 2008). In terms of non-physical methods, chemical solvents are used for the extraction of algal oils. Surveys have revealed that several methods for the extraction of oil from various vegetables and algae exist. Some of the methods cited are: enzymatic extraction, supercritical fluid extraction (SFE), osmotic shock and extraction using solvents such as hexane, methanol, benzene, diethyl ether, dichloromethane, and ethanol. These methods involve the extraction of both wet and dry biomass. Benzene and ether have been used relatively infrequently, but hexane extraction is widely used in the food industry and is relatively inexpensive. The disadvantage of using solvents for oil extraction are the dangers involved in working with the chemicals which could be classified as corrosive, explosive, or carcinogenic. In terms of equipment used in solvent-based extraction, the soxhlet extraction method has been used for the extraction of oils (*Algaculture*, 2008).

Solubility of lipids is an important criterion for the extraction of lipids and depends heavily on the type of lipids present and the proportion of polar and non-polar lipids in the sample. Hence, several solvent systems may be considered depending on the type of sample and its components (Shahidi, 2001). An ideal lipid extraction process for microalgal biodiesel production needs to be not only lipid specific but also selective towards desirable lipid fractions. The search for a cost effective and efficient method for the production of biodiesel has been the forefront of technology being developed since the late 1950's (Lewis *et al.*, 2000; Matile *et al.*, 1999). The first step in this process requires that a method or methods be developed for cell/cell wall disruption and the extraction of lipids with some degree of efficiency. Subsequent steps would involve the esterification and production of biodiesel.

Extraction methods like accelerated solvent extraction (ASE), supercritical fluid extraction (SFE) and microwave assisted extraction (MAE) are relatively expensive and sometimes time-consuming. Solvent extraction techniques like soxhlet extraction and MAE are probably some of the few methods that can be upscaled for mass production of biodiesel with relative ease. However, optimization of these techniques for upscaling has not been comprehensively investigated. Since the amount of lipids in microalgae is relatively small (on average between 15% -30% depending on the algal species), it is crucial that the selected extraction procedure is efficient enough to extract the maximum quantity of lipids possible (Lewis *et al.*, 2000; Matile *et al.*, 1999). The lipids, are made up of a diverse group of biological substances which are both polar and non-polar (Manirakiza, 2001). The extraction of lipids from microalgal biomass is a challenging task which is critical in the determination of the overall economics of biodiesel production (Govindarajan *et al.*, 2009). Although lab-scale extraction of lipids is fairly routine, the variables affecting lipid extraction from microalgae are not well understood and make up-scaling for commercial production a greater challenge (Halim *et al.*, 2011).

With regard to methods of extraction, the Folch or its variant the Bligh and Dyer method have been used extensively in the quantitation of lipids using solvents such as chloroform, methanol and hexane (Christie, 2013). The same can be noted for the Soxhlet extraction method. Soxhlet extraction has been used with many solvents either individually or in combination for the extraction of lipids. However, there are no reports of the use of a range of solvents and solvent mixtures via the soxhlet extraction method, in a single study (Christie, 2013). This, therefore, motivated the investigation of a broad range of solvents of different polarities being used for the extraction of lipids from algal biomass. The basis of the investigation was that any solvent which showed high extraction efficiencies would be chosen for further experimentation using optimized conditions determined during analysis. This part of the study examined the use of thirteen solvents for their efficiency in the extraction of lipids from algal biomass.

It is generally known that solvents used for the extraction of lipids tend not to discriminate between various compounds present in algae. Therefore apart from lipids, solvents would extract compounds like chlorophyll, the next most abundant constituent of microalgae, carotenoids, pheophytins and other associated degradation products in small measure. In total the latter may make up approximately 1 to 2 % of the total algal mass as dry weight (*ESS Method 150.1: Chlorophyll - Spectrophotometric*, 1991). Most plants may contain chlorophyll *a, b, c* and to a smaller extent chlorophyll *d*, but for the algal species, selected for this project, chlorophyll *a* and *b* predominate. Since different solvents produce varying solvent efficiencies for both lipids and chlorophyll, and because this has an influence on the amount of lipid extract measured as dry weight, the amount of chlorophyll present in algal biomass was determined. A UV method was used to photometrically determine the amount of chlorophyll in the algal biomass studied (Dere *et al.*, 1998).

The American Oil Chemists' Society (AOCS) lipid library broadly defines lipids as “fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds” (Christie, 2013). In a much broader sense, lipids are made up of steroids, sterols, fatty alcohols, phospholipids, triglycerides, diglycerides, monoglycerides, fatty acids and some may include sphingolipids (Plante *et al.*, 2011). The term lipid is sometimes used as a synonym for fat. Fats are classified as triglycerides or triacylglycerols. They generally consist of molecules containing carbon chains from C12 to C24. For the production of biodiesel, fatty acids are the compounds of prime interest. However, not all fatty acids can be completely converted to biodiesel. It is therefore pertinent to determine which fatty acids favour the production of biodiesel. Fatty acids can be separated, quantified and analyzed by Gas chromatography (GC) without any derivatization (Scragg *et al.*, 2003).

Gas chromatography is the method of choice for the simultaneous determination of glycerol, mono-, di-, and tri-glycerides in oil. In principle, these compounds can be analyzed on highly inert columns coated with a polar stationary phases, without

derivatization (Nel, 2008). A chromatographic method was thus optimized for the identification of lipids significant for use in biodiesel production. A set of selected fatty acid standards was prepared for the calibration of the GC before the analysis of samples (Ruppel and Huybrighs, 2008).

The sources of the oils (vegetable or animal fat) determine the composition and nature of fatty acids present in triglycerides. The type and concentration, of the fatty acid used, has a marked effect on biodiesel stability which in turn influence its storage and its oxidative properties. Thus, it is important to know the characteristics of the oils being used to produce biodiesel. When producing biodiesel, triglycerides are reacted with methanol in a reaction known as transesterification or alcoholysis. Transesterification produces methyl esters of fatty acids (FAME), i.e. biodiesel, and glycerol (Chisti, 2008).

A catalyst is usually used to improve the reaction rate and yield. Since the reaction is reversible, excess alcohol is used to shift the equilibrium to the products side. Some of the alcohols that may be used for transesterification are methanol, ethanol, propanol, butanol and pentanol. Methanol is used more frequently because of its low cost and its physical and chemical advantages. NaOH dissolves easily in alcohols and reacts quickly with triglycerides. To complete a transesterification stoichiometrically, a 3:1 molar ratio of alcohol to triglycerides is needed. In practice, a higher ratio is needed to drive the equilibrium to a maximum ester yield. The reaction can be catalyzed by alkalis, acids, or enzymes. Some of the alkalis used are NaOH, KOH, carbonates and corresponding sodium and potassium alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide and sodium butoxide.

The acid catalysts are generally sulfuric acid, sulfonic acids and hydrochloric acid (Christie, 2013). Alkali-catalyzed transesterification is much faster than acid-catalyzed transesterification and is more often used commercially (Illman *et al.*, 2000). Biomass-derived fuels share many of the same characteristics as their fossil fuel counterparts. Once formed, they can be substituted in whole or in part for

petroleum-derived products. In the African context, for economic reasons, fuels may be blended with ethanol for cleaner-burning diesel (Amigun *et al.*, 2006). Since esterification protocol is significant for the quality of biodiesel produced, this prompted an exercise in determining the most appropriate method for biodiesel production from the lipids extracted. Experiments were chosen to include one acid catalyzed method and three alkali catalyzed method. The reason for this choice was that in general it was found that acid catalyzed methods were more time consuming and required complex and expensive reagents while alkali catalyzed methods were faster and less corrosive. (Demirbas, 2008; Schuchardt *et al.*, 1998). For the algal strains studied, it was decided that the final method of choice that would be recommended for future analysis would be on the basis of its speed of conversion of the lipids to biodiesel and the yields produced.

The focus of this study was the analytical evaluation of algal biomass cultivated and harvested locally with a view to adopting it for industrial scale biodiesel production. This was conducted as follows:

- Sampling of algae was carried out from a freshwater laboratory raceway pond and a large capacity raceway pond south of Durban.
- Analytical techniques chosen were optimized for the extraction and conversion of algal lipids to biodiesel.
- This was followed by the characterization of the biodiesel produced. For the purpose of this research, biomass obtained from indigenous strains of algae was examined. The strains of algae tested were predominantly of the chlorella *sp* and scenedesmus *sp*.
- Various solvents were tested for their efficiency in the extraction of lipids (algal oil) from biomass.
- A single physical method and three chemical methods were selected on the basis of simplicity of the methods, costs involved and availability of equipment. Esterification methods were optimized for the conversion of the lipids to biodiesel. Instrumental methods, FTIR and GC were used to

separate, identify and quantify lipids and the biodiesel produced at laboratory scale.

- The extraction protocols using thirteen solvents and the binary mixtures proposed, including the design of experiments, optimization of esterification procedures conducted and the applications using the instrumental techniques have not been conducted in this manner prior to this study. However, there may be some overlap of technical methods performed.

The overall focus of this study was based on evaluating technology for the chemical aspect of producing biodiesel from the time the algae is harvested until biodiesel is produced.

This project can be considered novel and makes a significant contribution to the body of knowledge for the following reasons:

- The range of solvents used for extraction with the technologies investigated during this study has not been conducted previously.
- There is no evidence of the microwave method being optimized and investigated to the degree performed by this study.
- The initial drying and ageing studies for biomass was unique to this project.
- The chromatographic techniques used for the identification of specific lipids and biodiesel for the two indigenous species of algae have not been conducted prior to this study.

The various aspects concerned with the use of microalgae in the production of biodiesel was reviewed under the following headings in the next chapter:

- Algae selection, cultivation and harvesting
- Sampling and sample preparation
- Methods for extraction and optimization
- The production of biodiesel
- Quantitation
- Biodiesel characterization

Aims and Objectives

Aim:

To develop and optimize techniques for the extraction and conversion of micro algal lipids to biodiesel and characterize biodiesel produced.

Objectives:

1. Evaluate, select and optimize techniques for the extraction of lipids from algal biomass.
2. Identification, quantification and esterification of lipids extracted from microalgae.
3. Chemical and physical characterization of biodiesel and feasibility for large scale application.

CHAPTER 2

LITERATURE REVIEW

2.1 Algae selection, cultivation and harvesting

Algae species are found in both fresh water and marine environments. Both microalgae and macroalgae are found in these environments. The focus of this study was on fresh water microalgae. Table 2.1 is an illustration of some of the species of algae found in fresh water systems.

Table 2.1 Oil content and habitats of some freshwater microalgae.

Microalga	Oil content (% dry weight)	Habitat
Botryococcus braunii	25–75	Fresh water/estuary
Monodus subterraneus UTEX 151	16.1	Freshwater
Chlorella vulgaris CCAP 211/11b	19.2	Freshwater
Chlorococcum *sp*. UMACC112	19.3	Freshwater
Scenedesmus *sp*. F&M-M19	19.6	Freshwater
Scenedesmus *sp*. DM	21.1	Freshwater
Chlorella *sp*.	28–32	Freshwater
Neochloris oleoabundans	35–54	Fresh water

Modified from Chisti (2007), Rodolfi et al., (2009). (Mutanda *et al.*, 2011)

The species of algae were selected based on their potential for large scale cultivation. Eukaryotic microalgae are preferred to prokaryotes since they are known to store more lipids (Williams and Laurens, 2010). Many algae species exist world-wide and these have been investigated for their feasibility in producing lipids for biodiesel. However, some algal strains tend to be unique to a particular country. Not all species of algae investigated by researchers have shown proficiency in producing lipids for biodiesel (Rawat *et al.*, 2013). However, some species are better producers of lipids.

Two of these species which are relatively common world-wide, but which do not necessarily share the same genotype are: chlorella *sp.* and scenedesmus *sp.* Both these species have proved to be good producers of lipids conducive to the production of biodiesel (Rawat *et al.*, 2013) . Locally, both species tend to predominate on a seasonal basis. The selection of appropriate algal strains is important in ensuring the overall success of biofuel production from microalgae. As a general rule, an ideal algal strain for biofuel production should have at least some or all of the following characteristics:

- have a high lipid productivity
- be dominant over wild strains in open pond production systems
- have limited nutrient requirements
- be tolerant to a wide range of temperatures
- have a fast productivity cycle (Brennan and Owende, 2010).

The quality of oil used for the production of biodiesel has a great impact on the quality of the biodiesel produced. The alkyl ester content dictates the stability and performance of the fuel. This is also important in meeting international fuel standards. The lipid profile of an algal species will remain consistent provided it is grown under similar conditions. However, every algal species will have its own lipid profile. It is therefore important to utilize species that have a suitable lipid profile for the production of biodiesel (Schenk *et al.*, 2008). The lipid content of microalgae can vary remarkably depending on the state of the culture, environmental conditions and inherent traits of the algal species (Axelsson, 2012). Indigeneous strains of chlorella *sp.* and scenedesmus *sp.* proliferate at a local raceway pond where sampling was conducted. Hence these species were the focus of this study.

Chlorella *sp.* (Figure 2.1) is a single-celled green algae. It belongs to the class of *Chlorophyceae*. It is a primary alga because it grows autotrophically. Chlorella *sp.* occurs in both fresh and marine water. Chlorella *sp.* may be found in various habitats. They are generally found in fresh water ponds and ditches, in moist soil or other damp situations such as the surface of tree trunks, water pots and damp walls.

Its cells are solitary, about 2 to 10 µm in diameter and spherical, globular or ellipsoidal in shape.

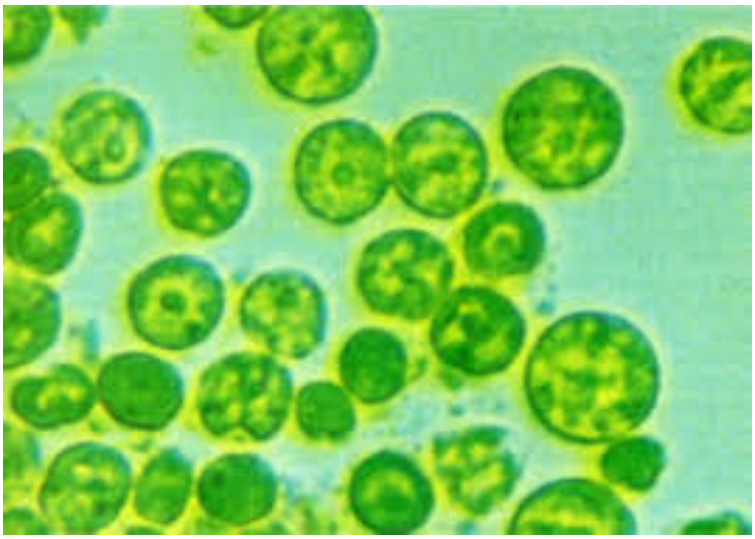


Figure 2.1 Light microscopy of *Chlorella sp.*

(Accessed 2014: http://chlorella sp.joyau-vert.ch/images/others/Chlorella sp_02.jpg)

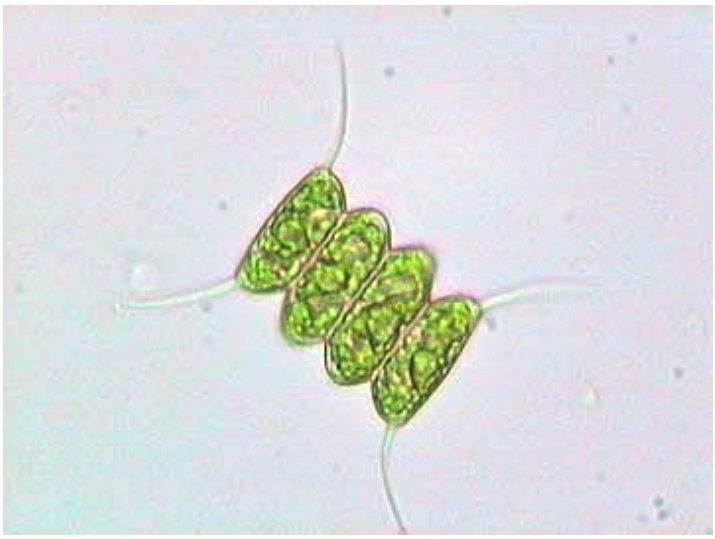


Figure 2.2 Microscopic image of scenedesmus *sp.*

(Accessed 2014: http://www.microscopy-uk.org.uk/mag/artoct05/mmdesmid.html)

Scenedesmus *sp.* (Figure 2.2) is a fairly common freshwater alga. However, the extremely diverse morphologies found among species make identification and understanding of their mechanisms difficult. Scenedesmus *sp*. can exist in a unicell stage; however, they are also frequently found in coenobia of four or eight cells (Lürling, 1999).

Scenedesmus obliquus is a freshwater microalga that can grow in industrial wastewaters of different origins showing good adaptation ability and it is a very versatile microalga as raw material for biofuels production. It is also considered one of the best candidates for biodiesel production among microalgal species, with a lipid content ranging between 18.8 and 29.3 % dwt (dry weight) for a nutrient-replete medium and up to 42 % dwt for a nutrient-deficient medium (Gouveia and Oliveira, 2009; Ruiz J. *et al.*, 2013).

Several methods for harvesting of microalgae exist. The commonly used methods are: filtration, gravity sedimentation, centrifugation, flotation, and flocculation. Biomass harvesting can potentially contribute to 20-30% of the total biomass production costs (Mata *et al.*, 2010). The choice of a harvesting technique is dependent on characteristics of microalgae, e.g. size, density, and the value of the target products (Vicente *et al.*, 2004). Harvesting in a two-step process may assist in conserving energy and reduce costs. In the first step, the algae may be concentrated, often by flocculation. This will concentrate the dilute cultures to about 1-5% solids. In the second step, the cells can be further concentrated by centrifugation, filtration or micro straining to get a solids concentration of 15-25% (Alabi *et al.*, 2009).

2.2 Sampling and Sample Preparation

2.2.1 Sampling methods

The gravity sedimentation and centrifugation methods were available for the harvesting of algae at laboratory scale. The probability of using these or any other

method for the harvesting of algae was not an objective of this study, however the methods mentioned were used for the collection of biomass.

2.2.2 Sample Preparation

It is important that the sample chosen for analysis is representative of the lipids present in the original sample, and that its properties are not altered prior to the analysis. While some samples, like nuts and seeds, may allow extraction of lipids by physical pressure, with algae more rigorous solvent or nonsolvent extraction methods are needed. The preparation of a sample for solvent extraction may involve the following steps:

Sample drying

Samples have to be dried prior to solvent extraction. Since many organic solvents cannot easily penetrate foods containing water, they may reduce the efficiency of extraction.

Particle size reduction

Dried samples are usually finely ground prior to solvent extraction to produce a more homogeneous sample. The grinding also increases the surface area of lipid exposed to the solvent. Grinding is often carried out at low temperatures to reduce the tendency for lipid oxidation to occur (McClements, 2013).

2.2.3 Drying of algae

Three commonly employed techniques for the drying of algae are sun drying (Figure 2.3), oven drying (Figure 2.4), and freeze drying. Gulde et al., (2014) conducted sun drying on a drying bed lined with white plastic of 1.5 x 10^3 micron thickness at ambient temperature (25 – 30 °C) for 72 h. For freeze drying, wet biomass was frozen overnight at -84 °C and lyophilized using freeze dryer. Oven drying was carried out using a hot air oven for 12 h at 60 °C.

Figure 2.3 Large scale sun drying of harvested algae.

The lipid yields of *Scenedesmus sp*. biomass dried by freeze drying, oven drying and sun drying were found to be 29.65 ± 1.05 %, 28.63 ± 0.425 %, and 28.33 ± 1.37 % lipid.g^{-1} DCW respectively (Guldhe *et al.*, 2014).

Sun drying requires the least energy but the most time. Freeze drying requires the most energy and about the same time as oven drying. Freeze drying has one advantage over the other two methods and that is it produces powdered crystals of biomass which can be used for extraction without any further intervention. Sun and oven dried methods produce flakes of biomass which are required to be pulverized before they can be used for extraction (Guldhe *et al.*, 2014).

Figure 2.4 Lab-scale oven drying of harvested algae.

One advantage of processing dried biomass is better percolation of the solvents in the biomass, leading to an increase in lipid extraction. However drying is not considered an economical option for biomass pre-treatment for a biofuels production process because of the high-energy requirements of the drying process. Alternative pre-treatment methods include cell rupture of the harvested biomass, which could aid in the lipid extraction process by avoiding or reducing the use of solvents. A range of cell rupture techniques, mechanical, chemical and enzymatic, has been described in the literature as applied to oilseeds and algae. However, no such method has been adopted for algae on a large-scale. This is, most likely, because the effectiveness of the cell rupture methodology depends heavily on the physical properties of the algal cell wall and thus will change or have to be adapted with particular species used in the culture. The composition and strength of the cell wall varies considerably across species and throughout the algal growth cycle. For example, Chlorella *sp*. and Scenedesmus *sp*. both have robust cell walls with high cellulose content, rendering the cells difficult to digest or rupture (Williams and Laurens, 2010).

2.2.4 Storage of algae

It is anticipated that most harvested algal biomass will be used for the production of biodiesel within 24 hours. However, continuous harvesting of feedstock for biodiesel production could result in over production of dried biomass which would need to be stored for future use. One of the challenges arising from this is the ability to store and preserve dried biomass for reuse. Furthermore, storage and preservation temperatures would require more energy for storage at constant temperature and would not be as feasible as storage at room temperature. It was with this in mind, a pilot study was conducted for approximately 3 months and the dried biomass was tested over this period for its ability to maintain biodiesel producing lipids with reasonable consistency. The overall study examined lipid profiles for biomass ageing over seven consecutive days followed by weekly analysis of lipids for up to three months. Search of the available literature found no reports on this aspect of the present study.

2.2.5 Qualitative analysis of lipids using Nile red

Nile red is a benzophenoxazone dye. In older chemical and histochemical literature the dye is sometimes referred to as Nile blue A-oxazone. Nile red has low solubility in water but it does dissolve in a wide variety of organic solvents. The dye is intensely fluorescent in all organic solvents. Depending upon the relative hydrophobicity of the solvent, the excitation and emission maxima of Nile red fluorescence can vary over a range of 60 nm; the fluorescence colours range from golden yellow to deep red. Measured at the respective fluorescence maxima, the relative fluorescence intensities of the dye are approximately equal in all organic solvents. In contrast, the fluorescence of the dye is quenched in water. Thus, Nile red exhibits the properties of a fluorescent hydrophobic probe (Greenspan *et al.*, 1985).
New technologies are being developed to help facilitate selection of microalgae strains. For in-stance, a study of several different *C. vulgaris* strains used Nile red, for simple, rapid, and sensitive screening of lipid content. Cells stained with Nile red were found to have a characteristic yellow fluorescence of about 572-582 nm, the intensity of which was directly correlated to intracellular lipid content under spectrofluorometeric analysis. This technique is faster than traditional gravimetric analysis, and may be detected even at a 0.1% difference in lipid content (Raehtz, 2009)

Nile red, a lipid-soluble fluorescent dye, has been frequently employed to evaluate the lipid content of animal cells and microorganisms, and microalgae. However, most of the studies have provided only a qualitative or semi-quantitative analysis of the lipids. (Chen *et al.*, 2009). This method was used to check for the presence of lipids in algae harvested for analysis. The method was used only as a qualitative and semi-quantitative basis.

2.3 Methods for Extraction and Optimization

In all of the uses of microalgae some form of extraction or conversion is required before the algae can be used as a biofuel source (Meher *et al.*, 2004). Algae oils have a variety of commercial and industrial uses, and can be extracted using a wide variety of methods. The simplest method is mechanical crushing. Since different strains of algae vary widely in their physical attributes, various press configurations such as the screw, expeller and piston work better for specific algae types (*Oilgae-Oil & Biodiesel from Algae*, 2008). Chemical solvents can be used for the extraction of algae oils. Surveys have revealed that several methods for the extraction of oil from various vegetable matter and algae exist. Some of the methods cited are: enzymatic extraction, supercritical fluid extraction (SFE), osmotic shock and extraction using solvents such as hexane, methanol, benzene, diethyl ether, dichloromethane, and ethanol. These methods involve the extraction of both wet and dry biomass. Benzene and ether have been used relatively infrequently, but hexane extraction is widely used in the food industry and is relatively inexpensive. The downsides to using solvents for oil extraction are the dangers involved in working with the chemicals which could be classified as corrosive, explosive, or carcinogenic. The soxhlet extraction method is also used for the extraction of oils. Oils from the algae are extracted through repeated washing, or percolation, with an organic solvent such as hexane or petroleum ether, under reflux in special glassware (*Algaculture*, 2008). Other methods of extraction also used are, enzymatic extraction, expression/expeller press, osmotic shock, supercritical fluid (SFE), and ultrasonic-assisted extraction. Algae generally need not be dried before oil extraction (Palligarnai and Briggs, 2008).

2.3.1 Separating funnel method

The separatory funnel is used for separation of substances by the use of extraction solvents. This method has been used for extractions in various applications. Its only advantage is that the extractions are generally conducted manually and require stringent extraction protocols to be followed for effective separation of compounds.

It is, however, not as effective as the automated methods like soxhlet and microwave extraction.

This technique is usually carried out using two immiscible solvents, one of which is invariably water. Typical organic solvents include ethyl acetate, hexane, chloroform, dichloromethane, and diethyl ether. Most extractions are conducted using either liquid-liquid phases or liquid-solid phases. The basis of extraction techniques is the "like dissolves like" rule. Water dissolves inorganic salts (such as lithium chloride) and other charged species, while organic solvents (ethyl acetate, dichloromethane, diethyl ether, etc.) dissolve neutral organic molecules. The method relies on partitioning whereby the preferential dissolution of a compound into one solvent over another applies (*The Separatory Funnel*, 2013). In particular, if a specific compound is allowed to mix freely with two immiscible liquids, it will partition between those two liquid phases, usually in such a way that more of the material will be dissolved in one of the solvents than is dissolved in the other. This situation can be described quantitatively in terms of a “distribution coefficient” which specifies the relative amounts of the solute dissolved in each of the solvent phases under specific conditions. Extractions are usually performed in a pear-shaped funnel with a stopcock at its base. Plastic stoppers are preferred to glass, whenever possible. When shaking the funnel, it is good practice to vent the apparatus every few shakes to prevent the build-up of pressure in the vessel. This precaution is especially important when using volatile organic solvents (such as ether or methylene chloride) or when using aqueous solutions containing carbonate or bicarbonate (which often release carbon dioxide gas). Initial trials were conducted using this extraction method to test the feasibility of using it for algal lipid extraction. It did prove to be very labour intensive when compared to other methods tested.

2.3.2 Soxhlet extraction

Since the amount of lipids in microalgae is relatively small (on average between 15% to 30% depending on the algal species), it is crucial that the selected extraction procedure is efficient enough to extract the maximum quantity of lipids possible

(Lewis *et al.*, 2000; Matile *et al.*, 1999). Lipids are made up of a diverse group of biological substances, some of which are polar while others are non-polar (Manirakiza, 2001). The extraction of lipids from microalgal biomass is thus a challenging task which is critical in the determination of the overall economics of biodiesel production (Govindarajan *et al.*, 2009). Although lab-scale extraction of lipids is fairly routine, the variables affecting lipid extraction from microalgae are not well understood and make up-scaling for commercial production a greater challenge (Halim *et al.*, 2011).

The solubility of lipids is an important criterion for the extraction of lipids. It depends heavily on the type of lipids present and the proportion of polar and non-polar lipids in the sample. Hence, several solvent systems may be considered depending on the type of sample and its components (Shahidi, 2001). Several extraction routes may produce liquid fuels from microalgal biomass (Torri *et al.*, 2012). The type of organism and the permeability of its cell wall will govern the choice of solvent system for lipid extraction and the extraction efficiency of solvent mixtures (Lee *et al.*, 2010). Afi et al., 1996, reported presence of polysaccharide wall and tri-laminar sheath (TLS) that were composed of highly aliphatic, non-hydrolyzable macromolecules (algaenan) in *Chlorella emersonii*. The resistant outer wall (sheath) was not present in *Chlorella vulgaris*. *Chlorella vulgaris* was reported to contain only a classical polysaccharide cell wall whereas, *Chlorella emersonii* contains both a classical cell wall as well as a resistant tri-laminar outer wall (TLS). TLS are composed of solvent insoluble macromolecules with unusually high resistance to chemical degradation (Afi *et al.*, 1996).

An optimum lipid extraction process for microalgal biodiesel production should be lipid specific, in order to minimize the co-extraction of non-lipid contaminants, and selective towards the required lipid fractions. The use of dry biomass may lead to a significant increase in energy costs since a drying step is required before the conversion step. Alba et al., have reported that a wet biomass-handling process, such as hydrothermal liquefaction (HTL) is more suited to the production of liquid fuels

from wet microalgae. This is supported by the fact that it reduces the high energy cost for thermal drying and the need for removal of water (Garcia Alba *et al.*, 2012). However, higher temperatures required for better extraction make this an energy intensive method. Even though the classic chloroform-based lipid extraction protocol (Folch method) is effective for the majority of microalgal lipid analyses, an alternative organic method which is more user friendly would be more suited for up-scaling (Halim *et al.*, 2011). The search for a cost effective and efficient method for the production of biodiesel has been in the forefront of technology being developed since the late 1950's (Lewis *et al.*, 2000; Matile *et al.*, 1999). The first step in this process requires that a method or methods be developed for cell/cell wall disruption and the extraction of lipids with some degree of efficiency. Subsequent steps would involve the esterification of the lipids and production of biodiesel. Nevertheless the solvent extraction technique is probably one of the few methods that can be up-scaled for mass production of biodiesel with relative ease. However, optimization of this technique for up-scaling has not been comprehensively investigated. It was anticipated that any solvent/s that extracted the maximum quantity of lipids under optimized conditions would be considered for further investigation. The solvent chosen should also be reasonably inexpensive and non-toxic.

The Folch method or its variant, the Bligh & Dyer method, have been used extensively in the extraction and quantitation of lipids (Christie, 2013). Although many solvents have been tested either individually or in combination for the extraction of lipids, there are no reports of a concerted study on the use of a range of solvents and solvent mixtures involving the soxhlet extraction technique.

Solvents used for the extraction of lipids tend not to discriminate when extracting compounds present in algae. This would imply that chlorophyll, carotenoids, pheophytins and associated degradation products would form part of the lipid extract and hence subsequently skew the results obtained for lipid quantities. In this study, an ultraviolet (UV) method was therefore used to determine the total amount of chlorophyll obtained after extraction by each of the solvents, since chlorophyll forms a major component of the so-called contaminants of lipids. Furthermore, a

chromatographic method was optimized for the identification and quantitation of lipids which are targeted for use in biodiesel production. Both these aspects are integral parts of the overall investigation which examines the use of a variety of solvents and their efficiency in the extraction of lipids from algal biomass via the soxhlet extraction method (Ramluckan *et al.*, 2014).

An efficient extraction requires that the solvent fully penetrates the biomass and matches the polarity of the targeted compound(s). This, in conjunction with the ability to make physical contact with the lipid material and solvate the lipid, makes for an efficient extraction solvent (Shen *et al.*, 2009).

2.3.3 Microwave-assisted solvent extraction (MAE)

The extraction step is the least evolved part of most analytical procedures. Soxhlet extraction (developed by F. Soxhlet in 1879) is still used in many routine laboratories. However, there has been an increasing demand for new extraction techniques, amenable to automation. These should have shortened extraction times and reduced organic solvent consumption, with minimum pollution in analytical laboratories and reduced sample preparation costs. Advances in sample preparation have resulted in techniques such as microwave-assisted extraction (MAE) being developed (Poole and Poole, 1996). One of the main advantages of using MAE is the reduction of extraction time when applying microwaves. This can mainly be attributed to the difference in heating performance employed by the microwave technique and conventional heating. Conventional heating requires a finite period of time to heat the vessel before the heat is transferred to the solution, while microwaves heat the solution directly. This keeps the temperature gradient to a minimum and accelerates the speed of heating. MAE also allows for a significant reduction in organic solvent consumption, as well as the possibility of running multiple samples. These are of course minimum criteria for modern sample preparation techniques and are all fulfilled to a great extent by MAE. Consequently MAE has become an attractive alternative to conventional techniques in recent years. Two types of microwave heating systems are commercially available for the

analytical laboratory, viz., open and closed vessel systems (Garc´ıa-Ayuso and Luque de Castro, 2000).

In recent years, numerous applications for the use of microwaves for assisting the extractions of organic and organometallic compounds from various matrices have been reported. The use of microwave energy for organic extraction was first achieved using conventional household systems in the late 1980s (Ganzler *et al.*, 1986; Kaufmann and Christen, 2002). Commercial microwave systems that are specifically designed for extraction is a relatively recent invention and has encouraged renewed interest in the technique. Over the past few years, numerous compounds have been extracted by microwave-assisted extraction (MAE) from several matrices, with special emphasis on environmental applications (Letellier and Budzinski, 1999).

Microwave-assisted extraction involves the heating of an extractant (mostly liquid organic solvents) in contact with the sample with microwave energy (Pare *et al.*, 1994). Owing to the hazardous nature of extraction using flammable organic compounds (such as solvents), it is strongly recommended that only equipment approved for MAE applications be used (Trivedi *et al.*, 2011). The application of microwave energy to the samples may be performed using closed vessels (under controlled pressure and temperature), or with open vessels (under atmospheric pressure) (Demesmay and Olle, 1997; Letellier and Budzinski, 1999). They are commonly named either pressurized MAE (PMAE) or focused MAE (FMAE), respectively. Although the traditional soxhlet and solvent extraction techniques are widely accepted, they have inherent limitations and problems (Wiesenberg *et al.*, 2004). Soxhlet extraction may require from 1 to 24 h for most extractions and has the disadvantage that it may consume a large amount of organic solvent. In contrast to these conventional methods, microwave-assisted extraction (MAE) can reduce the extraction time to less than 30 min with solvent volumes of lower than 50mL (Jin *et al.*, 1999). Temperature is of prime importance in ensuring efficient extraction. Elevated temperatures usually enhance the extraction, as a result of an increased diffusivity of the solvent into the internal parts of the matrix. The desorption of the

components from the active sites of the matrix is enhanced. In closed systems, pressure is also an important variable which is directly dependent on the temperature. Hence temperature is controlled to avoid degradation of the extracted compounds. (Hoogerbrugge *et al.*, 1997). The optimum temperature may depend on the matrix to be extracted (Pylypiw *et al.*, 1997). Most applications of microwave extraction have mainly concentrated on solid samples (Srogi, 2006).

The principle of heating using microwave energy is based on the direct effect of microwaves on molecules by ionic conduction and dipole rotation. In many applications these two mechanisms take place simultaneously. Ionic conduction is the electrophoretic migration of ions when an electromagnetic field is applied. The resistance of the solution to this flow of ions will result in friction and, thus, heat the solution. Dipole rotation means realignment of dipoles with the applied field. At 2450 MHz, which is the frequency, used in commercial systems, the dipoles align and randomize 4.9×10^9 times per second and this forced molecular movement results in heating (Veggi *et al.*, 2012).

The ability of a solvent to absorb microwave energy and pass it on in the form of heat to other molecules will partly depend on the dissipation factor (tan δ) which is given by the following equation:

$$\tan \delta = \varepsilon'' / \varepsilon'$$

where ε" is the dielectric loss (a measure of the efficiency of converting microwave energy into heat) and ε' is the dielectric constant (a measure of the polarizibility of a molecule in an electric field). Polar molecules and ionic solutions (usually acids) will absorb microwave energy strongly because they have a permanent dipole moment that will be affected by the microwaves. However non-polar solvents such as hexane will not heat up when exposed to microwaves. A simple comparison between methanol and water shows that methanol has a lower dielectric constant but a higher dielectric loss than water. This indicates that methanol compared to water, has lower ability to obstruct the microwaves as they pass through, but a higher ability to dissipate the microwave energy into heat. In closed vessels, the solvent can be heated

above its normal boiling point, thus enhancing extraction efficiency and speed (Veggi *et al.*, 2012).

MAE is a process of using microwave energy to heat solvents in contact with a sample in order to partition analytes from the sample matrix into the solvent. The ability to rapidly heat the sample solvent mixture is
inherent to MAE and the main advantage of this technique. By using closed vessels the extraction can be performed at elevated temperatures accelerating the mass transfer of target compounds from the sample matrix. A typical extraction procedure takes 15–30 min and uses small solvent volumes in the range of 10–30 ml. These volumes are about 10 times smaller than volumes used by conventional extraction techniques. In addition, sample throughput is increased as several samples can be extracted simultaneously. In most cases recoveries of analytes and reproducibility are improved compared to conventional techniques.

Since different chemical substances absorb microwave energy to different extents indicates that the heating imparted to the surrounding media will vary with the chemical substances used. Hence, for samples with non-homogeneous structural characteristics or different dielectric properties dispersed into a homogeneous environment, it is possible to produce selective heating of some areas or components of the sample. This phenomenon is sometimes called superheating (Eskilsson and Bjorklund, 2000).

Although microwave energy has great potential for rapidly heating materials,
microwave ovens have only recently appeared in analytical laboratories. In 1975, Abu-Samra et al were the first researchers ever to use a microwave domestic oven in the laboratory, performing trace analysis of metals from biological samples (Abu-Samra *et al.*, 1975). Since then microwave digestion methods have been developed for different sample types such as environmental biological, geological and metallic matrices, as well as for fly ashes and coal. Over the years procedures using microwave ovens have replaced some of the conventional hot plate and other

thermal digestion techniques which were predominant (Jin *et al.*, 1999).

2.3.4 Expeller

When algae are dried they retain their oil content. The oil can then be "pressed" out with an oil press. Many commercial manufacturers of vegetable oil use a combination of mechanical pressing and chemical solvents in extracting oil. While more efficient processes are emerging, a simple process makes use of a press to extract a large percentage (70-75%) of the oils out of algae (*Oilgae-Oil & Biodiesel from Algae*, 2008). This is a mechanical method which makes use of an expeller to press the algae. The raw materials are squeezed under high pressure in a single step. Expeller presses can recover 75% of the oil from algae. Expeller processing cannot remove the last trace of oil from algae (Toparea *et al.*, 2011).

2.3.5 Other methods

The methods specified here are used primarily at lab scale. Some of these methods may have greater efficiency than the methods evaluated in this study. These methods apart from being too expensive, require specialized equipment and would present greater challenges for upscaling. Hence, these methods were not selected for study, but are examined as possibilities for experimentation.

2.3.5.1 Supercritical fluid extraction

An extraction method that has gained acceptance in recent years is the use of supercritical fluids to extract high-value products from microalgae. This method produces highly purified extracts that are free of potentially harmful solvent residues. The extraction and separation are quick and safe for thermally sensitive products (Sahena *et al.*, 2009). Fractionation of specific compounds is also feasible. It may reduce separation costs (Herrero *et al.*, 2006 ; Mendiola *et al.*, 2007). Supercritical fluid extraction (SFE) relies on the fact that some chemicals behave as both a liquid and a gas, and have increased solvating power when they are raised above their critical temperature and pressure points. Carbon dioxide is used because of its

relatively low critical temperature (31.1ºC) and pressure (72.9 atm) (Cooney *et al.*, 2009). Supercritical CO_2 extraction efficiency is affected by four main factors viz., pressure, temperature, CO_2 flow rate and extraction time (Andrich *et al.*, 2006; Harun *et al.*, 2010; Xu *et al.*, 2008). These factors, along with the use of modifiers (most commonly ethanol as a co-solvent), can be altered and adjusted to optimize extractions. When ethanol is used as a co-solvent, polarity of the extracting solvent (in this case, CO_2) is increased. This also alters the viscosity of the fluid. The resulting effect is an increase in the solvating power of the CO_2, and the extraction requires lower temperature and pressure, making it more efficient (Herrero *et al.*, 2006 ; Mendes *et al.*, 2006; Mendes *et al.*, 2005; Mendiola *et al.*, 2007). Since CO_2 is a gas at room temperature, it is easily removed when extraction is completed. This makes it safe for food and it can be safely recycled, making it environmentally beneficial (Mendes *et al.*, 2006; Sahena *et al.*, 2009). One restriction to supercritical CO_2 extraction is the level of moisture in the sample. High moisture content can reduce contact time between the solvent and sample. This is because microalgal samples have a thick consistency and moisture acts as a barrier against diffusion of CO_2 into the sample, and diffusion of lipids out of the cells. Hence samples are dried prior to supercritical fluid extraction (Sahena *et al.*, 2009).

2.3.5.2 Ultrasound/sonication

Ultrasonic-assisted extractions use the process of cavitation to recover oils from microalgal cells. Cavitation occurs when vapour bubbles of a liquid form in an area where pressure of the liquid is lower than its vapour pressure. These bubbles grow when pressure is negative and compress under positive pressure. This causes a violent collapse of the bubbles. If bubbles collapse near cell walls, damage can occur and the cell contents are released (Harun *et al.*, 2010; Wei *et al.*, 2008). It has not been determined if ultrasonic methods may negatively impact oil quality and/or stability of polyunsaturated fatty acid rich oils. This technology may be difficult to scale up (Mercer and Armenta, 2011).

2.3.5.3 Accelerated solvent extraction or Pressurized liquid extraction

Pressurized liquid extraction (PLE), also referred to as pressurized fluid extraction (PFE), pressurized solvent extraction (PSE) or accelerated solvent extraction (ASE), is a technique that was introduced by Dionex corporation in 1995 (Richter *et al.*, 1996). The principle of the technique is based on the use of elevated temperatures (50 – 200 °C) and pressures (50-150 atm) to extract analytes from solid or semi-solid samples within short periods of time (5 – 15 min). The Dionex ASE® 200 system consists of a solvent delivery component controlled by an HPLC pump, nitrogen gas purge valve, a carousel for extraction cells and collection vials including a waste vial. Prior to extraction, a solid or semi-solid sample is placed in a stainless steel extraction cell lined with a filter paper disk on the outlet end to prevent passage of solid matter from the cell into the collection vial. The extraction cell is then placed onto a carousel and automatically drawn into the oven and filled with solvent. During extraction, the cell is heated, causing thermal expansion of the solvent and hence an increase of pressure inside the cell. The static and pressure relief valves function to regulate pressure inside the cell during static extraction by adding more solvent or opening the static valve to let solvent out of the extraction cell, whichever one is needed to maintain the desired pressure. After static extraction, some of the solvent inside the extraction cell can be replaced by fresh solvent for a subsequent extraction cycle. This solvent volume can vary from 5 to 150 % of the extraction cell. The introduction of fresh solvent increases the concentration gradient between the extraction solvent in the cell and the surface of the sample matrix. This results in improved mass transfer and better extraction efficiency compared to a single cycle extraction (Richter *et al.*, 1996). In the final step, pressurized nitrogen purges the remaining solvent from the cell and lines to a collection vial. Parameters that may be optimized in PLE are:

- Temperature (60 – 200 °C) - the increased temperatures disrupt the strong solute-matrix interactions caused by van der Waals forces, hydrogen bonding and dipole interactions of the solute molecules and active sites on the matrix. When the solvent is in contact with the matrix, the thermal energy in the heated solvent assists to desorb analytes from the matrix by overcoming cohesive (solute-solute) and

adhesive (solute-matrix) interactions. This decreases the activation energy required for the desorption process (Richter *et al.*, 1996).

• Pressure (50 – 100 bars) - The high pressures employed in PLE maintain the solvent in its liquid state even at temperatures above its atmospheric boiling point. The high pressure increases the solvation power and speeds up the extraction kinetics of solvents by forcing solvent into the pores of the matrix that normally would not be in contact with solvent at atmospheric pressure. This helps to solvate analytes trapped in matrix pores which have been "sealed" with water or air bubbles. The pressurized flow in PLE also assists to solubilize air bubbles surrounding analytes that are found on the surface of the matrix as well. For polar solvents such as water, increasing the temperature lowers the dielectric constant thus making it suitable for the extraction of less polar compounds (Turner et al., 2006). The dielectric constant of water at 25 °C is ~80, making it an extremely polar solvent. Increasing the temperature of water to 250 °C while applying sufficient pressure to maintain it in its liquid state reduces the dielectric constant to 27 which is midway between those of methanol ($\varepsilon = 33$) and ethanol ($\varepsilon = 24$) at 25 °C (Miller and Hawthorne, 1998). As a result, water at higher temperatures is more "miscible" or "soluble" in organic solvents and is often referred to as pressurised hot water extraction (PHWE) (Richter *et al.*, 1996).

2.3.5.4 Hydrothermal method

Because microalgae are generally collected as wet matrix, the hydrothermal method (HTT) is considered one of better ways for producing fuels from aqueous microalgae slurry by means of heat application followed by a phase separation. This process produces an oily phase (containing lipids and hydrophobic products from HTT), a variable amount of gas, water-soluble substances and a solid residue. The high nitrogen content can be a problem for the direct utilization of the oil as fuel with high temperature HTT. However, the method may face some limitations when implemented in a conventional way using commercial systems. There is strong evidence that flash pyrolysis produces a significant amount of non-GC detectable matter (Torri *et al.*, 2012).

2.3.5.5 The Bligh and Dyer (B&D) method

The primary advantage of the Bligh and Dyer method is a reduction in the solvent/sample ratio (1 part sample to 3 parts 1:2 chloroform/methanol followed by 1 or 2 parts chloroform). The Folch method, on the other hand, employs a ratio of 1 part sample to 20 parts 2:1 chloroform/methanol, followed by several washings of the crude extract. Despite this solvent reduction, the Bligh and Dyer method is expected to yield a recovery of ≥95% of total lipids. Although the procedure was developed using cod muscle, it can be applied to any tissue containing (or modified to contain) 80% water. The Bligh and Dyer method has undergone rigorous and favourable evaluations. Virtually all of these evaluations have been performed on samples containing less than 1.5% total lipid. Some studies report using a modified Bligh and Dyer method for lipid-rich samples; however, the modifications are often unspecified, making the evaluation and comparison of results difficult. In other cases, investigators report the use of the Bligh and Dyer method even with samples having high lipid contents, but do not indicate that any modifications have been made (Iverson *et al.*, 2001).

2.4 The Production of Biodiesel

2.4.1 Typical lipids that favour the production of biodiesel

The term “lipid” does not specify a particular chemical structure. Lipids are more chemically diverse. There are operational and structural definitions of lipids. Lipid analysts tend to have a firm understanding of what is meant by the term "lipid", but, there is no widely accepted definition. A common structural definition is that lipids are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds. It includes cholesterol and bile acids, but does not include other steroids, fat-soluble vitamins, carotenoids, terpenes or mineral oil, except in rare circumstances (Christie, 2013). Although the term lipid is sometimes used as a synonym for fats, fats are usually referred to as triacylglycerols, which is a subgroup of lipids (*Lipid*, 2014).

Lipids may also be defined as non-volatile substances that can be extracted from biological sources by solvents of low to medium polarity. The lipid extracted by solvents is also called “crude fat” or “extractable fat”. Crude fat is heterogeneous material, consisting of a mixture of triacylglycerols, phospholipids, fatty acids, sterols, waxes and pigments. The gravimetrically determined content of crude lipids is usually referred to as “total lipid”. Total lipid, as an estimate for energy content and nutritional values in biological material has been criticized because of the content of non-fat and non-digestible substances. The US Nutrition labelling and Education Act of 1990 (NLEA) has defined total fat as the sum of all fatty acids obtained from a total lipid extract expressed as triacylglycerols (Xiao, 2010).

Fatty acids are compounds synthesized in nature via condensation of malonyl coenzyme A units by a fatty acid synthase complex. Fatty acids act as building blocks of lipids. In general, they contain even numbers of carbon atoms in straight chains (usually in the range C_{14} to C_{24}) (Fahy *et al.*, 2005). Fatty acids can either be saturated, monounsaturated or polyunsaturated depending on the number of double bonds.

The most common and abundant saturated fatty acids in animal and plant tissues are straight chain compounds with 12, 14, 16 and 18 carbon atoms:
Lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0) (Figures 2.5, 2.6, 2.7, 2.8).

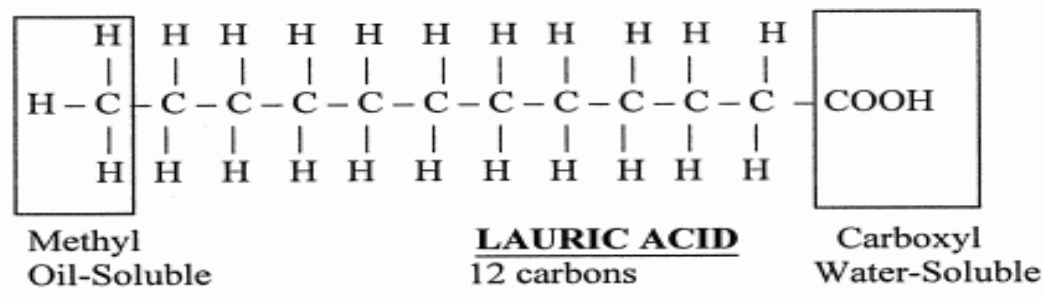


Figure 2.5 Structural diagram of Lauric acid.

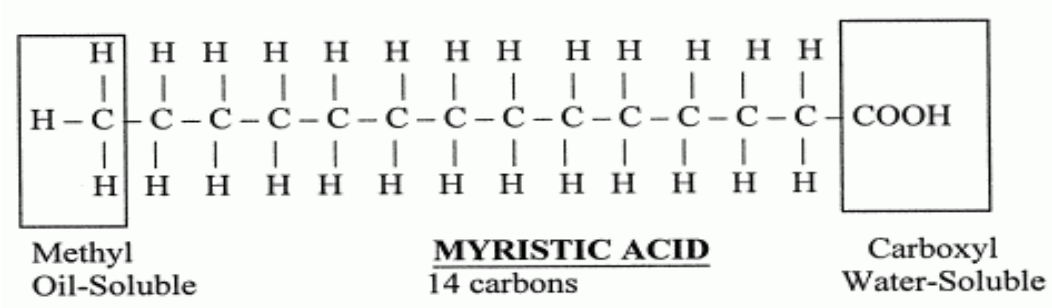


Figure 2.6 Structural diagram of Myristic acid.

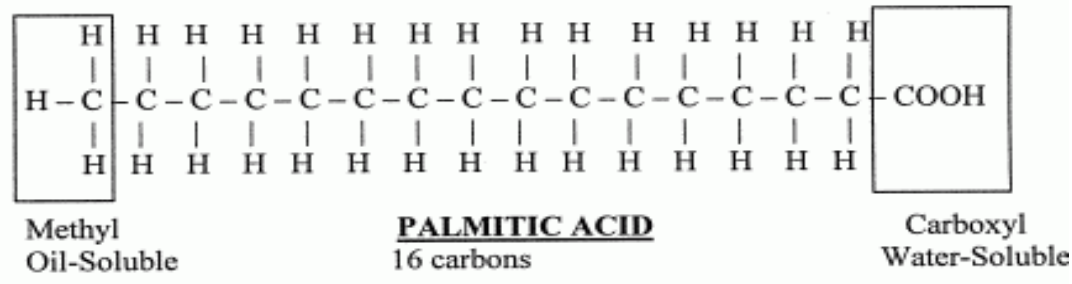


Figure 2.7 Structural diagram of Palmitic acid.

Structures courtesy of (Best, 2014)

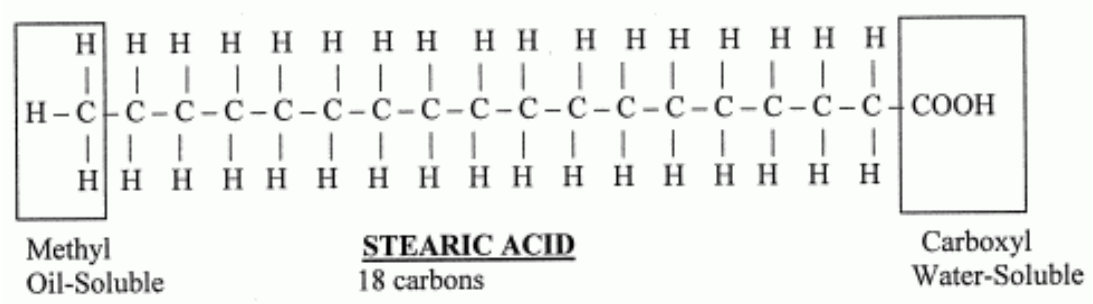


Figure 2.8 Structural diagram of Stearic acid.

Straight-chain even-numbered fatty acids with 10 to more than 30 carbon atoms and containing one cis-double bound have been characterized from natural sources. The most abundant monounsaturated fatty acid in tissue is cis-9-octadecenoic acid (18:1 n-9), also termed “oleic acid” (Figure 2.9).

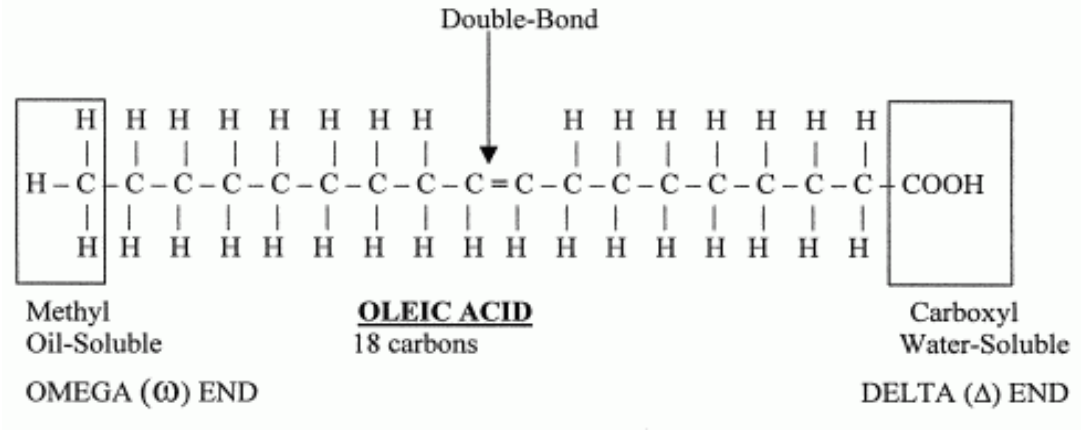


Figure 2.9 Structural diagram of Oleic acid.

Structures courtesy of (Best, 2014)

The polyunsaturated fatty acids (PUFAs) are fatty acids containing two or more double bonds. There are two principal families of PUFAs, the ω-3 and the ω-6. Their first double bond is located on the 3rd or 6th carbon-carbon bond, counting from the terminal methyl carbon toward the carbonyl carbon, and double bonds are separated by one methylene unit. The parent compound of the ω-6 family, linoleic acid (LA) (18:2 n-6) (Figure 2.10) is plentiful in nature. Alpha-linolenic acid (ALA) (18:3 n-3) (Figure 2.11), the parent compound of the ω-3 family, is far less common. Both can

be elongated and desaturated to long-chain PUFAs i.e. linoleic acid to arachidonic acid (AA) (20:4 n-6) (Figure 2.12) and linolenic acid to eicosapentaenoic acid (EPA) (20:5 n-3) (Figure 2.13) and docosahexaenoic acid (DHA) (22:6 n-3) (Figure 2.14). The fatty acids may be found in free form but in general they are combined in more complex molecules usually through ester bonds (Ruiz-Rodriguez *et al.*, 2010) .

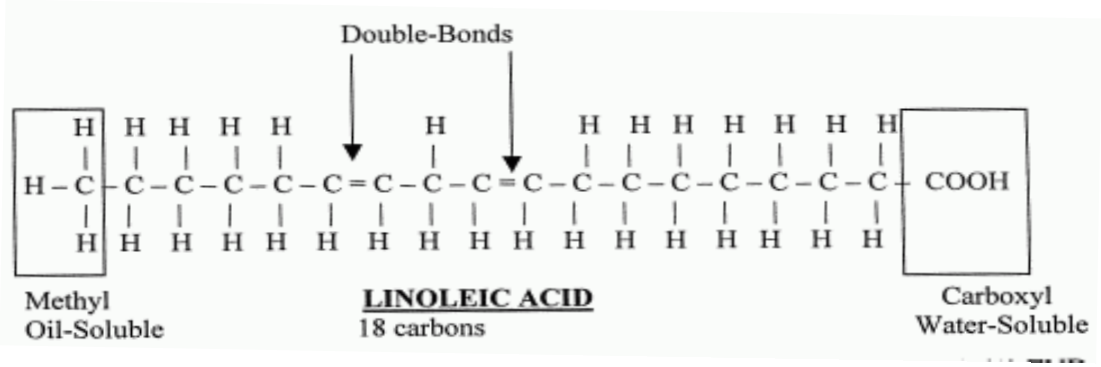


Figure 2.10 Structural diagram of Linoleic acid.

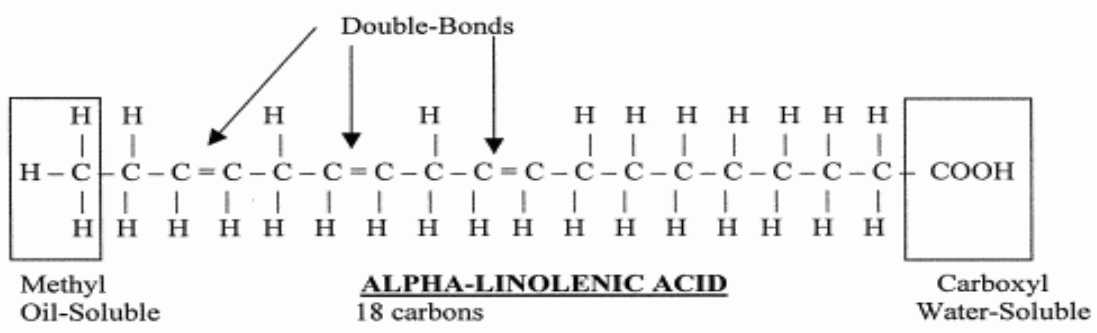


Figure 2.11 Structural diagram of Linolenic acid.

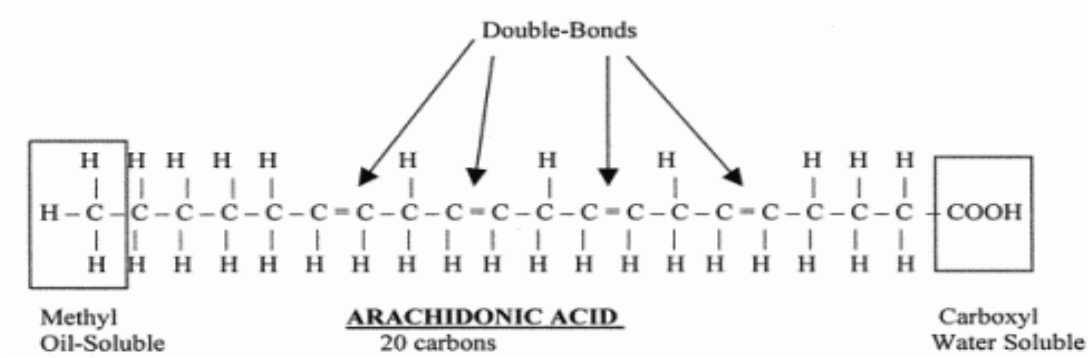


Figure 2.12 Structural diagram of Arachidonic acid.

Structures courtesy of (Best, 2014)

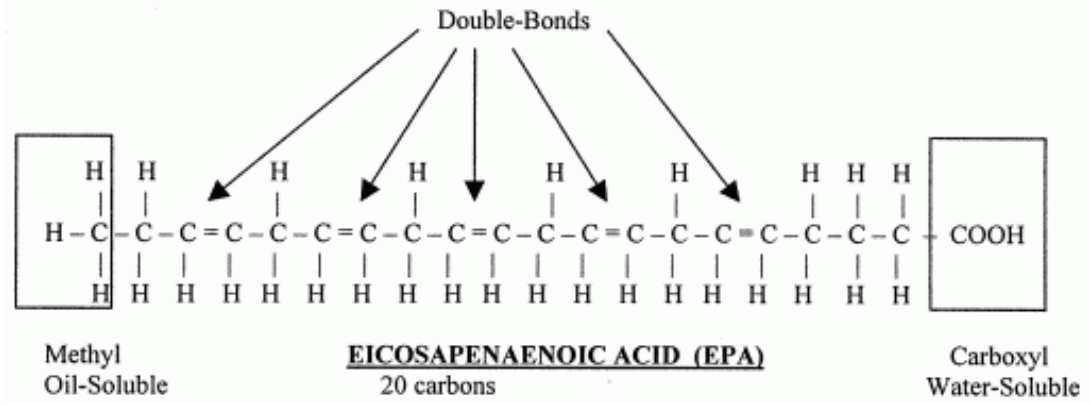


Figure 2.13 Structural diagram of Eicosapentaenoic acid.

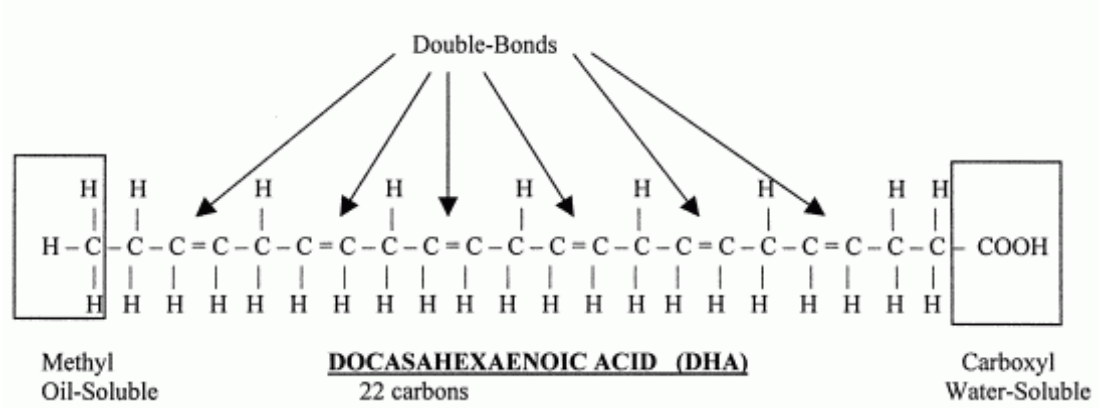


Figure 2.14 Structural diagram of Docosahexaenoic acid.

Structures courtesy of (Best, 2014)

The lipids are generally classified into two broad groups, viz., simple lipids and complex lipids. Simple lipids (including fatty acids, triacylglycerols, sterols, sterol and wax ester) are those which yield, on hydrolysis, at most two types of primary products per mole; complex lipids (including glycerophospholipids, glyceroglycolipids, ether lipids and sphingolipids) yield three or more primary hydrolysis products per mole Alternatively, the terms "neutral" and "polar" lipids respectively are used to define these groups, but they are less exact. For many purposes, the complex lipids are best considered in terms of either the

glycerophospholipids or phospholipids. These contain a polar phosphorus moiety and a glycerol backbone. The glycolipids, which contain a polar carbohydrate moiety, are more easily analysed separately (Christie, 2013).

Nearly all the commercially important fats and oils of animal and plant origin consist almost exclusively of triacylglycerols (TAG), (Figure 2.15). They consist of a glycerol moiety with each hydroxyl group esterified to a fatty acid.

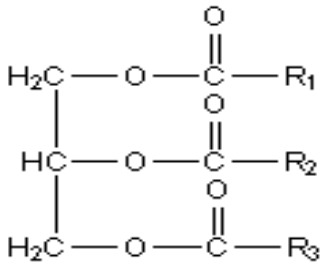


Figure 2.15 Triacylglycerols (TAG).

In their most common form, wax esters consist of fatty acids esterified to long-chain alcohols with similar chain-lengths. The latter tend to be saturated or have one double bond only. Free fatty acids are minor constituents of living tissue but are of biological importance as precursors of lipids, as an energy source and as cellular messengers. Large amounts of FFA are usually indicative of artefactual hydrolysis during storage or extraction of the tissues. Glycoglycerolipids, Sphingolipids and glycosphingolipids are of metabolic importance but are only present in trace amounts in most tissues. They were of limited relevance for this study.

The three main facets to any practical procedure for extracting lipids from tissue are:
1. Exhaustive extraction and solubility of the lipids in organic solvent,
2. Removal of non-lipid contaminants from the extracts, and
3. The potential toxicity of solvents to analysts.

Lipids occur in tissues in a variety of physical forms. The simple lipids are often part of large aggregates in storage tissues, from which they are relatively easily extractable. Complex lipids are usually constituents of membranes, where they occur in a close association with such compounds as proteins and polysaccharides, with

which they interact. They are not extracted so readily. Generally, lipids are linked to other cellular components by weak hydrophobic or Van der Waal's forces, by hydrogen bonds and by ionic bonds. Pure lipids will dissolve in a variety of solvents, depending on the relative strengths of the interactions between the solvent and either the hydrophobic or the hydrophilic regions of the molecules. Lipids with functional groups of low polarity only, such as triacylglycerols or cholesterol esters, are very soluble in hydrocarbon solvents like hexane, cyclohexane or toluene, and in solvents of somewhat higher polarity, such as chloroform or ethers. They tend to be insoluble in polar solvents such as alcohols and methanol. Solubility in polar solvents increases as the chain-length of the fatty acid moieties in these lipids decrease or as the chain-length of the solvent alcohol increases. Unsaturated lipids tend to dissolve in most solvents more readily than saturated and higher-melting analogues. In contrast, the polar complex lipids tend to be only sparingly soluble in hydrocarbon solvents. The dissolution can be aided by the presence of other lipids, but they do dissolve readily in more polar solvents such as chloroform, methanol and ethanol (Rustan and Drevon, 2005). In order to extract lipids from tissues, it is necessary to find solvents that will not only dissolve the lipids readily but will disrupt the interactions between the lipids and the tissue matrix. This was primarily the reason for selecting the range of thirteen solvents for the extraction protocols.

2.4.2 Selection of solvents for the extraction of lipids

The ideal solvent for lipid extraction would be one which completely extracts lipid components, while leaving all the other components behind. In practice, the efficiency of solvent extraction depends on the polarity of the lipids present compared to the polarity of the solvent. Polar lipids (such as glycolipids or phospholipids) are more soluble in polar solvents (such as alcohols), than in non-polar solvents (such as hexane). On the other hand, non-polar lipids (such as triacylglycerols) are more soluble in non-polar solvents than in polar ones. The fact that different lipids have different polarities means that it is impossible to select a single organic solvent to extract them all. Thus the total lipid content determined by solvent extraction depends on the nature of the organic solvent used to carry out the

extraction: the total lipid content determined using one solvent may be different from that determined using another solvent. Solvents used for extraction should also be inexpensive, have a relatively low boiling point (so that it can easily be removed by evaporation), and be non-toxic and non-flammable (for safety reasons). It is difficult to find a single solvent which meets all of these requirements. Ethyl ether and petroleum ether are cited as commonly used solvents, but pentane and hexane are also used for some foods (*Analysis of Lipids*, 2014).

Soxhlet extraction yields are influenced by solvent composition. Lipid content determined by Ishida and Chapman using methanol was more than two times higher than the content determined by extraction with less polar solvents like hexane and acetone. This is most likely because non-lipids were also extracted. The amount of lipids and non-lipid material extracted increases with the polarity of solvents used. Solvent mixtures containing a polar and a non-polar solvent extract a greater amount of lipids. Ryckebosch et al (2011), have shown that chloroform-methanol 1:1 gave the highest lipid yield while chloroform-methanol 2:1 extracted only 76.5%. Neutral or storage lipids are extracted with relatively non-polar solvents such as diethyl ether or chloroform. Membrane-associated lipids are polar and require solvents like ethanol or methanol to disrupt hydrogen bondings or electrostatic forces (Ishida and Chapman, 2009).

Alcohols are good solvents for most lipids, methanol and ethanol being the most popular. Chloroform is a popular solvent, particularly for lipids of intermediate polarity and when mixed with methanol it becomes a general extraction solvent mixture. It is not very stable, forming phosgene and HCl in air. It is sold after addition of preservatives, ethanol, amylene or cyclohexene. Dichloromethane is a similar extractant but less oxidizable. Hexane is the most popular solvent among hydrocarbons. However it is only good for lipids of low polarity. Its main use is to extract neutral lipids from mixtures of water with alcohols. Hexane can be replaced by petroleum ether which is a mixture of various hydrocarbons with 5 to 8 carbon atoms. Cyclohexane is sometimes used to store lipid extracts in the cold without

danger of evaporation since it freezes at about 6°C. Benzene is no longer used since it is now considered as a potent carcinogenic substance. It may be replaced by toluene which is generally more difficult to evaporate (Manirakiza, 2001). With this in mind, solvents chosen for extraction for this study were of the polar, non-polar and intermediate polar variety.

2.4.3 Chlorophyll

Chlorophyll is present in many organisms including algae and some species of bacteria. Chlorophyll *a* is the most abundant form of chlorophyll within photosynthetic organisms which gives plants their green colour. Other forms of chlorophyll, coded b, c, and d, may be present in all photosynthetic organisms but vary in concentrations (*The Basics of Chlorophyll Measurement*, 2014). Chlorophyll is formed in the presence of light. It is a very complex substance containing carbon, hydrogen, magnesium, nitrogen, and oxygen. It is insoluble in water, but readily soluble in alcohol, chloroform, or ether.

In chlorophyll the central ion is magnesium, and the large organic molecule is a porphyrin. The porphyrin contains four nitrogen atoms that form bonds to magnesium in a square planar arrangement. There are several forms of chlorophyll. The structures of two forms, chlorophyll *a* and *b* are shown (Figure 2.16).

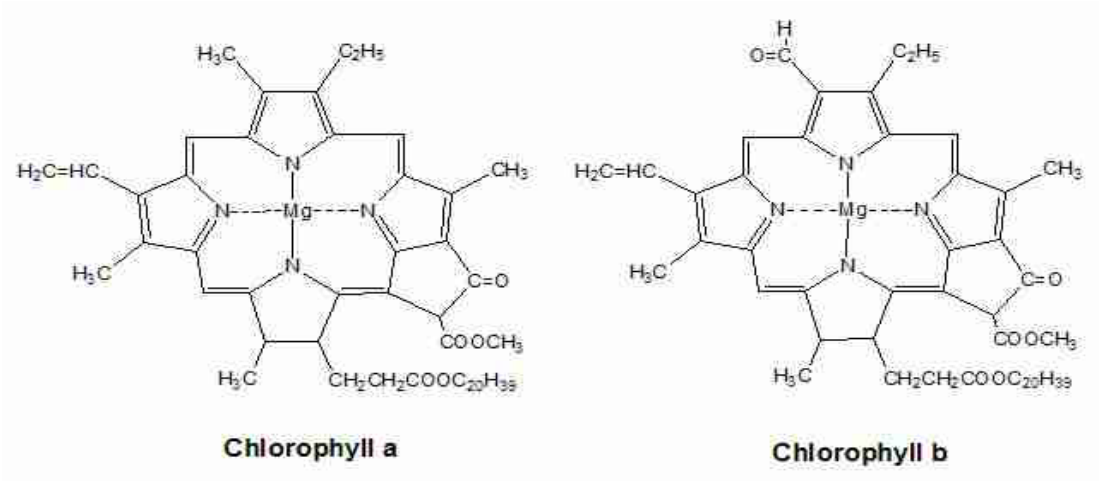


Figure 2.16 Structural diagrams of Chlorophyll a ($C_{55}H_{72}O_5N_4Mg$) and Chlorophyll b ($C_{55}H_{70}O_6N_4Mg$).

Chlorophyll exhibits two major light absorption bands, one on the blue side of the visible spectrum (< 460 nm) and the other in the red (630–670 nm) (Aminot and Rey, 2000). Since it absorbs red and blue-violet light, the light it reflects and transmits appears green (*Chlorophyll*, 2010). The content of chlorophyll *a* relating to the pigment level is almost the same in all algae groups, but chlorophyll *b* and *c* change depending on the algae species and environmental conditions (Dere *et al.*, 1998).
UV spectrometry, fluorimetry and high performance liquid chromatography are recommended methods for the determination of chlorophyll.

Little is known about the effects of chlorophyll on the conversion of lipids to biodiesel. Since chlorophyll is an integral part of the extracts obtained from algal biomass, esterification can be performed with or without the removal of chlorophyll. The quantification of chlorophyll was performed using all the solvents studied to establish the efficiencies of the various solvents in extraction of chlorophyll.

2.4.4 Transesterification methods

In the making of biodiesel, triglycerides are reacted with methanol in a reaction known as transesterification or alcoholysis. Transesterification produces methyl esters of fatty acids, i.e. biodiesel, and glycerol (Cook and Beyea, 2000). A catalyst is usually used to improve the reaction rate and yield. Since the reaction is reversible, excess alcohol is used to shift the equilibrium to the products side. Among the alcohols that can be used in the transesterification process are methanol, ethanol, propanol, butanol and pentanol. Methanol is used more frequently because of its low cost and its physical and chemical advantages (polar and shortest chain alcohol). NaOH dissolves easily in alcohols and reacts with triglycerides. To complete a transesterification stoichiometrically, a 3:1 molar ratio of alcohol to triglycerides is needed. In practice, the ratio needs to be higher to drive the equilibrium to a maximum ester yield. The reaction can be catalyzed by alkalis, acids, or enzymes. The alkalis include NaOH, KOH, carbonates and corresponding sodium and potassium alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide

and sodium butoxide. Sulfuric acid, sulfonic acids and hydrochloric acid are usually used as acid catalysts. Alkali-catalyzed transesterification is much faster than acid-catalyzed transesterification and is most often used commercially (Benemann, 1997). Biomass-derived fuels share many of the same characteristics as their fossil fuel counterparts. Once formed, they can be substituted in whole or in part for petroleum-derived products. In the African context, for economic reasons, fuels may be blended with ethanol for cleaner-burning diesel (Mackay and Probert, 2001). This transesterification process generally makes use of acid or base catalysis for the conversion of triglycerides to esters. Methods abound in literature, but not all are suited to the conversion of algal lipids to biodiesel. The general reaction mechanism is shown in Figure 2.17.

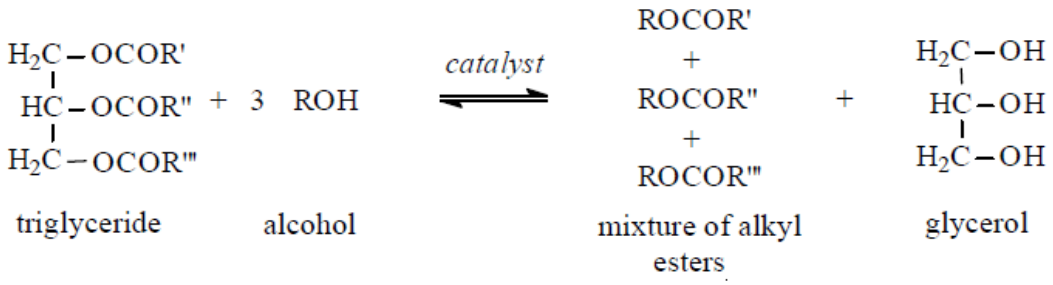


Figure 2.17 Transesterification of vegetable oils.

The following is an examination of some of the methods evaluated for use in this study.

2.4.4.1 Direct acid esterification

An advantage of acid catalysts is that they can directly produce bio-diesel from low-cost lipid feedstock's, generally associated with high FFA concentrations (low-cost feedstock's, such as used cooking oil and greases, commonly have FFAs levels of >6%). For acid-catalyzed systems, sulphuric acid, HCl, BF_3, H_3PO_4, and organic sulfonic acids, have been used by various researchers (Vyas *et al.*, 2010).

Freedman et al. (1984) compared the transesterification of soybean oil with methanol, ethanol and butanol using 1% concentrated sulphuric acid based on the weight of oil. In preliminary experiments with 6:1 M and 20:1 M ratios at 3 h and

18h, respectively, conversions to ester were unsatisfactory. However, a molar ratio of 30:1 resulted in a high conversion to the methyl ester. Each alcoholysis was conducted near the boiling point of the alcohol.

The transesterification process is catalyzed by Brønsted acids, preferably by sulfonic and sulphuric acids. These catalysts give very high yields of alkyl esters, but the reactions are slow, requiring, typically, temperatures above 100 °C and more than 3 h to reach complete conversion. The methanolysis of soybean oil, in the presence of 1 mol% of H_2SO_4, with an alcohol/oil molar ratio of 30:1 at 65 °C, takes 50 h to reach complete conversion of the vegetable oil (> 99%), while the butanolysis (at 117 °C) and ethanolysis (at 78 °C), using the same quantities of catalyst and alcohol, take 3 and 18 h, respectively (Freedman *et al.*, 1984). The alcohol/vegetable oil molar ratio is one of the main factors that influence the transesterification. An excess of the alcohol favours the formation of the products. On the other hand, an excessive amount of alcohol makes the recovery of the glycerol difficult. Therefore, the ideal alcohol/ oil ratio has to be established empirically, considering each individual process.

The mechanism of the acid-catalyzed transesterification of vegetable oils is shown in Figure 2.18, for a monoglyceride. However, it can be extended to di- and triglycerides. The protonation of the carbonyl group of the ester leads to the carbocation II which, after a nucleophilic attack of the alcohol, produces the tetrahedral intermediate III, which eliminates glycerol to form the new ester IV, and to regenerate the catalyst H^+. According to this mechanism, carboxylic acids can be formed by reaction of the carbocation II with water present in the reaction mixture. This suggests that an acid-catalyzed transesterification should be carried out in the absence of water, in order to avoid the competitive formation of carboxylic acids which reduce the yields of alkyl esters (Schuchardta *et al.*, 1998).

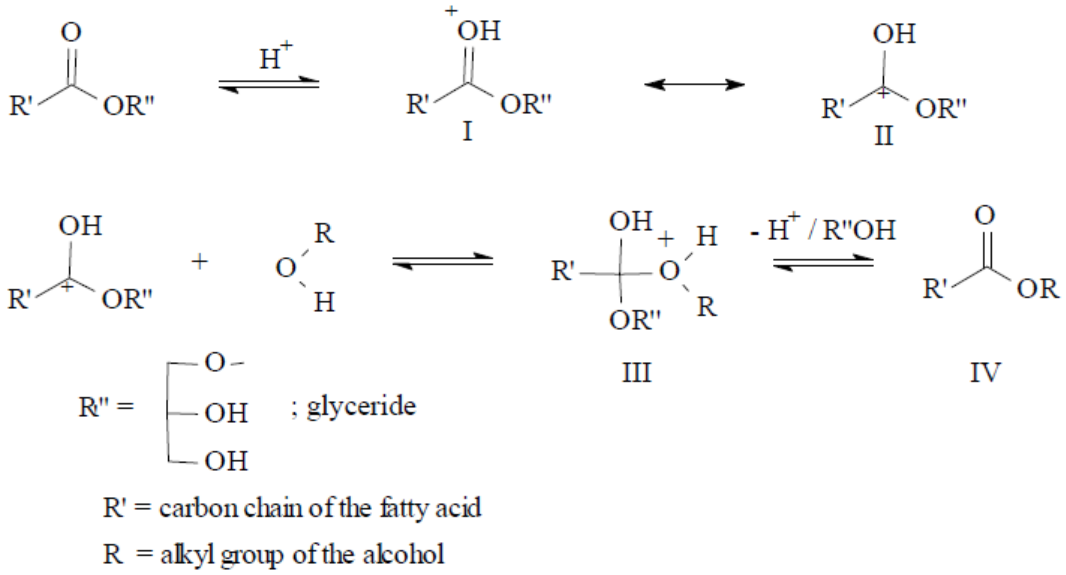


Figure 2.18 Mechanism of the acid-catalyzed transesterification of vegetable oils.

1. Methanolic hydrogen chloride

The transesterification is performed with the acidic reagent made up of 5% (w/v) anhydrous hydrogen chloride in methanol. It is most often prepared by bubbling hydrogen chloride gas into dry methanol. The hydrogen chloride gas is commercially available in cylinders or can be prepared by dropping concentrated sulphuric acid slowly onto fused ammonium chloride or into concentrated hydrochloric acid.

This method is best suited to bulk preparation of the reagent. The hydrogen chloride gas can be obtained by adding acetyl chloride (5 mL) slowly to cooled dry methanol (50 mL).

2. Methanolic sulphuric acid

Vegetable oils are transesterified very rapidly by heating in 10% sulphuric acid in methanol until the reflux temperature is reached. A solution of 1–2% concentrated sulphuric acid in methanol has almost identical properties to 5% methanolic hydrogen chloride and is very easy to prepare.

3. Boron trifluoride-methanol (BF_3)

The Lewis acid, boron trifluoride, in the form of its coordination complex with methanol is a powerful acidic catalyst for esterification of fatty acids. Boron trifluoride in methanol (12–14% w/v) is a very popular catalyst for transesterification. It is often utilized as a rapid means of esterifying free fatty acids. When it is compared critically with some of the other acidic catalysts under similar conditions, it does not even appear to be any more rapid in its reaction (Demirbas, 2008).

2.4.4.2 Direct alkali esterification

Transesterification reaction can be catalyzed by both homogeneous (alkalies and acids) and heterogeneous catalysts. The most commonly used alkali catalysts are KOH, NaOH and CH_3ONa (Gemma *et al.*, 2004). The reaction mechanism for alkali-catalyzed transesterification was formulated as three steps. The alkali-catalyzed transesterification of vegetable oils proceeds faster than the acid-catalyzed reaction. In the alkali catalytic methanol transesterification method, the catalyst is dissolved in methanol by vigorous stirring in a small reactor. The oil is transferred to a biodiesel reactor and then the catalyst/alcohol mixture is pumped into the oil. The final mixture is stirred vigorously for 2 h at 340 K at ambient pressure. A successful transesterification reaction produces two liquid phases: ester and crude glycerol. Crude glycerol, the heavier liquid, will collect at the bottom after several hours of settling. Phase separation can be observed within 10 min and can be complete within 2 h of settling. Complete settling can take as long as 20 h. After settling is complete, water is added at the rate of 5.5% by volume of the methyl ester of oil and then stirred for 5 min, and the glycerine is allowed to settle again. Washing the ester is a two-step process, which needs to be performed with extreme care. A water wash solution at the rate of 28% by volume of oil and 1 g of tannic acid per litre of water is added to the ester and gently agitated. Air is carefully introduced into the aqueous layer while simultaneously stirring very gently. This process is continued until the ester layer becomes clear. After settling, the aqueous solution is drained, and water

alone is added at 28% by volume of oil for the final washing (Demirbas, 2005; Demirbas, 2008).

Gemma et al., (2004) selected four different alkaline catalysts i.e. NaOH, KOH, CH_3ONa, CH_3OK for alkali-catalyzed transesterification of Sunflower oil. The biodiesel purity was near 100 wt. % for all catalysts. High bio-diesel yields were obtained by using the sodium or potassium methoxide (99.33 wt. % and 98.46 wt. %, respectively). However, when sodium or potassium hydroxides were utilized as catalysts, biodiesel yields decreased to 86.71 wt. % and 91.67 wt. %, respectively. This is due to the presence of the hydroxide group that produced soaps by triglyceride saponification. Owing to their polarity, the soaps dissolved into the glycerol phase during the separation stage after the reaction. Since base-catalyzed transesterification proceeds faster than the acid-catalyzed reaction and the fact that the alkaline catalysts are less corrosive than acidic compounds, industrial processes usually favour base catalysts (Freedman *et al.*, 1986; Freedman *et al.*, 1984; Schuchardta *et al.*, 1998; Schwab *et al.*, 1987). The mechanism of the base-catalyzed transesterification of vegetable oils is shown in Figure 2.19. The first step (Eq.1) is the reaction of the base with the alcohol, producing an alkoxide and the protonated catalyst. The nucleophilic attack of the alkoxide at the carbonyl group of the triglyceride generates a tetrahedral intermediate (Eq.2), from which the alkyl ester and the corresponding anion of the diglyceride are formed (Eq.3). The latter deprotonates the catalyst, thus regenerating the active species (Eq.4), which is now able to react with a second molecule of the alcohol, starting another catalytic cycle. Diglycerides and monoglycerides are converted by the same mechanism to a mixture of alkyl esters and glycerol.

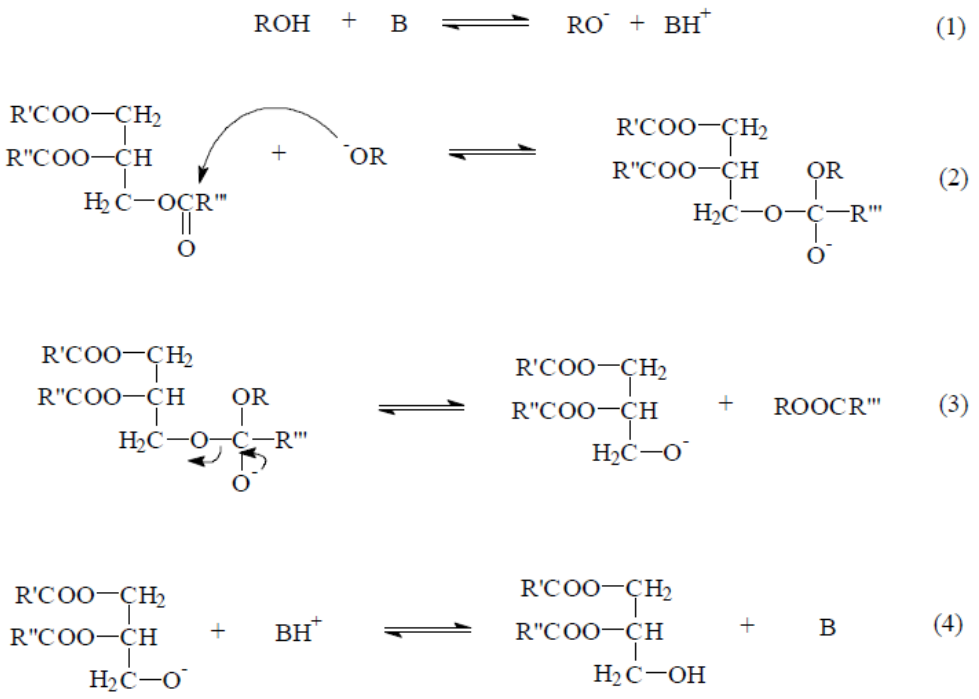
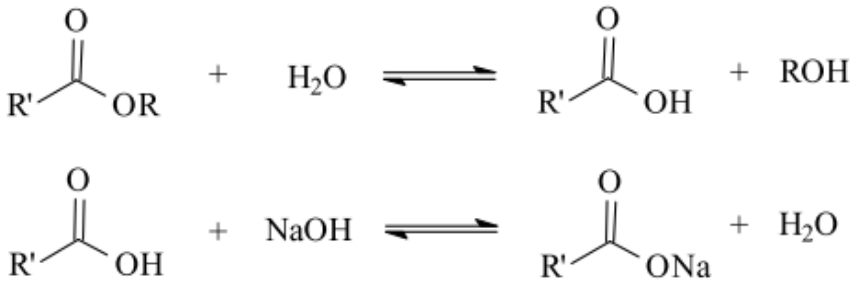


Figure 2.19 Mechanism of the base-catalyzed transesterification of vegetable oils.

Alkaline metal alkoxides (as CH_3ONa for the methanolysis) are the most active catalysts, since they give very high yields (> 98%) in short reaction times (30 min) even if they are applied at low molar concentrations (0.5 mol%). However, they require the absence of water which makes them inappropriate for typical industrial processes. Alkaline metal hydroxides (KOH and NaOH) are cheaper than metal alkoxides, but less active. They are, however, good alternatives since they can give the same high conversions of vegetable oils just by increasing the catalyst concentration to 1 or 2 mol%. If a water-free alcohol/oil mixture is used, some water is produced in the system by the reaction of the hydroxide with the alcohol. The presence of water gives rise to hydrolysis of some of the produced ester, with consequent soap formation (Figure 2.20).



R' = carbon chain of the fatty acid

R = alkyl group of the alcohol

Figure 2.20 Saponification reaction of the fatty acid alkyl esters produced.

The undesirable saponification reaction reduces the ester yields and makes it difficult to recover the glycerol due to the formation of emulsions (Freedman *et al.*, 1984). Potassium carbonate, used in a concentration of 2 or 3 mol% gives high yields of fatty acid alkyl esters and reduces the soap formation. This can be explained by the formation of bicarbonate instead of water (Figure 2.21), which does not hydrolyse the esters (Schuchardta *et al.*, 1998).

R = alkyl group of the alcohol

Figure 2.21 Reaction of potassium carbonate with the alcohol.

For sodium methoxide catalyzed transesterification, 100 g of vegetable oil is transesterified in toluene (80 mL) and methanol (200 mL) containing fresh sodium (0.8 g) in 10 min at reflux. The reaction between sodium methoxide in methanol and a vegetable oil is very rapid. It has been shown that triglycerides can be completely transesterified in 2–5 minutes at room temperature. The methoxide anion is prepared by dissolving the metals in anhydrous methanol. Sodium methoxide (0.5–2 M) in methanol effects transesterification of triglycerides much more rapidly than other transesterification agents. At equivalent molar concentrations with the same triglyceride samples, potassium methoxide effects complete esterification more quickly than does sodium methoxide. Because of the dangers inherent in handling

metallic potassium, which has a very high heat of reaction with methanol, it is preferred to use sodium methoxide in methanol. The reaction is generally slower with alcohols of higher molecular weight. As with acidic catalysis, inert solvents must be added to dissolve the simple lipids before methanolysis will proceed (Christie, 1990).

Some natural glycerides contain higher levels of unsaturated fatty acids. They are liquids at room temperature. Their direct use as biodiesel fuel is precluded by high viscosities. Fats, however, contain more saturated fatty acids. They are solid at room temperature and cannot be used as fuel in a diesel engine in their original form. Because of the problems, such as carbon deposits in the engine, engine durability and lubricating oil contamination, associated with the use of oils and fats as diesel fuels, they must be derivatized to be compatible with existing engines (Ma and Hanna, 1999). The use of raw vegetable oils for diesel engines causes numerous engine-related problems in the form of severe engine deposits, injector coking, and piston rings sticking (Perkins *et al.*, 1991). However, these effects can be reduced or eliminated through transesterification of the vegetable oil to form an alkyl ester. Therefore, biodiesel fuel can be used in diesel engines with little or no modification. Biodiesel contains no aromatics or sulphur, thus reducing the emissions of carbon monoxide (CO), hydrocarbon (HC) and particulate matter (PM) in the exhaust gas compared to diesel fuel (Canakci, 2005).

The use of microwave heating as a tool for preparative chemistry is continuing to grow. By using microwave irradiation it is often possible to reduce reaction times significantly and improve product yields. The preparation of bio-diesel using microwave offers a fast, easy route to this valuable biofuel. It has advantages of a short reaction time, a low oil/ methanol ratio, ease of operation, a drastic reduction in the quantity of by-products, and reduced energy consumption. Table 6 shows the comparison of energy consumption for the preparation of bio-diesel using conventional and microwave heating. Several examples of microwave irradiated transesterification methods have been reported using homogenous alkali catalyst,

acid catalyst and heterogeneous catalyst. Nezihe et al, (2008) reported 93.7% (for 1.0% (w/w) KOH) and 92.2% (for 1.0% (w/w) NaOH) yield of bio-diesel at 313 K temperature within 1 min under microwave heating (Nezihe and Aysegul, 2008). Michael et al., (2007) used continuous-flow microwave methodology for the transesterification and reported that continuous-flow microwave methodology for the transesterification reaction is more energy-efficient than using a conventional heated apparatus (Michael Barnard *et al.*, 2007). Microwave assisted transesterification of castor bean oil was carried out in the presence of methanol or ethanol, using a molar ratio alcohol/castor bean oil of 6:1, and 10% w/w basic alumina (in relation to the oil mass) as catalyst. A 95% conversion was obtained under basic conditions (Al_2O_3/50% KOH) using methanol at conventional (60ºC, stirring, 1 h) or microwave conditions (5 min). Aside from the great advantages of microwave-assisted reactions, there are also a few drawbacks. Microwave synthesis is not easily scalable from laboratory small- scale synthesis to industrial scale production.

Table 2.2 Comparison of energy consumption for the preparation of bio-diesel using conventional and microwave heating

Reaction conditions	Energy consumption (kJ/L) [a]
Conventional heating, [b]	94.3
Microwave, continuous-flow at a 7.2 L/min feedstock flow	26.0
Microwave, continuous-flow at a 2 L/min feedstock flow, [c]	60.3 (92.3) [d]
Microwave heating, 4.6 L batch reaction, [e]	90.1

a Normalized for energy consumed per litre of bio-diesel prepared.
b On the basis of values from the joint US Department of Agriculture and US Department of Energy 1998 study into the life cycle inventory of bio-diesel and petroleum diesel for use in an urban bus.
c Assuming a power consumption of 1700W and a microwave input of 1045 W.
d Assuming a power consumption of 2600W and a microwave input of 1600 W.
e Assuming a power consumption of 1300 W, a microwave input of 800 W, a time to reach 50ºC in 3.5 min, and a hold time at 50ºC in 1 min.

The most significant limitation of the scale up of this technology is the penetration depth of microwave radiation into the absorbing materials, which is only a few centimetres, depending on their dielectric properties. The safety aspect is another reason for not using microwave reactors in industry. Table 2.2 shows the work carried out for bio-diesel production from various feedstock's under varied conditions using microwave irradiation (Vyas *et al.*, 2010).

2.4.4.3 In-situ transesterification

This method involves the simultaneous extraction of lipids and transesterification via catalysis. As an alternative to the conventional process, it is considered to have potential of reducing the processing units and costs of the fuel conversion process. The in-situ process facilitates the conversion of the biomass oil to FAAE directly from the oil bearing biomass, thereby eliminating the solvent extraction step required to obtain the oil feedstock. This biodiesel production scheme could therefore aid in the simplification of the fuel conversion process, potentially reducing the overall process cost, hence lowering the final fuel product costs. This method may be especially advantageous for use with microalgae, since the extraction of microalgae lipids is usually accomplished via solvent extraction and not with the use of cheaper physical extraction methods (for example, expellers) as utilised for conventional oil crops. The alcoholysis of the oil in the biomass directly has been shown to result in increased biodiesel yields, compared to the conventional route. Process wastes and pollution could also be reduced by this method (Ehimen *et al.*, 2010). In-situ transesterification refers to the direct transesterification of lipids in a biomass matrix without prior lipid extraction and offers the advantage of quantifying all fatty acids as fatty acid methyl esters (FAMEs), irrespective of the lipid extraction efficiency. This process is gaining recognition as a lipid measurement procedure for algae (Laurens *et al.*, 2012).

2.5 Quantitation

2.5.1 FTIR

Verifying the FAME content of diesel fuel blends is an important aspect of quality control and auditing of blending and distribution operations. Because FAME has a strong infrared absorption at around 1745 cm^{-1} (due to the ester carbonyl bond), infrared spectroscopy is ideally suited to this analysis (Perston and Harris, 2009).
Infrared spectra of biodiesel blends exhibit a non-linear behaviour (peak shifts) with respect to concentration. This is due to interactions between the components of the mixture. As a result of this, it is impossible to have a single, simple linear model spanning the full range of possible blends. This is a method for whole organism analysis using intact cells which involves the measurement of infrared absorption in relation to a range of molecular vibrational modes. Specific molecular groups can be identified by their absorption bands, allowing macromolecules (including proteins, lipids, carbohydrates, and nucleic acids) to be quantified. A few studies have begun to demonstrate the potential of FTIR as a tool to identify changes in cellular components, including lipids, in response to a nutrient stress, such as low-N and low-P. Two bands were of particular interest, the band at 1740 cm^{-1} which was associated with ν(C=O) of ester groups, primarily from lipids and fatty acids, and the region from 1200– 950 cm^{-1} associated with ν(C–O–C) stretching of polysaccharides (Dean *et al.*, 2010). Preliminary experiments were conducted on selected samples to examine the feasibility of this as a quick qualitative or quantitative method for analysis. Thereafter the method was used for analysis of lipids and biodiesel produced in this study.

2.5.2 Chromatography

Gas chromatography (GC) is a popular method for fatty acids analysis. It has been the method of choice in fatty acid analysis for half a century. Within a few years after the first separations of individual volatile fatty acids performed, GC had become widely adopted as a highly applicable tool in micro-scale analytical work in a number of research areas of fatty acids (Seppänen-Laakso *et al.*, 2002). The method

has several advantages. Some of these are, a high efficiency of separation of components in small quantities of samples (0.1-50 μL), accuracy, reproducibility and speed. For greater volatility, fatty acids analyzed by gas chromatography are usually esterified as a first step. Fatty acids can also be analyzed in non-esterified form i.e. without derivatization (Oveisi *et al.*, 2006).

Gas Chromatography is also a useful tool to identify and quantify individual components in a mixture. Using individual standards and reproducible conditions enables peak identification by retention time. In most cases this is absolute unless there are two peaks with exactly the same retention time under the analysis conditions. This property cannot be applied to quantitation which is affected by numerous variables. Quantitation uses chromatographic data to determine the amount of a given component in a mixture. This data can be in the form of either peak height or peak area which is obtained from an integrated chromatogram. It is very important that this data is gathered accurately. It is best if the peak is totally resolved from any neighbouring peaks. A co-elution or other anomalies such as tailing or fronting will distort or obscure the beginning and ending points of the peak making it difficult to accurately determine the area of the peak. There are several types of quantitation methods commonly used. The five most common are area percent, single point external standard, multiple point external standard, single point internal standard, and multiple point internal standard. Area percent is the simplest quantitation method. This method assumes that the detector responds identically to all compounds. This assumption, however, is not always valid. To calculate area percent, the area of an analyte is divided by the sum of areas for all peaks. This value represents the percentage of an analyte in the sample. (Cole *et al.*, 2008; Lee *et al.*, 2010). In chromatography, the information about the chemical compounds in a mixture is transferred as a sequence of peaks spread along the time axis. The task of signal processing methods is to estimate the intensity of such peaks. The selection of a mathematical method and models should depend on what is known about the signal and noise (Kilg and Smit, 1996). The GC analysis of FAMEs with FID remains the most frequently used. The results from GC/FID are often expressed as a relative

percentage of total fatty acids, which may potentially contribute to error in data interpretation. The accurate quantification of FA's in biological samples depends on proper extraction, methylation of FA's into FAME's, optimized GC run conditions and calculation of their concentration using internal and external standards (Xu *et al.*, 2010). GC can be used to analyze fatty acids either as free fatty acids or as fatty acid methyl esters. Because the differences between cis isomer FAME's and trans isomer FAME's of the same carbon length and degree of unsaturation are very small, very efficient capillary GC columns with highly polar phases are required. The high polarity of the SP-2380 column allows the separation of geometric (cis/trans) isomers as a group. The phase is stabilized, providing a maximum temperature slightly higher than the popular SP-2560 column (Fatty Acid/FAME Application Guide, 2014).

Vegetable oil or animal fat source determines the composition and nature of fatty acids present in triglycerides. The type and concentration of fatty acid has a marked effect on biodiesel stability. This will influence its storage and oxidative properties. Thus, it is important to know the characteristics of the biodiesel being produced. Fatty acids are suitable for separation, quantification and analysis by GC without any derivatization. Gas chromatography is the method of choice for the simultaneous determination of glycerol, mono-, di-, and tri-glycerides in vegetable oil methyl esters. In principle, glycerol, mono-, di-, and tri-glycerides can be analyzed on highly inert columns coated with a polar stationary phases without derivatization. Chromatographic analysis have been used in many ways in quantifying and identifying individual components in biodiesel samples , such as the identification of contaminants and Fatty acids methyl esters. Chromatography is vital in modern quality control analysis of biodiesel hence, its wide application in the study of biodiesel composition. (Yahaya *et al.*, 2013)

Chromatography was identified as the method for primary quantification of fatty acids and biodiesel for this study. Standards and initial GC parameters were selected from manufacturer's specifications (Fatty Acid/FAME Application Guide, 2014;

Shimadzu-Corporation, 2012; Sigma-Aldrich, 1998) for the column and chromatograph used.

2.6 Biodiesel Characterization

Biodiesel, like petroleum diesel, comprises properties that are related to engine performance. A range of quality analyses is necessary to ensure the functionality of the fuel. Regulatory agencies have established standard specifications for various analyses in a many countries. The American biodiesel specification, ASTM D 6751, and the European specification EN 14214, are most commonly applied and utilized as a base for development of new specifications and methods. In some cases, specifications for petroleum diesel are transferred to biodiesel standards. However, due to differences in properties not all methods are suitable for biodiesel analysis and modifications or development of new methods have been necessary (Westberg, 2012). Numerous tests are required for the characterization of biodiesel. Conducting all of these tests would be necessary if biodiesel is produced on a large scale. The test conducted would also depend on the use and application of the biodiesel produced. For lab-scale production, as done in this study, a limited number of tests were conducted to evaluate the quality of biodiesel. These were selected from the standards identified by the American and European specifications.

This study has endeavoured to pursue various technologies applied to algal biodiesel, but diversifies into aspects of certain technologies which have not been investigated. Decisions were made regarding, types of cultures suited to the South African context, sampling methods suited to our environment, extraction technologies, esterification and characterization of biodiesel produced. For the purpose of this research, biomass obtained from indigenous strains of algae was examined. Various solvents have been tested for their extraction efficiency in the extraction of lipids (algal oil) from biomass. All the extraction methods chosen were examined for the feasibility of their application for large scale production and commercialization. A physical method, the expeller press, was also examined for the extraction of algal oils from biomass. Since

esterification methods abound in chemistry, some methods chosen were optimized for the conversion of the lipids to biodiesel. Chromatographic (GC) methods were used to separate, identify and quantify the lipids and biodiesel produced at laboratory scale. Prior research shows evidence of the use of solvents for the extraction of algal lipids, but not to the extent and detail provided by this study. The technologies optimized for the extraction and production of biodiesel are unique to this study.

CHAPTER 3

EVALUATION, SELECTION AND OPTIMIZATION OF TECHNIQUES FOR EXTRACTION OF LIPIDS FROM MICROALGAE.

3.1 Introduction

This chapter deals with the evaluation, selection and optimization of techniques used for the extraction of lipids from algal biomass. Methods that were proposed for this study were chosen with a view to examine both the cost and efficiency of the extraction methods. The first priority was to ensure that samples and sampling methods were adequate for the analyses planned. Harvesting of algal biomass was conducted using gravity sedimentation followed by centrifugation as cited in Rawat et al (Rawat *et al.*, 2013). Thereafter lipid extraction methods were examined. A range of both physical and chemical methods are available for extraction of lipids from algal biomass. Examples of physical methods are: expeller press, ultrasonic assisted extraction (UAE), and bead beating. Chemicals methods include solvent extraction by the separating funnel method, the soxhlet extraction method, microwave assisted extraction (MAE), supercritical fluid extraction (SFE), accelerated solvent extraction (Smedes and Thomasen, 1996), and other less well-known methods are enzymatic extraction, osmotic shock, pressurized fluid extraction (PFE) and hydrothermal methods. Since it was not feasible to examine all the extraction methods listed, the expeller press, the separating funnel, the soxhlet extraction and microwave assisted extraction methods were chosen for this study, as equipment for these methods was readily available.

The Büchi B 811 Soxhlet extraction system offers four modes of extraction, viz., Soxhlet Standard, Soxhlet warm, Hot extraction and Continuous flow. Initial trials using the Standard method and Continuous flow method were conducted as these used the least amount of energy in comparison to the other methods. The Continuous flow method was chosen as it proved more efficient than the Standard method for the samples analyzed (See Figure 3.1).

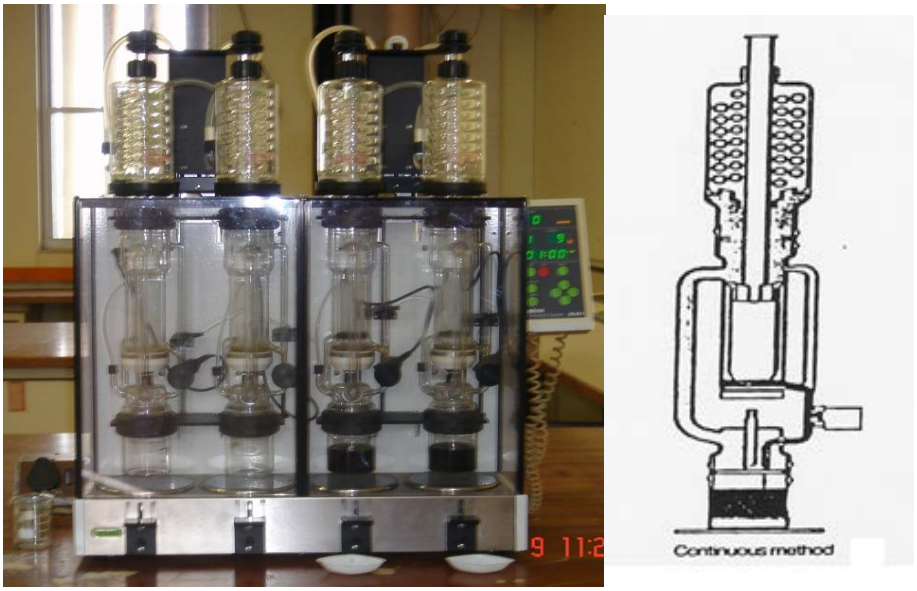


Figure 3.1 The Büchi Soxhlet extractor on the left with a cut-away diagram illustrating the continuous mode of extraction.

The Continuous mode involves the following (See Appendix A):

(a) The solvent evaporates, rises up to the condenser and condenses. The glass valve is-open, the optical sensor is inactive.

(b) When the programmed extraction time expired, the system goes to the next step.

In the Soxhlet method, the solvent beaker is heated and as the solvent evaporates it moves up into the condenser where it is converted into a liquid that trickles into the extraction chamber containing the sample in the sample tube. Eventually, the solvent builds up in the extraction chamber and completely surrounds the sample. As the solvent passes through, the sample extraction of the lipids is effected. The extraction

chamber is designed so that when the solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling solvent beaker. The lipid extract then remains in the beaker because of its lower volatility. The solvent continues to volatilize and recondense into the extraction chamber producing a continuous extraction process. At the end of the extraction process the beaker containing the solvent and lipid extract is removed. The solvent was evaporated and the mass of lipid extract remaining was measured (McClements, 2005).

Figure 3.2 shows the Microwave accelerated reaction system, (MARS 5, CEM Corporation, USA) for extraction (MARS Operation Manual, 2006). It incorporates the use of inert Teflon tubes of maximum volume of 70 mL with 5 mL ullage for expansion of liquids. The extraction vessels (teflon tubes) are placed diametrically opposed into sleeves within a carousel for balanced and even heating during analysis.

Figure 3.2 Microwave Accelerated Reaction System (MARS5) extractor with teflon sample tubes and carousel illustrated.

The main purpose of this section was to determine the optimum for the experimental parameters used. These parameters would then be applied to test the efficiency of MAE. The basis of the method made it possible to optimize four parameters, viz.,

volume of solvent, time of extraction, mass of algal biomass and temperature of measurement. The three solvents were chosen on the basis of their extraction efficiencies shown with the soxhlet method. The optimizing of the binary solvent ratios was dependent on the optimized parameters obtained for each of the individual parameters above. The sequence of optimization of the parameters was random. Each parameter had its own determinants and was restricted by the instrument's limitations. The volume of solvents had six variants from 10 to 60 mL with a 10 mL increment for each set of extractions. Volumes less than 10 mL did not cover the weighed biomass adequately to perform an efficient extraction, hence volumes below 10 mL were not tested. Volumes greater than 60 mL could not be used as they provided no ullage for the maximum tube volume of 75 mL. It was essential to leave a space for expansion of the solvent and a vapour phase interaction during the heating process. Time was varied from 5 minutes to 60 minutes based on suggestions from the MARS5 manual and some authors (Eskilsson and Bjorklund, 2000). Mass was limited to between 0.5 and 10 g, as masses greater than 10 g prevented the efficient and complete extraction of lipids. Temperature was varied from ambient (~25℃) to 200℃.

The microwave method is a relatively recent and novel method for the extraction of lipids from algal biomass. A collective study of algal biomass incorporating the optimization of parameters like mass, volume, temperature and time has not been performed using this method as shown by a search of the relevant literature. Traditional methods for experimentation involve a one factor at a time (OFAT) method. Two common methods used as alternatives to statistical experimental design are OFAT and matrix experimentation (Anderson, 2013). Each of these can be used for problem solving, but they are not considered economical or efficient. The OFAT approach is to fix all of the variables except one and then study the behaviour of the system at several levels of that variable. For each variable the best value is found, and the process repeated until all the variables have been considered. Although this method may be effective in some situations, it may also be inefficient. It may involve too many experiments to arrive at a conclusion. The second traditional approach to

empirical problem solving is to lay out a matrix of all the interesting combinations of the variables being investigated. All the combinations in the matrix are investigated until the solution is found. Although this method has the advantage of thoroughly exploring the experimental space, it requires an unnecessarily large number of measurements which can be time consuming (Haaland, 1989). Response surface methodology is a design method that shows correlations between one or more measured dependent responses with a number of independent factors. The process is designed to identify the optimum values of continuous variables and to maximize or minimize a given response by sampling a minimum number of discrete points (Burrows *et al.*, 2008). The statistical problem solving approach uses a series of small, meticulously designed experiments. Each experiment carefully explores the experimental space while studying many variables using a small number of observations. This statistical design method is thus efficient and effective. This, therefore, led to the application of this strategy to a three factor system for the extraction of lipids in algal biomass.

Solvents for extraction were chosen on the basis of their affinity for lipids from algal biomass and of their polarities. Solvents, which are known carcinogens and those that having extremely high toxicity and are generally unsafe to handle, were avoided. The hypothesis was that all or most solvents were able to extract lipids, but the primary choice of a solvent would be determined by its ability to achieve an optimum extraction of lipids within the parameters chosen. The range of solvents chosen for this study is given in Table 3.1. This range was chosen randomly, but after studying the reports in literature. In the light of searches conducted, it can be said that there is no evidence of a single study that has examined the range of solvents selected for the extraction of algal lipids for the indigenous species.

Table 3.1 Solvents used for extractions showing their relevant properties in order of increasing polarity index

	Solvent	Polarity Index	Boiling point	Density @ 25°C	Formula	Molar mass
		Units	°C	g/mL		g/mol
1	Petroleum Ether	0.1	35.0 - 60.0	0.640	multiple	
2	Hexane	0.1	69.0	0.659	C_6H_{14}	86.20
3	Cyclohexane	0.2	80.7	0.779	C_6H_{12}	84.16
4	Isooctane	0.4	99.2	0.690	C_8H_{18}	114.23
5	Toluene	2.4	110.0 - 111.0	0.865	$C_6H_5CH_3$	92.14
6	Benzene	2.7	80.0	0.874	C_6H_6	78.11
7	Diethyl ether	2.8	34.6	0.706	$(CH_3CH_2)_2O$	74.12
8	Dichloromethane	3.1	39.8 - 40.0	1.325	CH_2Cl_2	84.93
9	Isopropanol	3.9	82.0	0.785	$CH_3CH(OH)CH_3$	60.11
10	Chloroform	4.1	60.5 - 61.5	1.492	$CHCl_3$	119.38
11	Acetone	5.1	56.0	0.791	CH_3COCH_3	58.08
12	Methanol	5.1	64.7	0.792	CH_3OH	32.04
13	Ethanol	5.2	78.0	0.789	C_2H_5OH	46.07
	Water (For comparison only)	10.2	100.0	1.000		

(*Aldrich Handbook of fine chemicals*, 2009-2010).

In addition to the above, a range of other analyses were performed involving the extraction protocols observed with the extraction methods.

They were:

- **Chlorophyll analysis**

Chlorophyll is an integral part of algae. A characteristic algal pigment contains approximately 1−2% dry weight of planktonic algal mass (*ESS Method 150.1: Chlorophyll - Spectrophotometric*, 1991). However, little is known about the actual

effects of chlorophyll on the production of biodiesel from algal biomass. Furthermore, it is considered as a contaminant. It was therefore decided that knowledge of the quantity of chlorophyll in the algal biomass analyzed would be relevant in the determination of lipids with greater accuracy. This prompted the determination of chlorophyll present in the species studied.

- **Freeze-dried versus oven-dried biomass**

The method of drying of biomass prior to the application of extraction protocols became a contentious issue within the laboratory. It was therefore decided that the feasibility of both the freeze drying method and oven drying method would be tested. The only difference between the two methods was that the freeze dried method produced granular powder after the freeze drying process, while the oven dried method produced flakes which had to be pulverized.

- **Ageing of biomass**

Since no evidence was available with regard to the duration, one can continue to extract biomass without losing extraction efficiency. It was decided to conduct an investigation involving the ageing of biomass. This was done by retaining the biomass at room temperature and conducting extraction using an optimized method on a weekly basis for a duration of approximately 3 months.

- **Comparison of the optimized method with the Bligh and Dyer method**

The Bligh and Dyer method is probably the most referenced method for the determination of lipids from algal biomass (Smedes and Thomasen, 1996). This, therefore, motivated a comparative study of the Bligh and Dyer method with the optimized solvent extraction method determined by this study. Statistical evaluations were conducted where necessary.

3.2 Materials and Methods

3.2.1 Sampling and sample preparation

Samples for analysis were obtained as dried biomass. Since the ambit of this study does not include the growing, harvesting and drying of biomass, these processes will be described briefly. This function was performed in a separate laboratory. The algal biomass used for this study was obtained from a laboratory raceway pond of approximately 3 kL capacity designed specifically for the cultivation of algae. Algae samples were also taken from a 300 kL raceway pond south of Durban. The propagation of the algae was carried out using BG-11 medium, modified with controlled carbon dioxide sequestration (Stein, 1973). The biomass used for analytical work consisted of a mixed algal culture predominantly containing the chlorella vulgaris *sp*. and Scenedesmus *sp*. which are indigenous to KwaZulu Natal in South Africa. Pre-screening of the algal biomass for the presence of lipids was conducted using the Nile Red staining method (Chen *et al.*, 2009; Fowler *et al.*, 1987).

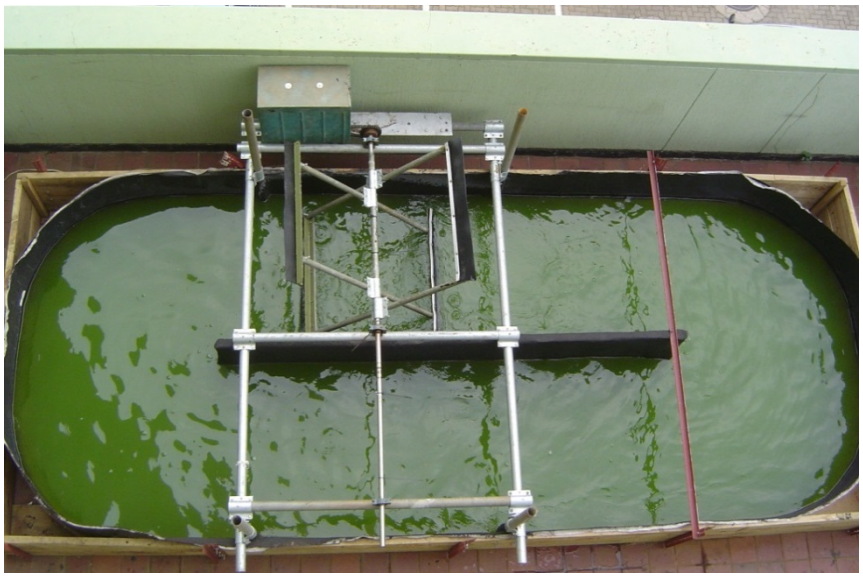


Figure 3.3 A typical raceway pond from which samples of algae were taken.

Algae, as wet biomass, was harvested from the 300 kL pond settling tank in 20 L buckets and transported to the laboratory, while algae samples from the 3kL raceway pond had samples removed manually though decanting into 20 L buckets for

transport to the laboratory. At the laboratory 1L aliquots were transferred to centrifuge tubes and centrifuged at 6000 rpm for approximately 10 minutes. Thereafter the supernatant was decanted and the biomass placed on aluminized drying pans. This was then placed in an oven set at 60°C ± 5°C for approximately 24 hours. The dried biomass flakes obtained were then pulverized using a standard stainless steel laboratory grinder until the flakes were few microns in diameter. This would produce a larger surface area for the lipid extraction processes used. The algal biomass in powdered form was used for all the extractions methods performed. The amount of algae obtained from this process was approximately 100 g. For the entire course of this study six samples were collected in this manner for analysis.

An important point to remember here is that all analysis conducted for the extraction experiments made use of biomass within a month of harvesting, except for those which were used for the ageing trials. It was assumed that in the absence of any data regarding the ageing of biomass and its subsequent lipid behaviour, the hypothesis was made that biomass would at least produce the same lipid quantities for a maximum shelf-life of one month. Furthermore, each batch of harvested biomass did not produce the same lipid quantities as this was dependent on its treatment and the dynamics of the raceway pond. It is for this reason variations in actual lipid quantities will be found in analyses performed. However, once again the assumption was made that the behaviour of all solvents towards lipids would be consistent during extraction procedures applied.

3.2.2 Separating funnel extraction method

Figure 3.4 The separating funnel extract showing supernatant with extract in suspension upon settling of biomass at the right (close-up view).

For this method, 5 g of biomass was weighed and transferred quantitatively to a separating funnel. All weighing was carried out on a calibrated analytical balance (Boeco, Germany). All analysis was carried out in duplicate. An aliquot of solvent was then added to the separating funnel. The separating funnel was stoppered and inverted a few times after which the vapour was vented with the tap facing upwards to relieve pressure built up in the flask owing to agitation. The flask was shaken continuously for 3 minutes, with venting of the solvent vapour pressure carried out every 30 seconds. After 3 minutes of extraction, the separating funnel was placed on a retort stand for 10 minutes to allow the extract to settle (Figure 3.4). Thereafter the solvent was drained into a pre-weighed 250 mL round bottom flask via a sintered glass funnel with porosity 3 (15 – 40 µm pore size). This procedure was repeated 3 times with the same volume of solvent being used each time. The funnel was rinsed 3 times using 5 mL of solvent for each rinse to remove residual extract from the funnel. A maximum of 25 mL was used for this rinsing process so that a total final volume of the extract was 100 mL. The solvent was then evaporated using a rotary vacuum evaporator (BÜCHI Universal Extraction System, 1996) with the water bath set at 70°C ± 2°C. The flask was then placed in a desiccator. After the flask was

cooled to room temperature, it was reweighed and the mass of the lipid extract was determined as percent dry weight (% dwt).

The range of solvents used for this experiment appears on Table 3.1. All solvents used were of HPLC grade with an assay ≥ 99.5% (Sigma Aldrich, USA). For this method ten solvents were initially selected; the excluded solvents were cyclohexane, isooctane and isopropanol which were included when conducting the soxhlet extraction experiment.

3.2.3 Büchi universal extraction soxhlet method

3.2.3.1 Single-solvent extractions

A Büchi B 811 Soxhlet (Labortechnik, B. (1996) BÜCHI Universal Extraction System, Switzerland) apparatus was used for the extraction of lipids (BÜCHI Universal Extraction System, 1996). All solvents used were of HPLC grade with purity ≥ 99.5% (Sigma Aldrich, USA). The solvent from each sample extract was evaporated using a rotary vacuum evaporator (BÜCHI Universal Extraction System, 1996). All weighing was carried out on a calibrated analytical balance (Boeco, Germany).

The analyses were performed as follows:

The procedure was conducted in triplicate for each solvent. The thirteen solvents used for extraction are shown on Table 3.1. One gram sample of pre-dried and pulverised biomass (microalgae) sample was weighed accurately into a Soxhlet glass sample tube. The sample tube was transferred to the extraction chamber in the Soxhlet apparatus. The extraction solvent (100 mL) was transferred into the solvent beaker and placed on the heating plates. The cooling water supply to the condensers was opened to ensure continuous recondensation and recycling of the solvent. The temperature was selected as per the Büchi manual for extraction in the Continuous mode (BÜCHI Universal Extraction System, 1996). Boiling point temperatures were programmed using boiling points of the solvents shown in Table 3.1. The extractions were conducted for three hours. On completion of the extraction process, the samples

were left to cool for at least 15 minutes after which the solvent beakers with the lipid extracts were removed and transferred quantitatively to pre-weighed 250 mL round bottom flasks. The solvent was evaporated using a rotary evaporator under vacuum. The flasks were then transferred to a desiccator until cool and then reweighed. The optimum conditions for temperature, solvent volume and mass used for the Soxhlet extraction were dictated by the manufacturer's manual for laboratory scale applications (BÜCHI Universal Extraction System, 1996).

3.2.3.2 Optimization of extraction time using selected solvents

From the multisolvent extractions, three solvents, viz. chloroform, ethanol and hexane were selected, as their lipid extraction efficiencies were overall much higher than those of the other solvents used for the extractions. These solvents were then used to determine their lipid extraction efficiencies by varying the time of extraction. The extractions were conducted with each solvent, in triplicate, at one-hour intervals from one to five hours. The extraction protocols used were the same as those used for the single solvent extractions. The Büchi method (BÜCHI Universal Extraction System, 1996) recommends 2 to 4 hours as a general extraction time for fats. However, since no optimum has been established for lipids, this experiment was used to establish an optimum extraction time.

3.2.3.3 Optimization of extraction using binary ratios of selected solvents

The Bligh and Dyer method (Smedes and Thomasen, 1996) and modifications show how the use of two solvents can be more efficient at extraction of lipids than a single solvent. Since lipid profiles vary from polar to non-polar, binary solvent mixtures are promoted as being better than single solvents. In view of this, this experiment uses chloroform, ethanol and hexane binary ratios for extraction of lipids as they appear in Table 3.7. The use of more than 2 solvent combinations was avoided as this would begin to present problems with miscibility, cost and safe handling for large scale applications. It was also decided to limit the binary mixture ratios to a maximum of 3:1/1:3. Extraction protocols were the same as those used for single solvent extractions. For each binary mixture in Table 3.7, the solvents were mixed

first, homogenized and then 100 mL volumes were used for triplicate analysis. The only difference from the single solvent extraction was that the heating rate for the soxhlet apparatus was determined using the average boiling points of the two solvents used (BÜCHI Universal Extraction System, 1996).

3.2.4 Microwave assisted extraction (MAE)

3.2.4.1 Experimental Procedure

The standard operating procedure (SOP) from the MARS Operation Manual was used to determine the level of variation for each parameter that would be used in this experiment. The process involved maintaining three parameters fixed while varying the fourth. On establishing the optimum for the varied parameter, this optimum was then used as a fixed value while establishing the next optimum parameter. This pattern was continued until all parameters were optimized. The volume of solvent used for the extractions was selected as the first parameter to be varied as it was the only parameter determined by the availability and cost of solvents.

3.2.4.2 Optimization of extraction volume

The following procedure was used in optimizing volume:

Pulverized algal biomass (0.5 g) was weighed accurately into a teflon tube. This average mass was weighed into nine tubes. The first incremental volume used was 10 mL. Since three solvents were used and triplicate analyses were performed, 10 mL of chloroform was transferred quantitatively into the first three tubes. Using the same procedure, ethanol was transferred to the next three tubes and hexane to the final three tubes. The teflon tubes were sealed using the stoppers and screw caps provided. The teflon tubes (extraction vessels) were then transferred into sleeves on the carousel. The carousel was then placed in the microwave oven. The microwave power was set to 1600 W. The temperature was kept at ambient (± 25ºC and the time was set to an initial 5 minute ramp with 5 minute hold time and a final 5 minute cooling time as required by the instrument program. On completion of the extraction process, the carousel was removed from the microwave. The lipid extract from each extraction vessel was then transferred to a clean Buchner flask via a sintered glass

funnel of porosity 3 using vacuum to speed up the process. The teflon tubes and funnels were rinsed a minimum of 3 times with small volumes (~ 10-20 mL) of extraction solvent to ensure that all the extract was transferred to the Buchner flask. The contents of the Buchner were then quantitatively transferred to a pre-weighed 250 mL round bottom flask. The solvent was then removed using a vacuum rotary evaporator with the water bath temperature set at 70°C ± 5°C. The flask was then removed and placed in a desiccator to cool to ambient temperature. Thereafter the flasks were reweighed and the percent mass dry weight for lipids was determined. This procedure was repeated using 10 mL increments from 10 mL to 50 mL (Table 3.8).

3.2.4.3 Optimization of extraction time

This part of the experiment involved varying time with the optimized volume kept fixed while the other parameters were set as per the standard operating procedure (SOP). Initial ramp time and cooling time was maintained at 5 minutes throughout all experiments. The hold time (actual extraction time) for this optimization experiment was varied from 5 minutes to 20 minutes at 5 minute intervals, thereafter to 60 minutes at 10 minute intervals (Table 3.9). The remainder of the procedure was conducted as stated under volume optimization.

3.2.4.4 Optimization of extraction mass

For this parameter, the optimized parameters for volume and time were fixed while the temperature was kept at ambient. Mass was varied from 0.5 g to 10 g. The actual masses tested were, 0.5 g, 1.0 g, 2.0 g, 5.0 g and 10.0 g. The rest of the procedure was the same as conducted under the volume optimization.

3.2.4.5 Optimization of extraction temperature

In this experiment, the optimized parameters for volume, time and mass were fixed to the optima determined previously. The temperature was varied from ambient (~25°C) up to a maximum of 200°C at 25°C intervals starting from 25°C to a maximum of 200°C. The temperatures used for this experiment are shown in

Table 3.11.

3.2.4.6 Extractions using binary mixtures of solvents

Extraction of lipids was also conducted using the three selected solvents in binary ratios as done for the soxhlet extraction method. The ratios used and the solvent variations appear on Table 3.12. This experiment was carried out after optimizations with the single solvents were completed. The modified method was as follows:

An average mass (1.0 g) of pulverized algal biomass was weighed accurately into each of the nine teflon tubes. Triplicate analyses were performed for each binary mixture. A volume of 30 mL each of the binary solvent mixture was transferred quantitatively into the teflon tubes. The first set of triplicate analysis contained the first binary mixture (chloroform: ethanol, 1:1), the second binary solvent mixture (chloroform: hexane, 1:1), was transferred to the next three tubes and a third binary solvent mixture (ethanol: hexane, 1:1), was transferred to the final three tubes. The teflon tubes were sealed using the stoppers and screw caps provided. The teflon tubes were then transferred into sleeves on the carousel. This was then placed in the microwave oven. The microwave power was set to 1600 W. The temperature was set at 100°C and the time was set to an initial 5 minute ramp with 10 minute hold time and a final 5 minute cooling time as required by the instrument program. On completion of the extraction process, the carousel was removed from the microwave. The lipid extract from each extraction vessel was then transferred to a clean Buchner flask via a sintered glass funnel of porosity 3 using vacuum to speed up the process. The teflon tubes and funnels were rinsed a minimum of 3 times with small volumes (~10-20 mL) of extraction solvent mixture to ensure that all the extract was transferred to the Buchner flask. The contents of the Buchner were then quantitatively transferred to a preweighed 250 mL round bottom flask. The solvents were then removed using a vacuum rotavapour. The flask was then removed and placed in a desiccator to cool to ambient temperature. Thereafter the flasks were reweighed and the percent mass dry weight for lipids was determined. This procedure was repeated for each set of binary ratios of solvents until the five sets were complete (Table 3.12).

3.2.4.7 Experimental design for the optimization of parameters

The determination of individual optimum parameters for any analysis is a complex process. Design of experiment (DOE) are statistical experimental methods that allow use of reduced experimental numbers in order to extrapolate optimum values for parameters tested. This may increase the probability of obtaining far more accurate results for any of the parameters used in a more efficient manner than the OFAT approach. A mathematical model describing data was fitted using multiple regressions. From previous OFAT experiments that showed possible important factors, the three factors chosen were time (X_1), mass (X_2) and volume (X_3). Response surface methodology (RSM), based on central composite experimental designs (CCD), was applied to optimize lipid extracts using point prediction solutions generated from quadratic equations. This was generated to describe the lipid extracts in the central composite design.

3.2.4.8 Response surface experiment to optimize important factors for microwave extraction of lipids

The three important factors identified in previous experiments (OFAT) were time, mass of algae and volume of extractant. These showed an interaction, hence a central-composite design (CCD) with six face centred points was designed. In addition to the low, high and centre points, two additional points at $\alpha = \pm 1.68179$ were included. A graphical representation of the design space is shown in Figure 3.5. All levels of the experimental design and factors used are indicated in Table 3.2.

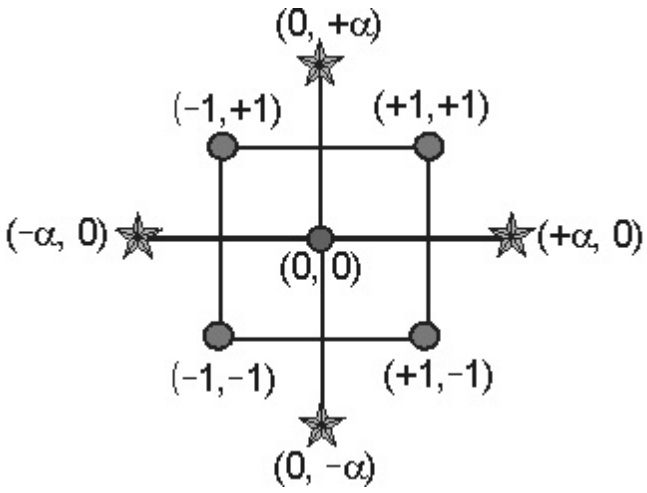


Figure 3.5 Representation of a central composite design for two factors showing six face-centred points around a replicated centre point. The four corners of the square represent the factorial (+/- 1) design points and the four star points represent the axial (+/-α) design points (*Stat-Ease*, 2014).

The levels of all three factors used to extract lipid from algae using MAE are indicated in Table 3.2. These comprise time of extraction, mass of algae and volume of binary solvent mixture (Chloroform/Ethanol, in the ratio 1:1).

Table 3.2 Concentrations of variables in the central composite experiment indicating low, high and centre points along with alpha values (α =±1.68179)

		Level				
Factor	**Symbol**	**-α**	**-1**	**0**	**+1**	**+α**
Time (min)	X_1	1.59	5.00	10.00	15.00	18.41
Mass (g)	X_2	-0.01	0.50	1.25	2.00	2.51
Volume (ml)	X_3	13.18	20.00	30.00	40.00	46.82

Experiments were conducted in 75 ml teflon tubes. The dried algal biomass was weighed accurately into a clean and dry tube, after which the required volume of the binary mixture was added. The tube was sealed and transferred to the microwave

carousel. The carousel was then transferred to the microwave and the program set as required by the factors determined in Table 3.3. Temperature was kept constant at 100ºC. The runs were conducted in one block and randomized to prevent experimenter bias. The run order indicates the random order in which each run was carried out. Standard order sorts the experiment into 8 runs, six centre points and six alpha values. The random experimental design is presented in Table 3.3.

Table 3.3 Experimental design of a three-factor central-composite experiment showing coded factor levels with randomized run order to determine effect on lipid extraction by three factors during microwave digestion

		Factor and coded levels		
Std Order	**Run Order**	***Time*** X_1	***Mass*** X_2	***Volume*** X_3
16	1	0	0	0
10	2	1.681793	0	0
5	3	-1	-1	1
20	4	0	0	0
17	5	0	0	0
2	6	1	-1	-1
12	7	0	1.681793	0
18	8	0	0	0
1	9	-1	-1	-1
4	10	1	1	-1
15	11	0	0	0
7	12	-1	1	1
14	13	0	0	1.681793
3	14	-1	1	-1
9	15	-1.68179	0	0
8	16	1	1	1
11	17	0	-1.68179	0
6	18	1	-1	1
13	19	0	0	-1.68179
19	20	0	0	0

The model fitted to the data above was a second-order polynomial function in the general form given below:

$$Y = c_0 + \sum_{i=1}^{n} a_i x_i + \sum_{j \leq i}^{n} b_{ij} x_i x_j \qquad (3.1)$$

where Y is the predicted percentage lipid, subscripts i and j take the number 1 to the number of variables (n=2), c_0 is the intercept value, a_i and b_{ij} are the linear and quadratic coefficients, and x_i and x_j are the levels of the independent variables. All designs were constructed and analysed on the platform Design Expert 8.0.7.1 (*Stat-Ease*, 2014).

An analysis of variance (ANOVA) was used to determine the goodness-of-fit of experimental data to the model. ANOVA is a statistical method based on the *F*-test that assesses the significance of the experimental results. It is used to evaluate the adequacy of the fitted model. The *p*-values were used as a tool to check the significance of each of the coefficients. This is essential to understand the pattern of the mutual interactions between the factors. The *F*-test with a low *p*-value for response indicates a high significance for the corresponding coefficient and/or regression model. *P*-values less than 0.05 indicate that the model terms are significant, whereas values greater than 0.1 are not significant (Boddy and Smith, 2009). A quadratic polynomial equation was generated to describe the data based on the model. Three-dimensional and contour graphs were used to illustrate the interactions between the factors used. A point-prediction was made to maximize the amount of lipid extracted while optimizing the three factors tested.

3.2.5 Expeller press

Figure 3.6 Hand operated oil expeller.

The expeller press (Figure 3.6), is an instrument that applies a physical method for the extraction of lipids. This instrument has been tested with kernels which are generally presented as solid seeds with high oil content. It has, however, not been tested on algal biomass. For this experiment, dried uncrushed biomass was added to the funnel of the expeller. The oil burner was switched on and the manual rotation of the arm for crushing was started. Waste material was collected separately. The method proved to be inadequate for this type of sample, as no oil could be extracted. This was probably due to the low oil content of algal biomass. To mimic oil bearing seeds, the decision was made to investigate the possibility of pelletizing of the algal biomass prior to introducing the samples to the expeller. However, pelletizing of algal biomass was not possible owing to the lack of proper equipment. The method was discontinued owing to its inability to break up algal cells effectively under the conditions applied.

3.2.6 Other analyses

3.2.6.1 Chlorophyll analysis

Solvent extraction is used as a general method to extract lipids, but it is also non-discriminatory i.e. it extracts other compounds present in algal biomass which have an affinity for the solvent in addition to the lipids. The second most proliferous compound in algal biomass is chlorophyll, which is part of the photosynthesis process that gives algae its characteristic green colour. Although it does not seem to have any influence on the production of biodiesel, it is necessary to quantify chlorophyll to obtain a more accurate quantitation of the lipid yields.

- **UV method**

Chlorophyll extraction was conducted by the same method used for lipid extraction. The total chlorophyll content was determined by UV spectroscopy. Three calibration standards, i.e. 1, 5 and 10 ppm were prepared by dilutions from a 100 ppm standard. used for calibration of the instrument at wavelengths scanned from 300 nm to 700 nm. The specific wavelengths for chlorophyll *a* and chlorophyll *b* were selected from the optima obtained from each scan. The average wavelength obtained for each chlorophyll standard was used for determination of the amount of chlorophyll in the samples. Standards were diluted in HPLC grade acetone prior to analysis. Chlorophyll analysis was conducted using the Cary 50 UV-Vis spectrophotometer (Varian, Australia). Chlorophyll analysis was conducted on both single solvents and binary mixtures of selected solvents used in the extraction of lipids from biomass.

- **Calculation of percentage of lipids and chlorophyll**

The mass of the total lipid extract was determined from the difference in the flask mass before and after extraction. The value was expressed as a percentage of the original mass of biomass weighed. The exact amount of the lipid extract was determined after subtraction of the chlorophyll content. The chlorophyll content was obtained from the standard calibration graphs for chlorophyll *a* and *b* and expressed as a percentage of the lipid extract obtained for each solvent or binary solvent mixture.

The quantity of lipid extract in the sample was calculated as follows:

$$\% \text{ lipids} = (M_L \times 100) / M_B - C \text{ where,}$$

M_L = Mass of lipid extract

M_B = Mass of biomass weighed

C = Chlorophyll content

3.2.6.2 Freeze-dried biomass versus oven-dried biomass

Freeze-dried and oven-dried samples were prepared for solvent extractions using the optimized binary solvent mixture, chloroform: ethanol, 1:1. As described previously, 1 g each of the freeze-dried and oven-dried samples was weighed into teflon vessels and 30 mL of the binary mixture added. Triplicate analysis was performed in each case. The vessels were then placed in the carousel and transferred to the microwave oven. The optimized conditions determined for extraction were used, viz.

Initial time	5 minutes
Extraction time	10 minutes
Final hold time (cooling)	5 minutes
Temperature of measurement	100°C

The final quantity of the lipid extract was determined using the parameters optimized in section 3.2.4.

3.2.6.3 Ageing of biomass

This experiment was conducted with a view to examine the shelf life of biomass. The samples were analyzed on a weekly basis using the same protocol, ensuring reproducibility, for approximately 3 months. The algal biomass sample was retained in a closed container at room temperature for the full duration of the analysis. Triplicate analysis of the sample was performed on the same day of each week using the optimized parameters determined and 1:1 chloroform: ethanol binary mixture.

3.2.6.4 Comparison of optimized solvent extraction system for MAE with Bligh & Dyer method.

A comparative study was conducted using the optimized chloroform: ethanol (1:1) and a modified Bligh & Dyer extraction method using chloroform and methanol mixture (1:1). This experiment was used to determine the efficiency of the optimized method versus the solvent system used by Bligh and Dyer. The modification involved using the solvent systems with the microwave extraction method. Mass, time, temperature and volume were used as determined by the optimization of these parameters. The extraction efficiency was based on the quantity of lipid obtained per gram of algal biomass expressed as a percentage, i.e.

$$\text{Extraction efficiency (expressed as \% m/m)} = \frac{\text{Mass of lipid extract}}{\text{Mass of algal biomass}} \times 100$$

The same method was applied for all analyses where efficiency of extraction are expressed.

3.3 RESULTS AND DISCUSSION

3.3.1 Separating funnel technique

This technique of separation has been effective in partitioning or separating compounds as required. Its primary application in this project was to determine whether it was possible to extract lipids and to check the efficiency of these extractions using the selected solvents (Table 3.4). Since all the extractions were performed at room temperature and manual input was required for the extractions, there was no guarantee that the results would be consistent over time. The method served as a good guideline for the extraction behaviour of the solvents. The method is also labour intensive and would not be feasible for upscaling. However, when compared to the more sophisticated soxhlet or microwave methods, some common ground can be observed for the extractions with chloroform, ethanol and methanol. The results for hexane do not compare favourably with the soxhlet method (i.e. 10.04% lipid extract by the soxhlet method versus 0.32% by the separating funnel method). The better efficiency for hexane extraction by the soxhlet method may be

partly due to the soxhlet method being conducted at the boiling point of hexane, i.e. 69ºC while the separating funnel extraction was done at room temperature (Figure 3.7).

Table 3.4 Results of algal lipid extraction using a range of solvents by the separating funnel (SF) method

No.	Solvent	% Lipid extracted
1	Ethanol	11.34
2	Methanol	6.02
3	Chloroform	5.90
4	Petroleum Ether	3.32
5	Acetone	2.40
6	Diethyl ether	1.54
7	Dichloromethane	0.67
8	Benzene	0.59
9	Hexane	0.32
10	Toluene	0.15

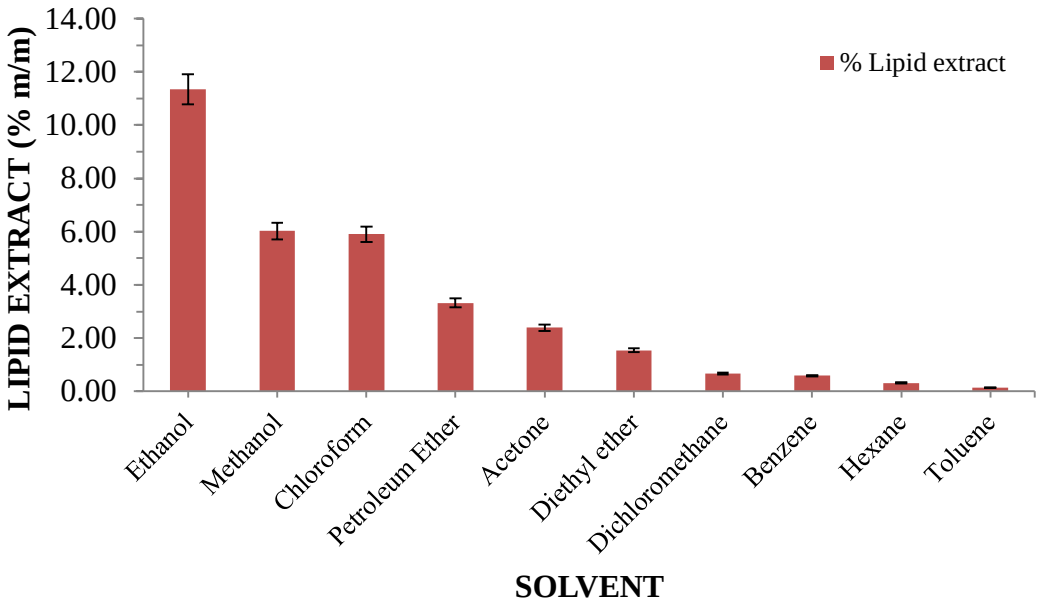


Figure 3.7 Graphical representation of % lipid extracted by various solvents using the separating funnel technique.

3.3.2 Büchi universal extraction soxhlet method

The results in Table 3.5 show the average of triplicate analyses conducted with each solvent. Figure 3.8 illustrates the amount of the lipid extracted in relation to the amount of chlorophyll for each solvent. The relative error for each solvent is also shown. The highest values of the lipid extracts i.e. those above 10% was achieved by extraction with chloroform, ethanol, and hexane. The remaining solvents gave lipid extracts between 2% and 10%. Although the toluene extract produced a lipid extract close to 10% it was not selected for further experimentation owing to its higher level of toxicity when compared with the other solvents. Since chloroform, ethanol and hexane each proved to have the highest efficiencies for the extraction of lipids from the biomass, it may be concluded that a range of lipids varying from polar to non-polar were present in the algal biomass. It was also significant to note that acetone was the solvent with the lowest efficiency, extracting an average of 2.32% of lipids, when compared to chloroform which extracted the highest quantity of lipids, with an average value of 10.78 % lipids (Table 3.5). This also serves to confirm chloroform as the choice as reported by Bligh and Dyer, Folch and Christie, for the extraction of lipids (D'Oca *et al.*, 2011). Chloroform, ethanol and hexane were selected for further trials owing to their efficiency being greater than those of the other solvents tested.

The solvents used were not expected to extract chlorophyll with the same efficiency as they would extract lipids. This is illustrated by the fact that chloroform extracted the highest quantity of lipids and methanol extracted the highest quantity of chlorophyll (Table 3.5). Several researchers have used ethanol and acetone as solvents for chlorophyll on the basis that these solvents provide the best efficiency (Aminot and Rey, 2000; Carlson and Simpson, 1996; Dere *et al.*, 1998; Ling and Subramaniam, 2007; Wasmund *et al.*, 2006). For the biomass sample used for this experiment, acetone showed the least efficiency in the extraction of both lipids and chlorophyll. Although methanol did not extract the highest quantity of lipids, it did extract the highest amount of chlorophyll when compared with the other twelve solvents. Dere et al, 1998, reported that methanol was the best solvent for extraction of chlorophyll. They postulated that this is due to the type of algae and its cell wall

structure. The use of methanol is not encouraged since it is more harmful than ethanol and acetone. It has been shown that the use of methanol as an extraction solvent results in an unstable solution and leads to the formation of chlorophyll *a* degradation products (Ryckebosch *et al.*, 2011).

Table 3.5 Algal lipid and chlorophyll concentration after Soxhlet extraction using thirteen solvents

No.	Solvent	Lipid extract	Chlorophyll concentration in Lipid extract
		% m/m	% m/m
1	Chloroform	10.78	1.32
2	Ethanol	10.26	0.54
3	Hexane	10.04	0.76
4	Toluene	9.89	0.35
5	Isooctane	8.78	0.43
6	Benzene	7.19	0.53
7	Diethyl ether	7.06	0.52
8	Cyclohexane	6.92	0.14
9	Methanol	6.79	1.49
10	Dichloromethane	6.09	0.11
11	Isopropanol	5.33	0.36
12	Petroleum Ether	4.85	0.06
13	Acetone	2.32	0.04

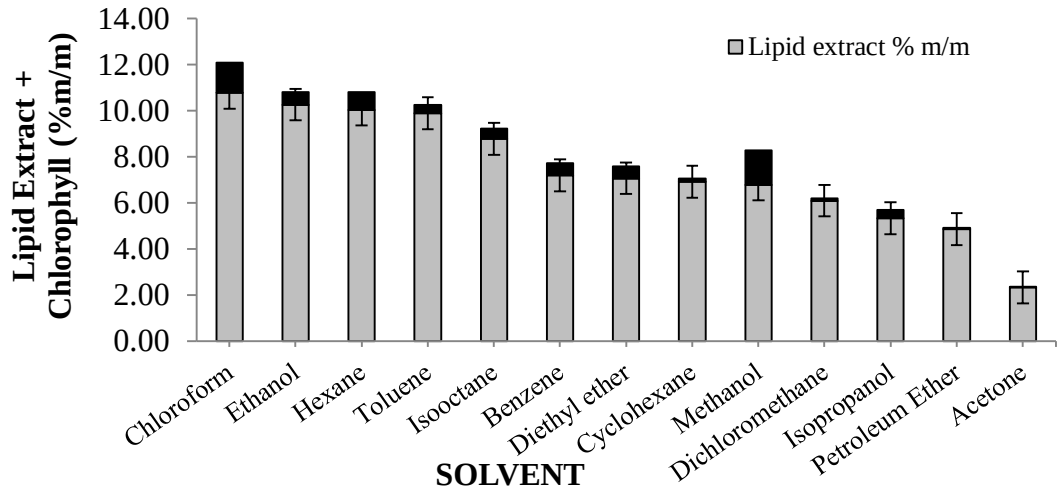


Figure 3.8 Quantities of lipids and chlorophyll extracted by the soxhlet method using the thirteen solvents.

3.3.2.1 Effect of time on extraction efficiency

The duration of the extractions for chloroform, ethanol and hexane were varied from one to five hours with hourly increments. Normal extraction trends would be expected to approach a maximum as extraction time increases after which it would be expected to plateau as the duration of extraction time increases. This trend, however, is not strictly followed by the solvents tested (Figure 3.9). Ethanol simulates the expected trend to a certain degree, but hexane shows a sharp decrease after the three hour maximum and chloroform shows a gradual decrease. The extraction behaviour of chloroform and hexane after three hours could be attributed to the formation of volatile degradation products which may have caused the decrease in the amount of lipids extracted (Phukan *et al.*, 2011). All three solvents show a decrease in extraction efficiency after three hours, but in hexane the decrease is more pronounced probably indicating that greater degradation of lipids with increase in time of extraction (Ryckebosch *et al.*, 2011). Chloroform, ethanol and hexane show distinct optima of 3.49 %, 5.71 % and 4.99% respectively after three hours of extraction (Table 3.6). The sudden decrease in the lipid content of the extract could also be attributed to inconsistencies arising from the homogeneity of lipids in the algae. Nile red tests by established methods have shown that not all algal cells contain the same quantities of lipids (Chen *et al.*, 2009; Fowler *et al.*, 1987). However, repeated trials on the same sample in this study showed similar trends in the extraction behaviour of the solvents thus reinforcing the fact that with extended time some degree of degradation could be taking place. This resulted in a gradual decrease in quantities of lipids extracted after three hours. The extraction time of three hours was thus considered as the optimum for the extraction of lipids.

Table 3.6 Actual values of lipid concentrations obtained for chloroform, ethanol and hexane solvents with varying time

No.	Time (Hour)	% Lipids		
		Chloroform	Ethanol	Hexane
1	1.0	0.74	1.77	0.46
2	2.0	2.03	3.78	3.67
3	3.0	3.49	5.71	4.99
4	4.0	2.70	5.22	1.26
5	5.0	1.53	5.02	1.28

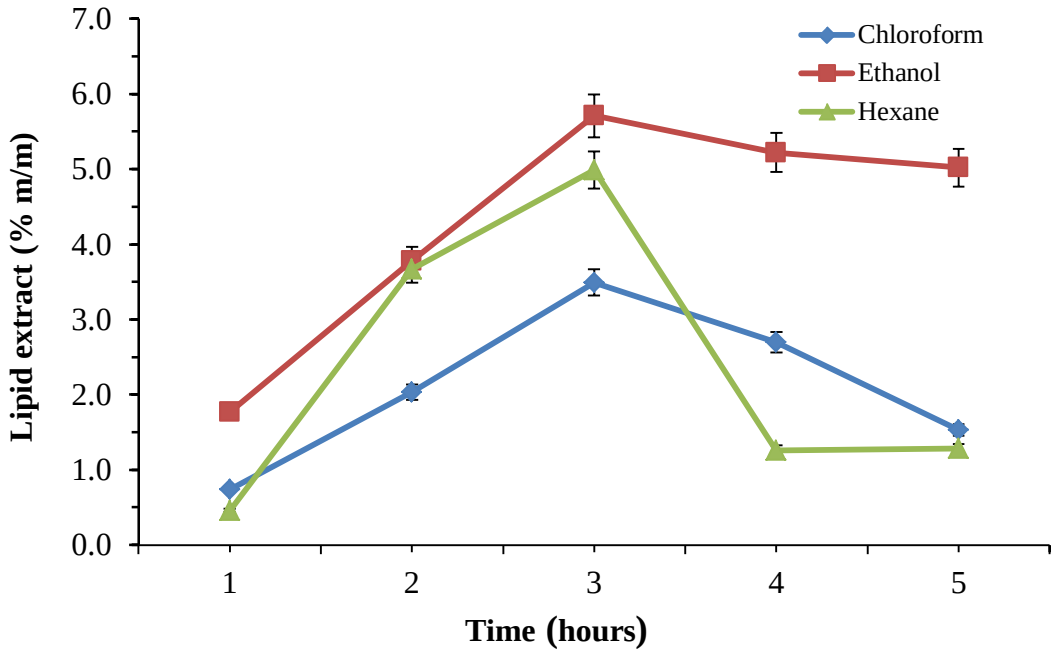


Figure 3.9 Illustration of lipid quantities obtained after extraction with chloroform, ethanol and hexane solvents with varying time.

3.3.2.2 Effect of binary solvents mixtures on extraction efficiency

A comparison of the various ratios of the three binary mixtures selected indicates that the 1:1 chloroform: hexane mixture extracted the least amount of lipids at 0.98% whereas the 1:1 chloroform: ethanol mixture recorded the highest quantity of lipids extracted at 11.76% (Table 3.7). The extraction by various ratios of chloroform: hexane show levels below 2% hence giving lower efficiency than the chloroform:

ethanol mixtures which varied from 2.5% to approximately 12%. Based on the principle of solvent extraction where “like dissolves like”, the lower results produced by the chloroform: hexane mixture could indicate that the algae contained smaller quantities of non-polar lipids. Chloroform: ethanol mixtures yielded extraction results which are indicative of larger quantities of polar and neutral lipids. The ethanol: hexane mixture of the 1:3 ratio extracted approximately 4% of lipids at its highest efficiency. It is interesting to note that when the extractions were performed using single solvents (Figure 3.8), the optimum extraction was obtained using chloroform and ethanol with lipid extract values greater than 10%. When comparing this with the values obtained in Figure 3.10, the highest lipid content was obtained using the chloroform: ethanol mixture (ratio 1:1) where an amount of 11.76% was extracted. The solvents show similar extraction efficiency to single solvents when they are in equal proportions, i.e. 1: 1 ratio, but as the ratios are varied, solvent efficiencies deteriorate and lower efficiencies become evident. This may be attributed in part to the intermolecular forces (Van der Waals forces) that exist between molecules of each of the solvents and the molecules of biomass presented for extraction. The extraction behaviour of the solvents when mixed depends not only on the result of intermolecular attractions, but also on the discrimination between different types of polarities. Changes in viscosity will also affect the solubilities of the mixtures depending on their polarity and the Van der Waals forces acting on them (Burke, 1984).

The best efficiency for lipids was given by a chloroform: hexane mixture in a 1: 2 ratio. This is less than half of the maximum produced by the ethanol : hexane mixture of 1:3 and six times less than the maximum produced by the 1:1 chloroform : ethanol mixture. However, the highest chlorophyll extract was produced by the 1:3 ethanol: hexane mixture. Once again, it can be observed that there is no consistency or relationship between the quantities of lipids extracted and the quantities of chlorophyll obtained (Table 3.7). This may also be attributed to solvent behaviour resulting from varying degrees of molecular interaction between the mixtures.

Table 3.7 Results showing quantities of lipids and chlorophyll extracted using binary mixtures of chloroform, ethanol and hexane at various ratios

BINARY SOLVENT	Ratio	Lipid extract	Chlorophyll conc. in Lipid extract
		% m/m	% m/m
Chloroform: Hexane	1:1	0.98	0.10
	1:2	1.85	0.09
	1:3	1.75	0.22
	2:1	1.66	0.22
	3:1	1.59	0.03
Ethanol: Hexane	1:1	2.94	0.48
	1:2	3.33	0.59
	1:3	3.99	0.91
	2:1	3.26	0.16
	3:1	1.45	0.26
Chloroform: Ethanol	1:1	11.76	0.33
	1:2	3.83	0.22
	1:3	5.33	0.08
	2:1	2.78	0.07
	3:1	2.58	0.01

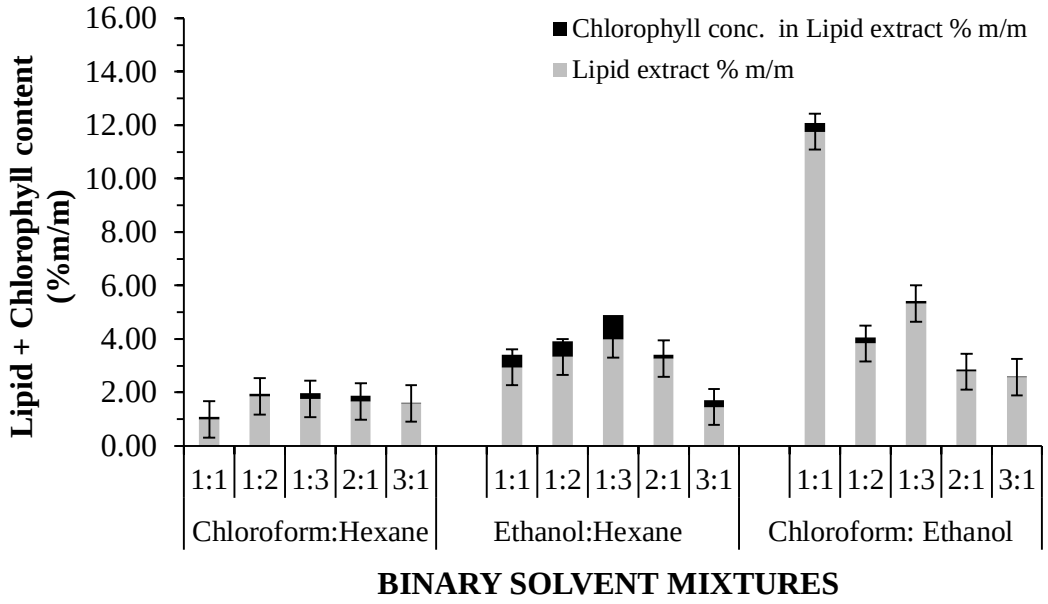


Figure 3.10 Comparison of lipid content obtained by soxhlet extraction of algal biomass using binary mixtures of solvents.

3.3.3 Microwave assisted extraction (MAE)

3.3.3.1 Optimization of extraction volume

This experiment makes use of three solvents, viz., chloroform, ethanol and hexane, which produced the highest lipid extracts for the soxhlet extraction method. Figure 3.11 shows that each of the solvents demonstrates an optimum extraction efficiency when 30 mL of the solvent was used for extraction. Application notes by CEM show SOP's using 30 mL for oil extraction, but do not indicate why this volume is used. The assumption is made that this is the optimum for extraction of oils/lipids. This experiment confirms this. Studies using MAE have shown that solvent volumes must be sufficient to immerse the entire sample for efficient extraction to take place. Further to this, higher volumes of solvent may produce lower recoveries (Eskilsson and Bjorklund, 2000). This may explain the reason for a decrease in lipid extracts beyond the 30 mL volume of solvent. Although ethanol shows a maximum efficiency at 30 mL, it does however, show little variation over the range of volumes chosen for testing when compared to chloroform and hexane.

Table 3.8 Lipid quantities obtained in the determination of optimum for varied volume of selected individual solvents

Optimizing MAE VOLUME parameter				
			% Lipids	
No.	Volume	Chloroform	Ethanol	Hexane
1	10.0	6.86	9.88	7.99
2	20.0	6.88	11.41	8.27
3	30.0	15.70	12.78	20.64
4	40.0	9.80	10.12	12.36
5	50.0	13.10	10.55	13.30

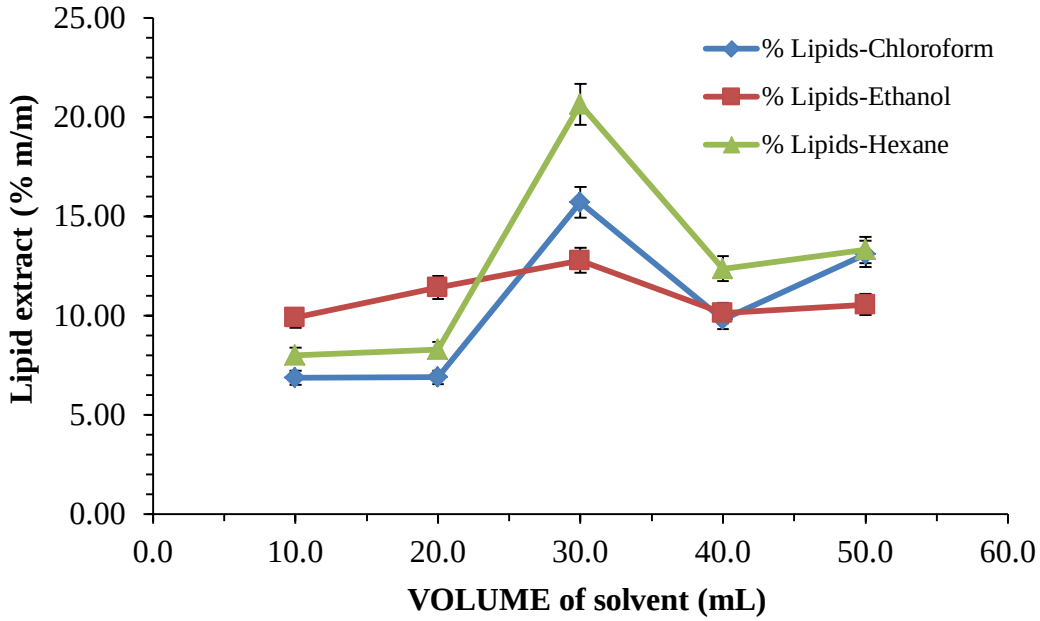


Figure 3.11 Determination of optimum volume for extraction of algal lipids by MAE.

3.3.3.2 Optimization of extraction time

The evaluation of time as a parameter is essential in ascertaining the speed with which an analysis may be performed. This experiment illustrates that not all solvents have the same capacity for extraction in relation to time. Chloroform and hexane produced 10.05% and 10.66% of lipids respectively after 10 minutes of extraction

(Table 3.9). Figure 3.12 shows that these were the optimum values for the two solvents. Ethanol on the other hand, produced 9.36% as an optimum after 5 minutes of extraction. The dielectric constant, a measure of the polarizibility of a molecule in an electric field, has an effect on the ability of a solvent to absorb microwave energy. Polar molecules will absorb microwave energy strongly because they have a permanent dipole moment that will be affected by the microwaves (Eskilsson and Bjorklund, 2000). This may explain why ethanol needs less time to extract the majority of the lipids compared to chloroform and hexane. Figure 3.12 shows a general decrease in the amount of lipids extracted over time. This may also be due to some degradation products being formed over time.

Table 3.9 Lipid quantities obtained in the determination of optimum for varied time with selected individual solvents

Optimizing MAE TIME parameter				
	Time	% Lipids		
No.	(minute)	Chloroform	Ethanol	Hexane
1	5.0	7.79	9.36	5.29
2	10.0	10.05	7.39	10.66
3	15.0	5.68	7.84	3.57
4	20.0	6.78	6.81	3.72
5	30.0	2.18	1.00	1.19
6	40.0	2.93	4.56	1.85
7	50.0	2.93	3.83	1.28
8	60.0	5.00	4.82	4.48

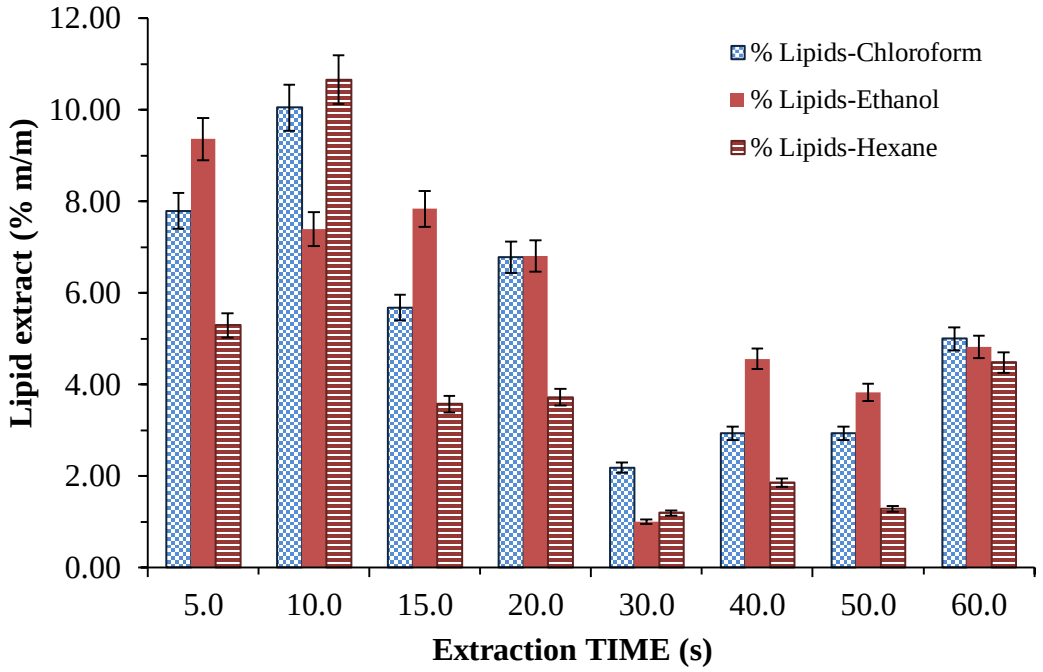


Figure 3.12 Determination of optimum time for extraction of algal lipids by MAE.

3.3.3.3 Optimization of mass of algal biomass

Sample mass is generally not a parameter selected for optimization as it is dependent on other factors used. The primary determining factor that influences mass would be the amount of lipids present. However, lab-scale applications are limited by the amounts (mass) and volume of substances that can be used. It was therefore decided to optimize this parameter as it would also be a factor that influences upscaling protocols. All three solvents, viz., chloroform, ethanol and hexane, showed the most consistent extraction profiles with the mass range chosen for this study. All solvents produced maximum lipid extracts at a mass of 1 g. Since other parameters were kept constant, the values obtained (Table 3.10) for the 0.5 g mass would indicate that a lower proportion of lipids would be present. But, when masses greater than 1 g are used, instead of an expected increase or plateau after the optimum mass, values of the lipid extracts decreased gradually. This may be explained by the fact that with increase in mass a proportionality rule will apply, i.e. as mass is increased, the volume of extraction solvent should be increased for optimal extraction of lipids. In

this experiment the volume was kept constant, hence it is possible that saturation point for extraction may have been reached. This, however, does not explain the decrease in the amount of lipid extract (Figure 3.13). This may be explained by the possibility that a larger quantity of the volatiles were extracted first resulting in a slightly greater loss in mass during the evaporation of the solvent.

Table 3.10 Lipid quantities obtained in the determination of optimum for varied mass with selected individual solvents

Optimizing MAE MASS parameter				
	Mass	% Lipids		
No.	(g)	Chloroform	Ethanol	Hexane
1	0.5	5.70	4.90	5.51
2	1.0	7.08	7.77	7.01
3	2.0	3.05	2.70	2.79
4	5.0	2.18	2.14	1.66

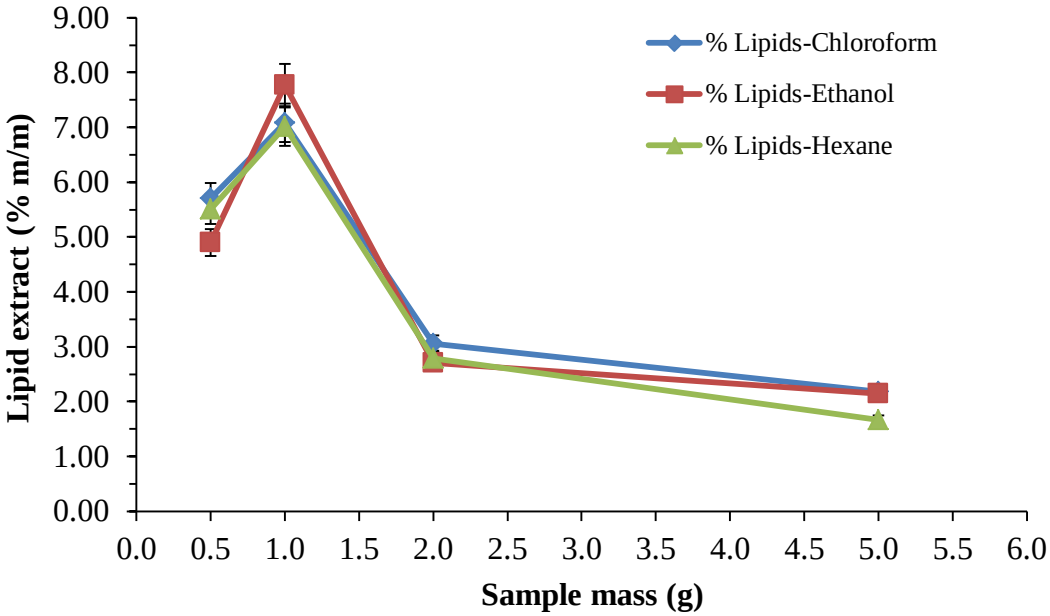


Figure 3.13 Determination of optimum mass for extraction of algal lipids by MAE.

3.3.3.4 Optimization of extraction temperature

Temperature is by far the most investigated parameter in MAE (Eskilsson and Bjorklund, 2000). It is an important factor for most technologies, but for MAE it is even more important since temperatures of solvents used in these extractions tend to go beyond the boiling points of the solvents. The positive effect of this is that the extraction efficiency of the solvents used is increased. Solvents generally have a higher capacity to solubilize analytes at higher temperatures. An overall result is the decrease in surface tension and viscosity will improve sample wetting and matrix penetration (Eskilsson and Bjorklund, 2000). As expected there is a general increase in the quantities of lipids extracted as temperature increases (Figure 3.14). Hexane has a relatively lower range of fluctuations in the lipid content. This may be explained by its low dielectric constant and its non-polar nature. Eskilsson and Bjorklund claim that non-polar solvents such as hexane will not heat up like polar solvents would when exposed to microwave radiation. Each solvent expressed a gradual overall increase in lipid quantities as the temperature was increased from 25ºC to 100ºC. Thereafter, only ethanol showed an increase in the lipid quantity extracted. Chloroform and hexane show a decrease in quantities of lipid extracted (Table 3.11). This decrease may be attributed to thermal degradation of the lipids at higher temperatures (Veggi *et al.,* 2012). At 200ºC the temperature proved to be too high to execute safe extraction of the samples. This was evident from the increased pressure within the teflon tubes resulting in greater loss of sample. The optimum temperature was taken as 100ºC.

Table 3.11 Lipid quantities obtained in the determination of optimum for varied temperature with selected individual solvents

Optimizing MAE TEMPERATURE parameter				
	Temperature	% Lipid extract		
No.	(^{o}C)	Chloroform	Ethanol	Hexane
1	25.0	4.64	3.94	3.98
2	50.0	4.72	5.52	4.79
4	100.0	9.14	9.20	5.05
6	150.0	5.02	9.37	4.33
7	200.0	not feasible		

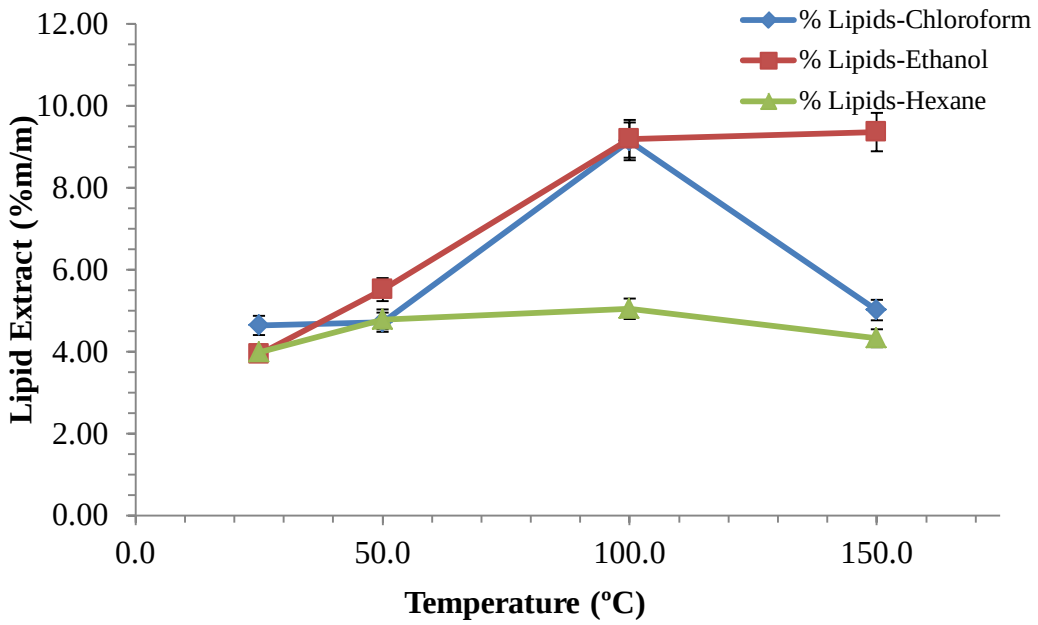


Figure 3.14 Determination of optimum temperature for extraction of algal lipids by MAE.

3.3.3.5 Optimization of binary solvent systems for extraction

It is a well-known fact that single solvent systems cannot perform as efficiently as multi-solvent systems in the extraction of lipids. The simple reason for this is that algal lipids may be polar, neutral or non-polar, hence multi-solvent systems may employ polar, non-polar and intermediate polarity solvents to increase their extraction efficiencies. The extraction behavior of solvents may depend on their polarities. Binary solvent systems are governed by their miscibility and behavior in solution. The three solvents chosen were mixed in ratios shown in Table 3.12. Mixing ratios greater than 3:1 or 1:3 were omitted owing to the greater probability of the major solvent dominating the extraction efficiency of the mixture. The results show that the 1:1 ratios for chloroform: ethanol (6.53%) and ethanol: hexane (6.18%) were the most efficient extraction mixtures in those groups while the 3:1 ratio for chloroform: hexane was the most efficient for that group with a lipid extract of 4.83%. The best extraction efficiency for the binary mixtures was shown by the 1:1 chloroform: ethanol binary mixture (Figure 3.15). It should be noted that the

variations observed in lipid content for different sets of analysis was dependent on time and date the sample was taken. The variation in the lipid content for different batches of harvested algal biomass is indicative of the influence of varying conditions on the growing of algae (Typical data are shown in Appendix B).

Table 3.12 Lipid quantities obtained in the determination of optimum for varied temperature with selected binary solvent mixtures

Optimizing MAE BINARY SOLVENT ratios				
No.	**Ratio**	**% Lipid extract**		
		Chloroform: Ethanol	**Chloroform: Hexane**	**Ethanol: Hexane**
1	1:1	6.53	3.61	6.18
2	1:2	3.83	3.14	2.73
3	1:3	5.69	4.65	2.36
4	2:1	5.57	4.03	4.18
5	3:1	4.54	4.83	4.67

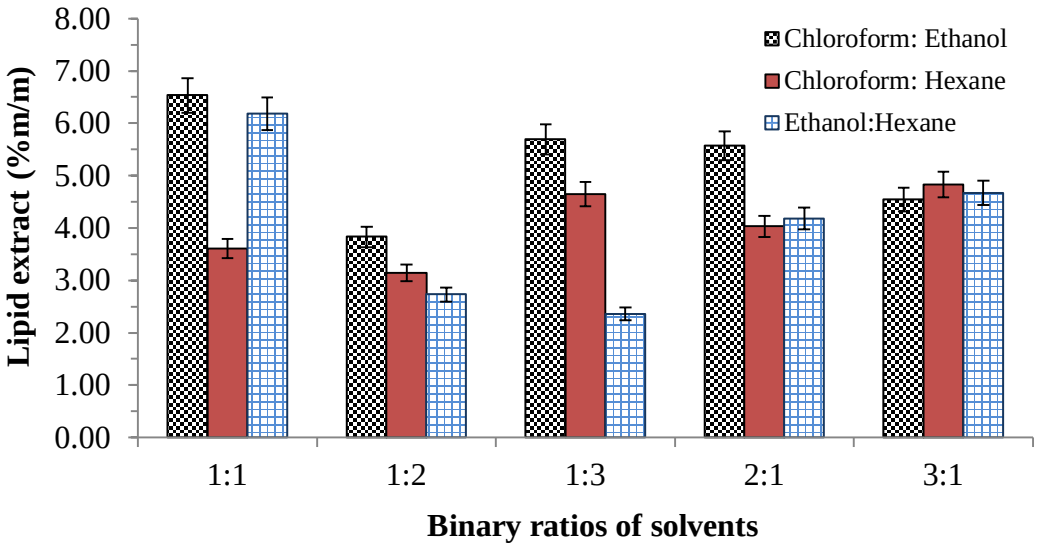


Figure 3.15 Determination of optimum binary ratio for the extraction of algal lipids by MAE.

3.3.3.6 Experimental Design

Central composite experiments were conducted to determine the interactions between factors and to predict maximum quantities of lipids extracted from algal biomass using three factors. In order to approach the proximity of the optimum values, a model which included the linear, interaction and squared terms (Eq. 3.2), was fitted to the data obtained from the CCD experiments. The values of the regression coefficients were calculated and the following equation was derived using the coefficients of the coded factors:

$$\text{Lipid (\% m/m)} = +11.61 +0.19*X_1 -0.93*X_2 +1.00*X_3 -0.69*X_1X_2 -0.41* X_1X_3 +0.085*X_2X_3 -0.96*X_1^2 -0.59 *X_2^2 -0.54*X_3^2 \quad (3.2)$$

From the equation above, volume of solvent (X_3) had the highest positive effect on the experiment as shown by the coefficient of +1.00 while time (X_1) had the lowest. Mass of algae (X_2) showed a small negative effect individually and was also involved in one negative interaction with time (X_1X_2). The strongest positive interaction was found to be mass of algae and volume. All three interactions are further investigated in Figures 3.16 to 3.18.

Experimental and predicted results using the above equation were calculated and statistically analysed for the goodness-of-fit of the equation generated to the experimental data. Experimental data along with predicted and residual lipid extraction percentages are indicated in Table 3.13.

Table 3.13 Experimental design of a three-factor central-composite experiment showing coded factor levels with randomized run order to determine effect on lipid extraction by three factors during microwave digestion

Std Order	Run Order	*Time* X_1	*Mass* X_2	*Volume* X_3	*Actual Value*	*Predicted Value*	*Residual*
1	9	-1	-1	-1	8.65	8.25	0.40
2	6	1	-1	-1	10.82	10.83	-0.01
3	14	-1	1	-1	8.05	7.60	0.45
4	10	1	1	-1	7.79	7.42	0.37
5	3	-1	-1	1	10.89	10.90	-0.01
6	18	1	-1	1	11.77	11.86	-0.09
7	12	-1	1	1	10.97	10.59	0.38
8	16	1	1	1	8.74	8.78	-0.04
9	15	-1.68179	0	0	8.02	8.57	-0.55
10	2	1.681793	0	0	9.26	9.22	0.04
11	17	0	-1.68179	0	–	–	–
12	7	0	1.681793	0	7.87	8.39	-0.52
13	19	0	0	-1.68179	7.86	8.41	-0.55
14	13	0	0	1.681793	11.81	11.78	0.03
15	11	0	0	0	11.49	11.61	-0.12
16	1	0	0	0	11.23	11.61	-0.38
17	5	0	0	0	11.92	11.61	0.31
18	8	0	0	0	11.92	11.61	0.31
19	20	0	0	0	11.68	11.61	0.07
20	4	0	0	0	11.52	11.61	-0.09

***S**tandard run 11 was ignored in the model due to the mass of algae being 0.01 with a subsequent lipid extract that was not measurable.

The analysis of variance (Table 3.14) shows that the Model *F*-value of 25.71 implies that the model is significant. There is a less than 0.01% chance that an *F*-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case X_2, X_3, X_1X_2, X_1X_3, X_1^2, X_2^2, X_3^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

Table 3.14 Analysis of variance for the three factor model used

ANOVA for Response Surface Quadratic model						
Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	48.58	9	5.40	25.71	2.26E-05	significant
x_1-Time	0.51	1	0.51	2.44	1.53E-01	
x_2-Mass	7.75	1	7.75	36.90	1.85E-04	significant
x_3-Volume	13.75	1	13.75	65.48	2.02E-05	significant
x_1x_2	3.84	1	3.84	18.27	2.07E-03	significant
x_1x_3	1.33	1	1.33	6.33	3.30E-02	significant
x_2x_3	0.06	1	0.06	0.28	6.12E-01	
x_1^2	12.63	1	12.63	60.14	2.84E-05	significant
x_2^2	2.90	1	2.90	13.81	4.79E-03	significant
x_3^2	3.96	1	3.96	18.84	1.88E-03	significant
Residual	1.89	9	0.21			
Lack of Fit	1.53	4	0.38	5.27	4.86E-02	significant
Pure Error	0.36	5	0.07			
Cor Total	50.47	18				

In Figure 3.16a and 3.16b, the three dimensional and contour plot showed that the mass and time factors exhibited optima at a mass of approximately 0.75 g with a time of approximately 12 minutes. In Figure 3.17a, the three dimensional plot showed that as volume and time decreased, the lipid quantities decreased, indicating lower efficiencies of extraction as these factors were reduced. The contour plot (Figure 3.17b) showed that an optimum extraction efficiency was reached at about 10 minutes with a solvent volume of about 38 mL as shown by the red zone.

However, the volume and mass factors (Figure 3.18a and 3.18b) showed that a volume of approximately 37 ml and a mass of just below 1 g gave optimum for lipid extracts. These variables have to be viewed collectively to eliminate doubts about interactions between them. Since it is anticipated that this technology will be applied on a large scale, interactions between the factors chosen will only become evident when trials are conducted. In the interim, the optimum conditions can be applied with a 95% confidence level at laboratory scale.

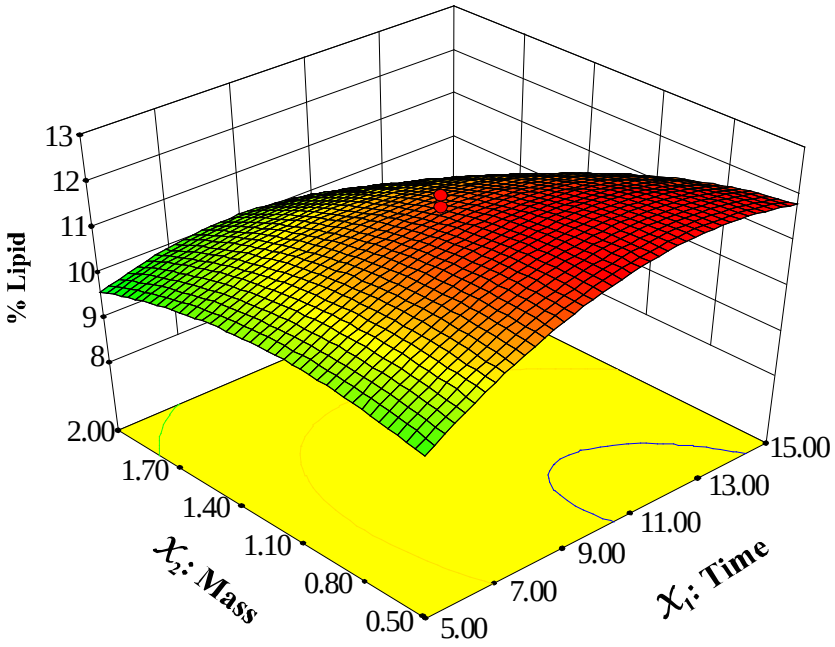


Figure 3.16a Three-dimensional response surface graph of lipid content affected by time of analysis and mass of algae.

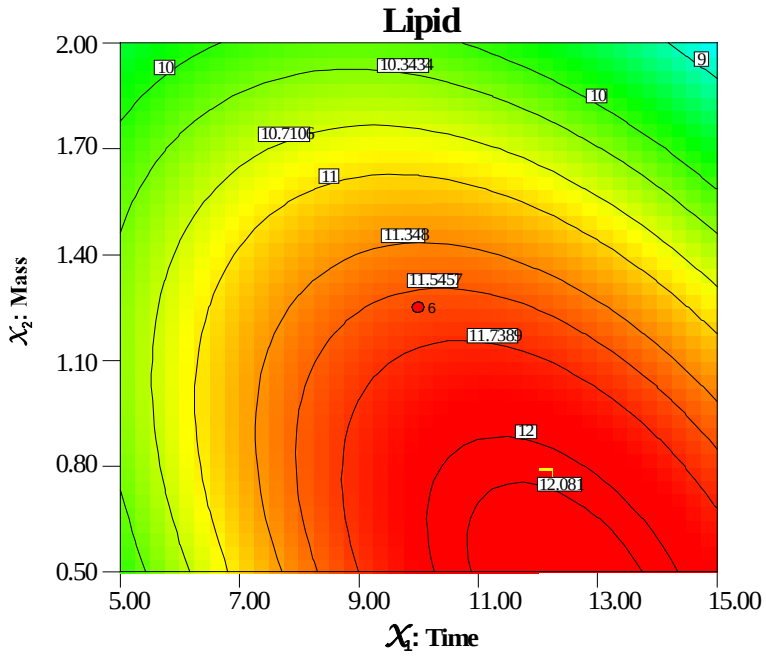


Figure 3.16b Contour plot showing graph of lipid content affected by time of analysis and mass of algae.

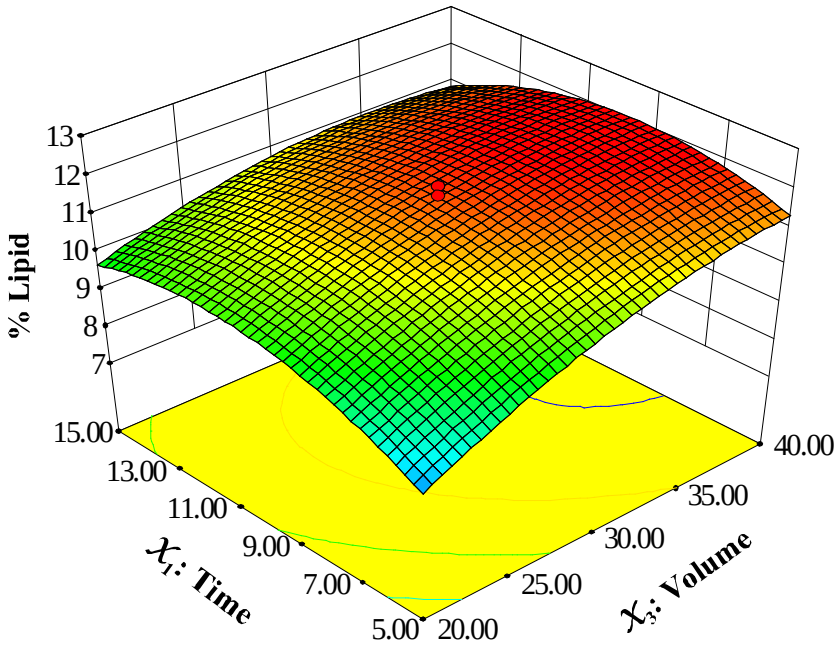


Figure 3.17a Three-dimensional response surface graph of lipid content affected by time of analysis and volume of solvent

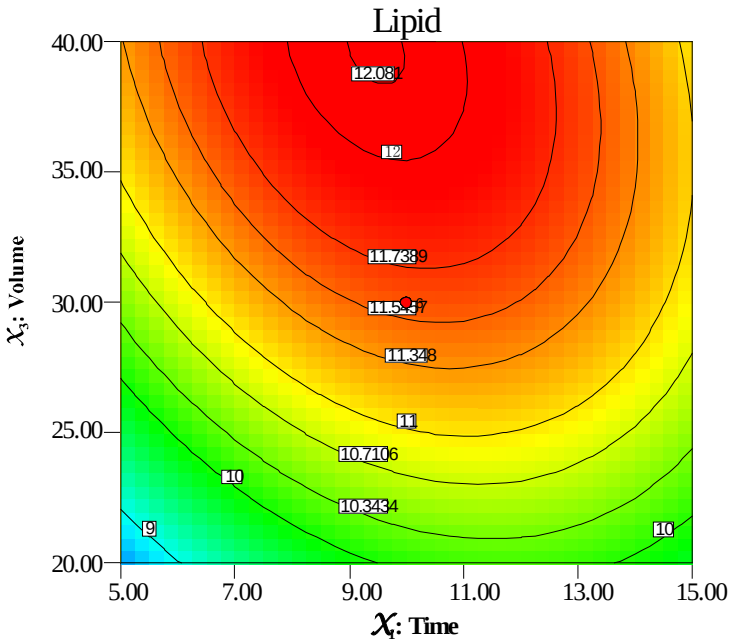


Figure 3.17b Contour plot showing graph of lipid content affected by time of analysis and volume of solvent.

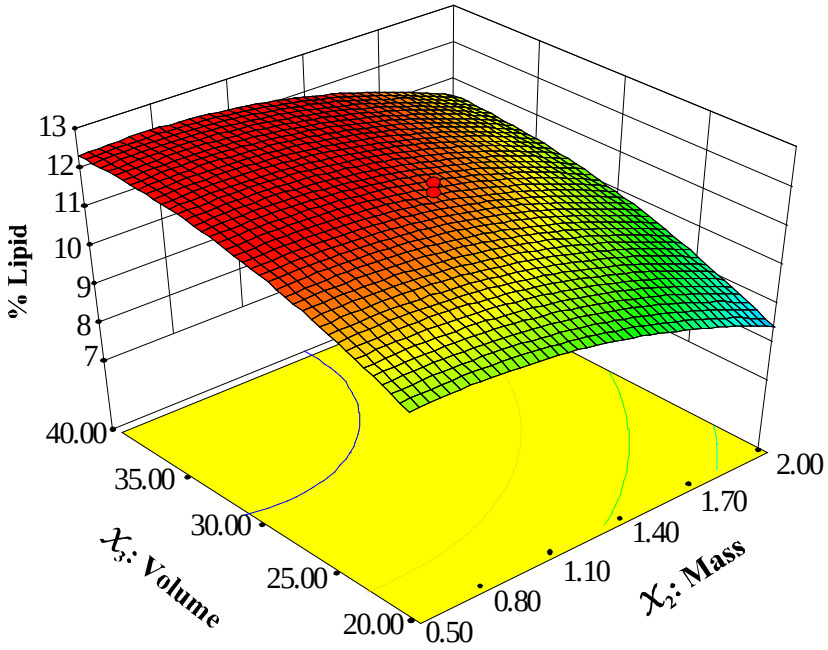


Figure 3.18a Three-dimensional response surface graph of lipid content affected by mass of algae and volume of solvent.

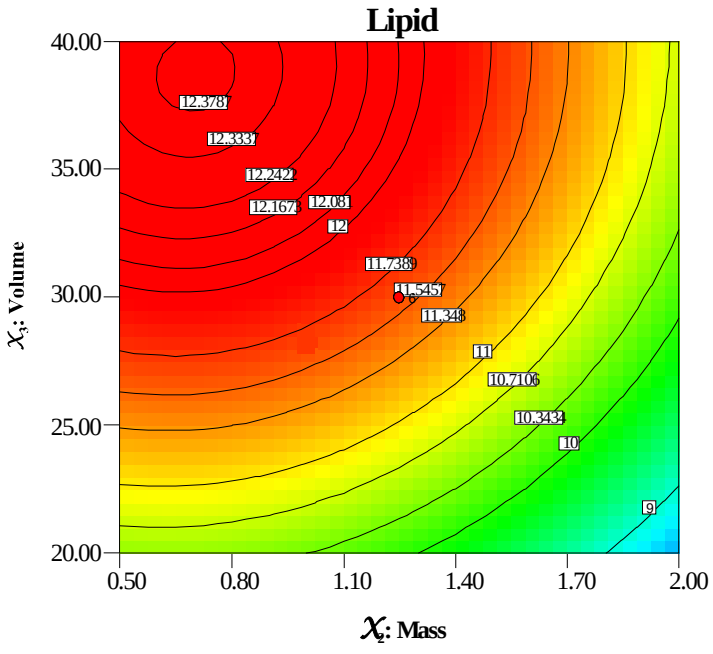


Figure 3.18b Contour plot showing graph of lipid content affected by mass of algae and volume of solvent.

From the above interaction graphs and equation describing the interactions an integral for lipid extraction maxima was calculated by the program (*Stat-Ease*, 2014) which provided 36 solutions shown in Table 3.15.

Table 3.15 Point prediction table showing optimized factors

Number	Time	Mass	Volume	Lipid (%)	Desirability
33	10.91	0.76	35.34	12.3680	1
10	11.09	0.91	37.55	12.3317	1
7	9.43	0.66	38.37	12.3308	1
2	10.6	0.99	35.55	12.2492	1
29	11.94	0.84	33.65	12.2451	1
12	9.54	1.06	38.34	12.2435	1
32	10.74	0.62	32.12	12.2346	1
26	12.78	0.51	31	12.2119	1
36	9.27	0.83	34.73	12.2032	1
21	12.34	0.65	31.04	12.1918	1
35	8.53	1.04	38.82	12.1804	1
25	8.42	1.04	38.65	12.1668	1
11	11.39	0.93	32.56	12.1497	1
6	13.36	0.55	30.38	12.1245	1
3	14.22	0.55	38.44	12.1008	1
20	10.26	1.16	35.44	12.1000	1
15	8.62	0.57	35.66	12.0922	1
34	11.59	1.13	38.58	12.0907	1
4	14.15	0.6	38.88	12.0762	1
27	13.64	0.7	31.77	12.0754	1
31	9.58	1.22	36.4	12.0676	1
9	8.19	1.03	36.35	12.0631	1
1	11.33	1.05	32.67	12.0454	1
19	7.81	0.94	36.8	12.0335	1
23	14.02	0.55	30.05	12.0292	1
5	9.37	1.29	38.02	12.0268	1
17	9.27	1.22	35.52	12.0148	1
8	13.37	0.92	34.26	12.0038	1
13	13.22	0.92	33.25	12.0016	1
14	8.88	1.22	35.48	11.9906	1
24	14.42	0.7	33.5	11.9873	1
22	13.54	0.58	28.66	11.9739	1
16	7.18	1.08	39.35	11.9546	1
39	12.76	0.68	28.35	11.9494	1
28	7.11	0.87	39.39	11.9466	1
38	10.33	0.68	29.15	11.9419	1
18	13.56	0.67	28.94	11.938	1
37	13.45	0.66	28.59	11.9304	1
30	11.72	1.24	36.67	11.9234	1

A mass of 1 g was used as a cut-off point and solution 12 from Table 3.16 was chosen for the prediction of high and low values for lipids extracted (Table 3.15). Sample 33 shows that the highest lipid value achieved, i.e.12.37%, lies within the predicted intervals for sample 12.

Table 3.16 Table for response 12 with 95% confidence intervals

Number	Response	Prediction	Std Dev	SE Mean	95% CI low	95% CI high	SE Pred
12	Lipid	12.24	0.46	0.19	11.81	12.68	0.50

A comparison was also drawn between the optimum for the DOE (Design of experiment) methods and the original OFAT method conducted on the same batch of sample and after DOE results were obtained. The quantities of lipids shown in Table 3.17 do indicate that all results appear within the 95% confidence interval.

Table 3.17 Comparison of optimum lipid quantities and for the OFAT method and the DOE method

Method	Time	Mass	Volume	Lipid (%)	Std dev.
RSM	9.54	1.06	38.34	12.24	0.46
OFAT	10.00	1.00	30.00	11.96	0.23

3.3.3.7 Chlorophyll

The amount of chlorophyll in algae ranges from 0.1% to 1.0% (Hosikian *et al.*, 2010). Little is known about the effect of chlorophyll on biodiesel production. It has, however, affected the colour of the final product. This was particularly noticeable with the in-situ esterification process. Here, it was necessary to clarify the biodiesel to produce lightly coloured biodiesel. Having knowledge of the quantity of chlorophyll present would make it easier to remove it more efficiently if required. Chlorophyll analysis was therefore performed using each of the thirteen solvents applied to lipid extraction. This was done to establish the quantities of chlorophyll extracted by each solvent. In the chlorella and scenedesmus species studied, chlorophyll *a* and *b* are predominant. Other types like chlorophyll *c* and *d* are found in trace quantities. Figure 3.19 shows the standard curves produced for chlorophyll *a* and *b*. The R^2 show excellent curve fit for standards analyzed.

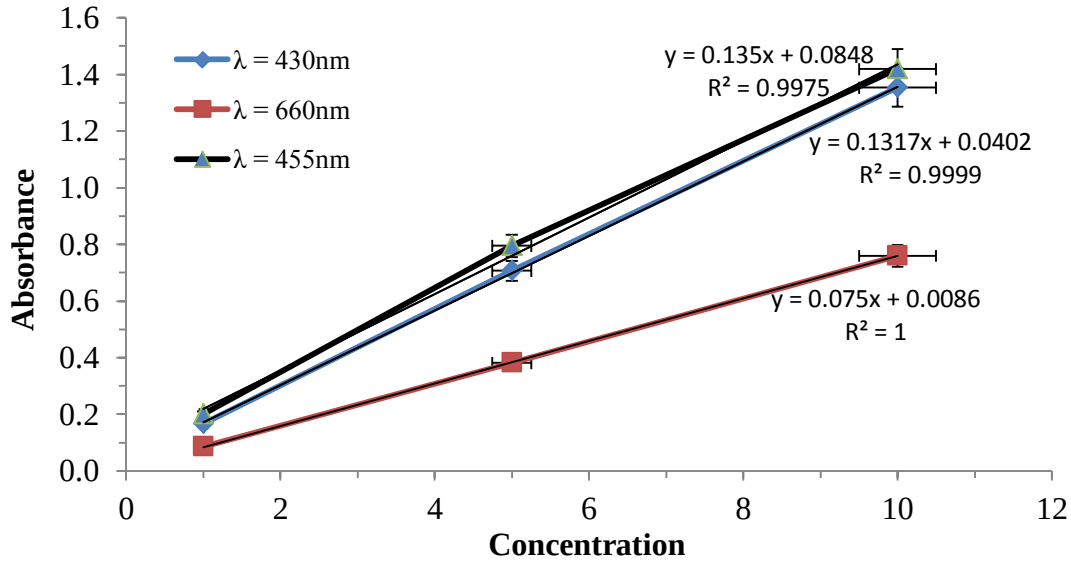


Figure 3.19 Graph of standards determined for chlorophyll *a* and chlorophyll *b* from which the concentrations of chlorophyll were determined.

The curves were used to determine the values of the samples obtained from the solvent extracts. These values for each solvent are shown in Table 3.18 and represented graphically in Figure 3.20. The EPA recommends the use of 90% acetone as a solvent for chlorophyll while some other researchers recommend methanol.

Table 3.18 Total chlorophyll concentration obtained for the individual solvents used in extraction

No.	Solvents	Total chlorophyll	
		%	ppm
1	Methanol	0.19	1929
2	Chloroform	0.11	1131
3	Hexane	0.08	788
4	Diethyl ether	0.07	695
5	Benzene	0.07	688
6	Isopropanol	0.06	630
7	Ethanol	0.05	499
8	Isooctane	0.05	474
9	Toluene	0.03	341
10	Cyclohexane	0.02	201
11	Dichloromethane	0.02	183
12	Acetone	0.02	166
13	Petroleum Ether	0.01	120

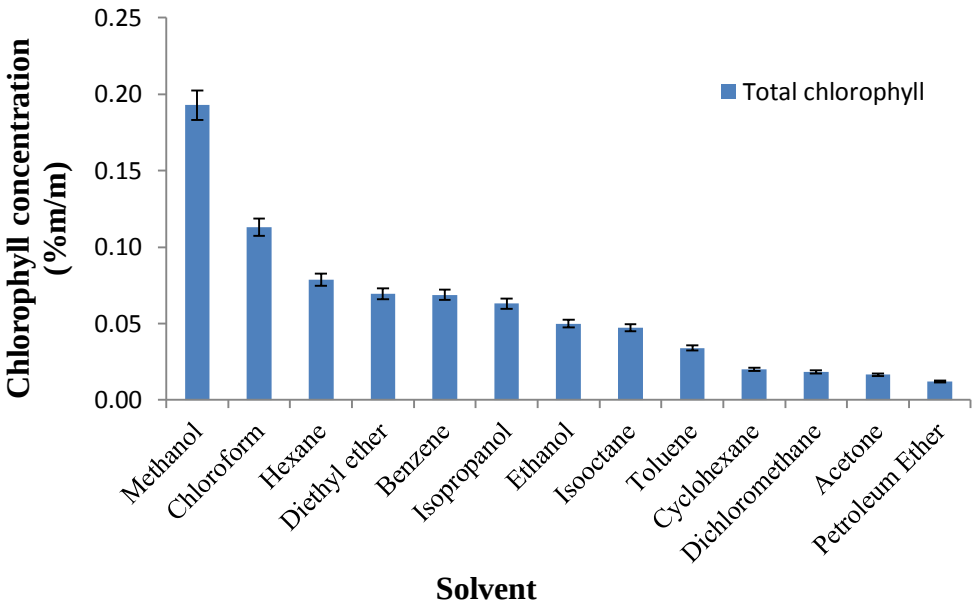


Figure 3.20 An illustration of the chlorophyll content obtained using individual solvents.

From Figure 3.20 it can be seen that a large variation in the extraction of chlorophyll

by the different solvents selected is evident. This study has shown that methanol extracted the highest quantity of chlorophyll (~0.2% m/m) compared to the other solvents studied. This is in line with studies by some researchers (Dere *et al.*, 1998; Hosikian *et al.*, 2010). The lowest quantity of chlorophyll was extracted by petroleum ether, at 0.01%m/m). Acetone extracted approximately 10 times less than the quantity extracted by methanol. These quantities were subtracted from the total lipids extracted by each solvent to give actual values of the lipids extracted (Figure 3.8).

Figure 3.21 shows the results obtained from experiments conducted using binary solvent mixtures. These mixtures were made from the three solvents optimized during the multiple solvent extractions conducted. The solvents were chosen on the basis that they were the solvents that produced the highest quantity of lipid extract from the solvents studied.

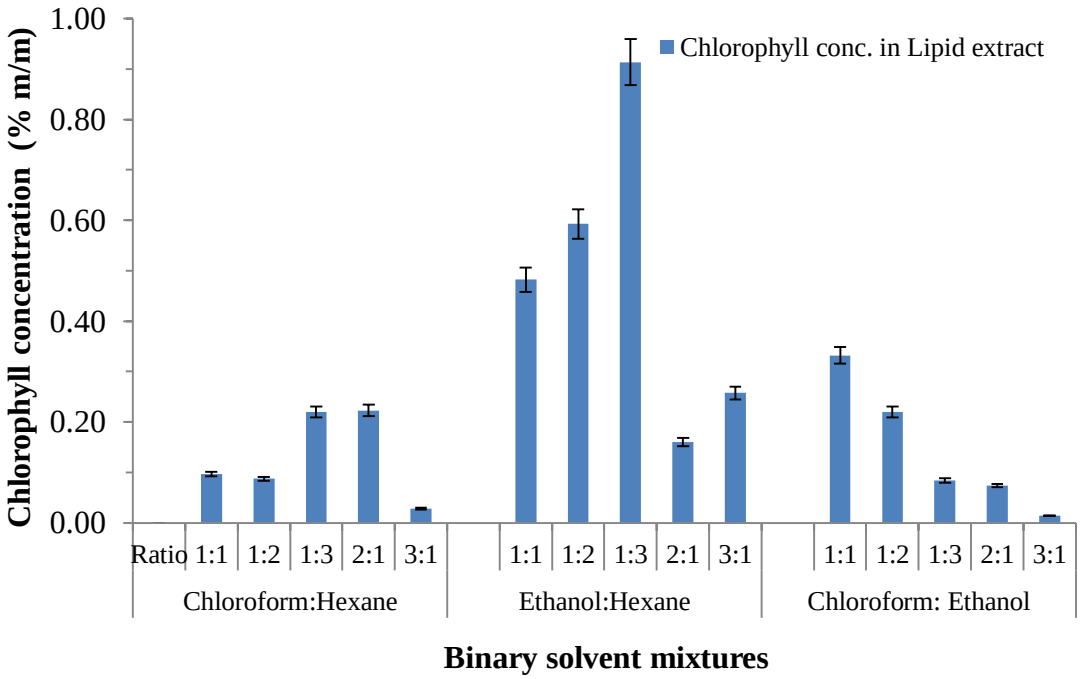


Figure 3.21 An illustration of the chlorophyll content obtained using binary mixtures of solvents (See data Table 3.7).

The ratios of the binary mixtures were the same used for the experiment conducted during the extraction of lipids (Figure 3.10). As there was no evidence of any parallel study conducted, conclusions can only be drawn on data obtained during this study. Examination of the lipid and chlorophyll extracted by the binary solvents will reveal that little correlation exists between the quantities of lipid and the quantities of chlorophyll extracted. While the 1:1 chloroform/ethanol mixture extracted the largest quantity of lipids, the mixture was only able to extract about one-third the largest quantity of chlorophyll extracted by the 1:3 ethanol/hexane mixture. The lowest quantity of lipids was extracted by the 1:1 chloroform/hexane mixture and the lowest quantity of chlorophyll was extracted by the 3:1 chloroform/ethanol mixture. Overall the ethanol/hexane binary mixtures were better at extracting chlorophyll than any of the other mixtures.

3.3.3.8 Freeze-dried versus oven-dried

A comparison of the results in Figure 3.22 show that a marginal variation exists between the freeze-drying method and the oven-drying method for the analysis of lipids.

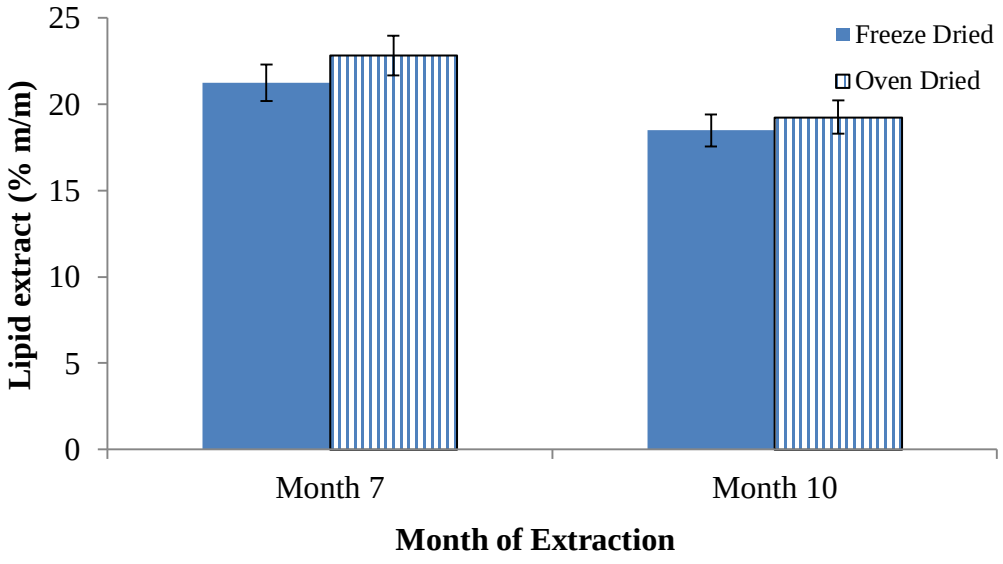


Figure 3.22 A comparison of lipid content obtained by the freeze-dried method and the oven-dried method analysed at two different intervals.

The only advantage that the freeze-drying method has over the oven-drying method is that biomass samples after freeze-drying are obtained readily in powdered form for analysis. The oven-dried samples, however, are obtained as dried flakes which have to pulverized before being presented for analysis. This added step is, however, not as energy consuming as the freeze-drying process. The freeze dried and oven dried biomass samples were analysed at two different periods at an interval of approximately three months. This was done to determine whether any significant change occurred with the two samples. They were stored under the same conditions in the laboratory. An F-test was conducted on the lipid quantities obtained in July and October as shown in Table 3.19. The calculated F-value for the July samples for the two drying methods was 6.53 while that for October was 1.50. The critical value of F for 2 degrees of freedom, $F_{crit.}$, was 19.00. This was at the 5% probability or 95% confidence level. Since $F_{calc.} < F_{crit.}$ For both periods, it can be said that the null hypothesis should be accepted, and it may be concluded that the two methods gave equivalent precision during the period analyzed. It should be noted that both the freeze dried and oven dried samples showed a decrease in the lipid content over the period analyzed, hence indicating a consistent loss of lipid content over the three month period. Figure 3.22 illustrates this pattern.

Table 3.19 Results of the comparison of the freeze-drying method versus the oven-drying method at different intervals

Month	July	October
Freeze dried (% lipid)	21.25	18.49
Std. dev.	0.09	0.82
S_F^2	0.0081	0.6724
Oven dried (% lipid)	22.82	19.25
Std. dev.	0.23	0.67
S_F^2	0.0529	0.4469
$F_{calc.}$	6.53	1.50
$F_{crit.}$ for 2 degrees of freedom	19.00	19.00

3.3.3.9 Ageing of biomass

Figure 3.23 gives a breakdown of the analysis conducted for standard fatty acids in algal lipids by chromatography. The fatty acids are typical fatty acids required for biodiesel raw material. The standards were analysed together with samples extracted over the period chosen for ageing of biomass. The graph does not show any significant variation or deterioration in the standards over ten weeks. However, Table 3.20 shows that stearic acid with a coefficient of variation of 11.4% was the only fatty acid that showed any significant variation during analysis. This is also supported by its standard deviation being the only value over 1.00.

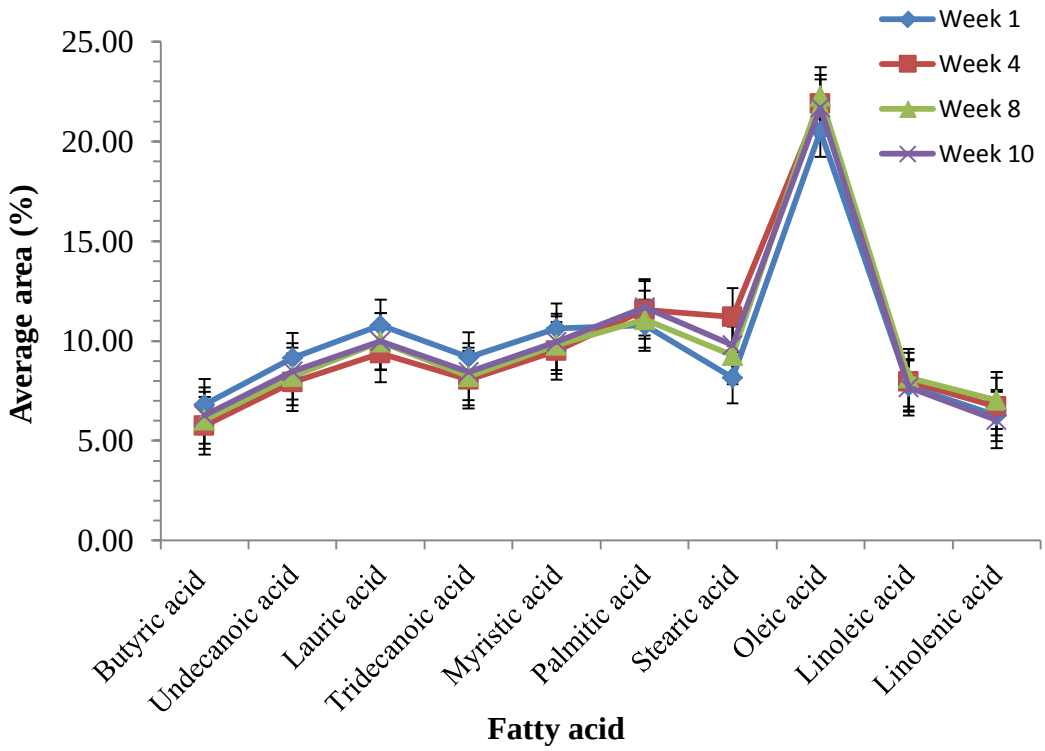


Figure 3.23 An illustration of the behavior of standards analysed during the course of testing ageing behavior over 10 weeks.

Table 3.20 Shelf life of standards conducted to determine if samples undergo ageing

Compound name	Average	Std. dev.	CV
Butyric acid	6.2106	0.39	6.3
Undecanoic acid	8.4400	0.44	5.2
Lauric acid	10.0306	0.51	5.1
Tridecanoic acid	8.4752	0.43	5.0
Myristic acid	9.9716	0.41	4.1
Palmitic acid	11.2847	0.36	3.2
Stearic acid	9.6099	1.10	11.4
Oleic acid	21.5876	0.66	3.0
Linoleic acid	7.8833	0.19	2.4
Linolenic acid	6.5069	0.39	6.0

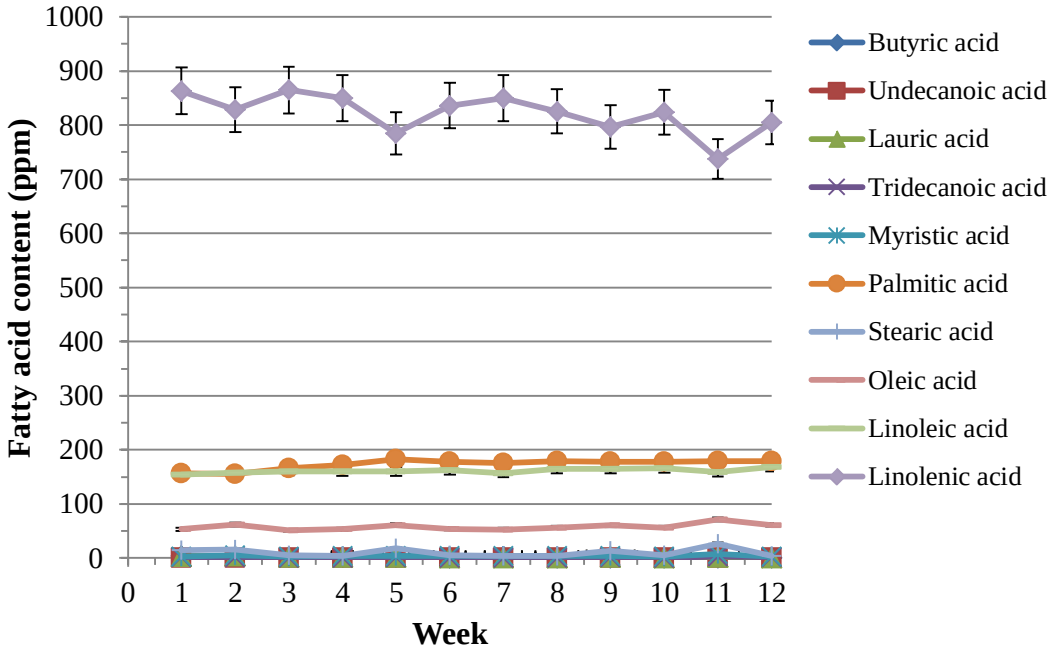


Figure 3.24 The quantities of each fatty acid found in algal lipids analysed over the 12 week of ageing.

Figure 3.24 exhibits the behaviour of lipids over approximately three months. The biomass was stored in a desiccator at room temperature for the duration of the

analyses. From the graph, the quantities of lipid extracted show little variation over the period of ageing. However, closer examination of Table 3.21 indicates that there was an overall gradual decline in the quantity of lipids extracted. The coefficient of variation (CV) is not a good indicator of precision for values less than one, but it can be seen that those lipids, e.g. linolenic acid, have far less variation in their lipid quantities with better CV values.

Table 3.21 Average quantities of lipids in ppm shown with standard deviations and coefficient of variation over the three months of ageing

Compound name	Average after 4 weeks	Std. dev.	CV	Average after 8 weeks	Std. dev.	CV	Average after 12 weeks	Std. dev.	CV
Butyric acid	2.5155	0.83	33.0	1.8885	0.98	52.2	1.4571	1.01	69.4
Undecanoic acid	0.4633	0.08	17.0	0.3147	0.16	51.8	0.2803	0.18	65.8
Lauric acid	2.4119	0.95	39.5	1.6836	1.01	60.0	1.4864	0.90	60.8
Tridecanoic acid	1.3814	0.56	40.6	1.2104	0.44	36.5	1.3026	0.61	46.7
Myristic acid	3.5065	0.90	25.6	3.6604	0.70	19.2	3.9279	1.14	28.9
Palmitic acid	162.6776	6.75	4.1	170.6687	9.49	5.6	173.3429	8.63	5.0
Stearic acid	10.0886	5.31	52.7	8.9219	5.92	66.3	10.1667	7.30	71.8
Oleic acid	55.0011	4.08	7.4	55.5629	3.65	6.6	57.8530	5.50	9.5
Linoleic acid	158.1241	2.53	1.6	159.5915	3.08	1.9	161.2073	4.06	2.5
Linolenic acid	851.5576	14.34	1.7	837.8350	24.27	2.9	822.0898	35.22	4.3

3.3.3.10 Comparison of optimized method versus Bligh and Dyer method

The Bligh and Dyer method (Iverson *et al.*, 2001) is perhaps the most applied one for extraction technologies. It has been a benchmark for the extraction of lipids using multisolvent mixtures of which methanol combined with chloroform is used for comparison by most researchers (Iverson *et al.*, 2001; Lee *et al.*, 2010; Smedes and Thomasen, 1996) . For this reason it was thought pertinent to compare the optimized method producing the best extraction for the binary solvents in this study with a

slightly modified Bligh and Dyer method. The modification was that both methods were analysed using MAE. This comparative study was conducted using the optimized chloroform: ethanol (1:1) and a modified Bligh & Dyer method using chloroform and methanol mixture (1:1). The results compared favourably with the Bligh & Dyer method which produced a slightly higher extract but with less than 1% difference between the two methods and a standard deviation of triplicate results for each method being <1.0 (Manirakiza, 2001).

Table 3.22 Results obtained for optimized method versus Bligh and Dyer method

	Optimized method (C: E, 1: 1)	**Bligh and Dyer (C: M, 1: 1)**
% lipids	13.18	13.26
Std. dev.	0.42	0.35
$F_{calc.}$	1.44	
$F_{crit.}$ for 2 degrees of freedom	19.00	

Furthermore, Table 3.22 shows that $F_{calc.} < F_{crit}$ by a large margin, hence we may conclude that there is no significant difference between the precision of the two methods. This is further illustrated by Figure 3.25.

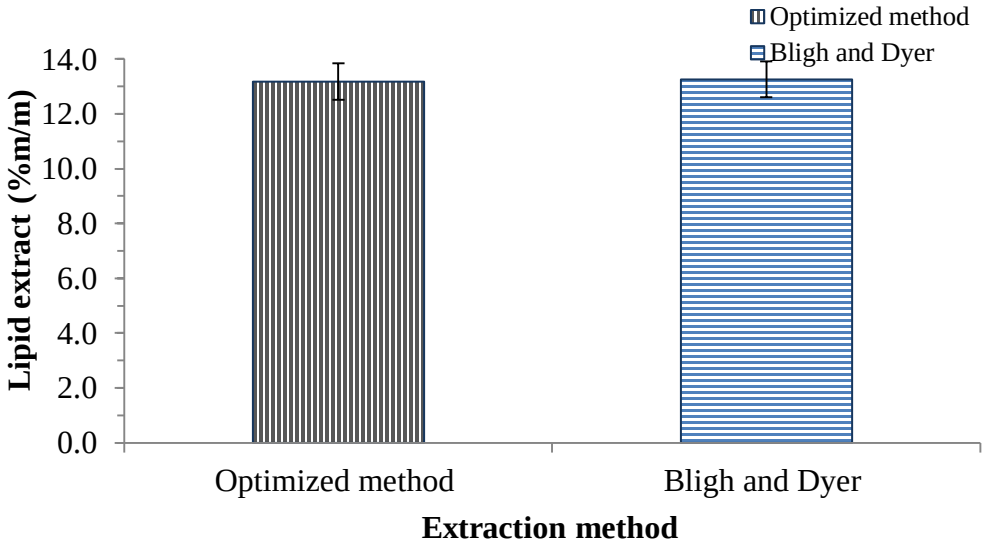


Figure 3.25 Comparison of the optimized and Bligh and Dyer method

3.3.4 Expeller press

This method could not provide any conclusive results for the analysis performed. The primary problem was with the method of presentation of the sample. The manufacturers of the instrument have used this instrument primarily for the extraction of oils from seeds with high oil content. Biomass used for this study under the best oil producing conditions can rarely produce more that 30% lipids. The design of the instrument is suitable for pelletized products with reasonably higher oil content than that produced by biomass used. Furthermore, the pelletizing process was considered to be very time consuming and expensive, hence it was not pursued.

3.4 Conclusions

- The thirteen solvents had varied extraction efficiencies for the extractions performed on the algal biomass. This characteristic would be linked to their polarities and their abilities to disrupt the algal cells at the boiling points of each solvent. Chloroform, ethanol and hexane produced the highest lipid yields for the single solvent extractions thus indicating the presence of polar and non-polar lipids. Further analysis by chromatography confirms the type of lipids being extracted by the selected solvents. This is shown in Chapter 4.

- A comparison of the soxhlet extraction method and the microwave assisted extraction method shows that MAE is superior in terms of the time it takes to complete an extraction. The optimized variable show that it takes 10 minutes to complete an extraction with the MAE method compared to 3 hours by the soxhlet method. The longer extraction time for the soxhlet method are likely to increase costs when considering the time it takes to complete an extraction and the volume of solvent required. The MAE method uses less than one third of the solvent volume equivalent of the soxhlet method. This would make the method more energy efficient or cost effective. The only problem may be the employment of microwave technology on a large scale.

- The binary mixture of 1:1 chloroform: ethanol showed the highest efficiency, producing a lipid quantity of 11.76% while the best single solvent, chloroform, produced 10.78%. The use of solvents involving greater than binary combinations are considered to be impractical for upscaling and would increase the costs of production of biodiesel.

- The oven-drying method is more cost efficient than the freeze-drying method. The only drawback of the method is that the biomass has to be pulverized prior to extraction. This added step requires some energy input, but it is overall far less than would be employed by the freeze-drying method. An option to employ the sun drying is available and involves virtually no cost, except for the fact that it may be time consuming (Guldhe *et al.*, 2013). Since there is very statistical advantage shown by either method, the cheaper option of oven drying may be preferred.

- The method optimized for extraction by MAE compares favourably with the Bligh and Dyer method with a less than 1% difference being obtained by the extraction protocols.

- The statistical design methods showed that it was possible to achieve a slightly higher quantity of lipid (~12.37%) when compared to the OFAT method using similar optimized parameters (~11.96%).

- Further research on the expeller technique has to be done before it can be considered as a viable method. This study was not able to provide any conclusive evidence of this method being viable as a method for the extraction of lipids.

- The quantities of chlorophyll extracted by the solvents do not have a negative impact on the production of biodiesel and are relatively small compared to the quantities of lipid extracted.

- The experiment conducted on the ageing of biomass show that samples may be safely retained for approximately three months with only a slight deterioration in the ability of the biomass to produce lipids for biodiesel. This was subject to biomass being stored under controlled conditions.

CHAPTER 4

IDENTIFICATION AND QUANTIFICATION OF LIPIDS AND ESTERIFICATION TO FAME

4.1 Introduction

The successful production of biodiesel requires that the fatty acids obtained from the lipid extracts are esterified. Before this process is carried out, the type of fatty acids found in the lipid extracts needs to be identified and quantified. The reason for identification is to establish the presence of fatty acids that are conducive to their conversion to biodiesel; and quantification is required to give an indication of the yields of biodiesel that may be produced.

The process of transesterification of lipids is carried out by both acid-catalysed and alkali-catalysed methods. This may involve both direct and in-situ methods. The direct method involves extraction, collection of the lipid extract and esterification of the lipid to produce biodiesel. The in-situ method requires the addition of algal biomass and the esterification reagents in a single vessel followed by filtration and purification or clarification steps to produce the biodiesel. Alcohols such as methanol, ethanol, propanol, butanol and pentanol may be used in the transesterification process. Methanol is preferred owing to its low cost and its desirable physical and chemical properties, i.e. polar and short chain alcohol (Dennis *et al.*, 2010).

The alkali-catalysed method is preferred since it is much faster than the acid-catalysed method and is more often used commercially. NaOH, KOH, and carbonates are also relatively easily dissolved in the alcohols (Helwania *et al.*, 2009; Vicente *et al.*, 2004). Acid esterification methods may take from 3 to 50 hours to

complete while alkali methods are faster and may take from 1 minute to approximately 3 hours (Schuchardta *et al.,* 1998). Alkali methods do however have a tendency to saponify the ester and form soap. It is therefore necessary to ensure that reactions conditions are controlled and ratios of catalyst and alcohol are carefully monitored. For an esterification method to be viable for upscaling, it has to be reasonably fast and produce satisfactory yields of biodiesel.

The simplest esterification method would involve the reaction of a triglycerol or triglyceride with an alcohol catalysed by an acid or a base to produce an ester and glycerol. This is illustrated by the general reaction below:

Triglyceride alcohol mixture of alkyl esters glycerol

Figure 4.1 A general reaction showing the conversion of a triglyceride to esters and glycerol after reaction with a basic alcoholic catalyst.

Since alkali methods tend to be more popular for the transesterification process, it was decided that trials would be run on selected alkali by varying their concentration in alcohol medium and then esterifying the lipids extracted with a view to obtaining the optimum concentration. The alkali considered and their concentrations are shown in section 4.2.1.1. After this experiment, a single acid catalysed method and two alkali catalysed methods were chosen for direct and in-situ transesterification (Laurens *et al.*, 2012).

The acid catalysed method employed the use of HCl/MeOH mixture while alkali-catalysed esterification methods included NaOH or KOH in methanol (Vicente *et al.*, 2004). The in-situ methods were investigated using selected catalysts. Methanol was used as the primary solvent for all the esterification processes. After esterification of

the extracted lipids was complete, the biodiesel yields were calculated. The reason for the choice of several esterification methods was to establish a suitable method for the production of biodiesel at a reasonable cost. In addition to this, it was necessary to determine which method produced the highest lipid yields and provided the greatest efficiency during the process. It was also essential to know what types of lipids were being produced by the biomass and whether they could be used to produce biodiesel that was of an acceptable standard, and was marketable.

Instrumental methods such as FTIR, GC and GC-MS have been found to be suitable for the identification and quantitation of both lipids and biodiesel produced from algal biomass. The FTIR (Fourier transform infra-red) method may be used for both qualitative and quantitative analysis of the fatty acids in lipids and esters in biodiesel. It is limited by the fact that it is only able to indicate the presence of compounds or give total quantities present in a sample. For the purpose of identifying individual fatty acids (FA) or fatty acid methyl esters (FAME), chromatography is most suitable (David, 2005; Ruppel and Huybrighs, 2008; Shimadzu-Corporation, 2012; Sigma-Aldrich, 1998). The FTIR method gave an indication of the presence of FAME collectively found in biodiesel (Perston and Harris, 2009; Shimadzu-Corporation, 2013). Hence, it was used as a preliminary measure to determine the quantity and type of fatty acids that were present in extracts.

Gas Chromatography is an established technique for the analysis of fatty acids and FAME. The GC-MS method, however, gives greater accuracy in both qualitative and quantitative analysis. Several researchers present the GC method as being adequate for the quantitation of fatty acids and FAME (Christie, 1990; Dodds *et al.*, 2005; Laakso *et al.*, 2002; Paik *et al.*, 2009; Xu *et al.*, 2010). Gas Chromatography was therefore used for the identification and quantitation of the extracted lipids and the biodiesel (as FAME) produced. The analysis was made up of two parts. The first was to identify and quantify the fatty acids in the lipid extracts. Individual fatty acid standards identified for biodiesel production were prepared for the identification and quantitation of the fatty acids in the lipid extracts. The second was the determination

of the fatty acid methyl esters. For the lipid analysis an eleven component standard (Table 4.3) was prepared from individual standards, while for the FAME analysis, a 37-component standard obtained from Sigma Aldrich containing a range of FAME standards was used (Figure 4.16). An internal standard, heptadecanoic acid methyl ester, was also used for quantitation. These standards were used to optimize the methods for the qualitative and quantitative determination of the lipids and biodiesel produced. The quantitation method employed in this chapter is unique to the algal samples used in this study.

4.2 Materials and Methods

4.2.1 Transesterification of lipids

All reagents used for transesterification were of analytical reagent grade or higher with assays >99%. Both acid and alkali (base) catalysed methods are used for analysis by many researchers (Demirbas, 2009; Jain *et al.*, 2011; Laurens *et al.*, 2012; Leung *et al.*, 2010; Miao *et al.*, 2009; Milinsk *et al.*, 2008) and there are many procedural similarities and some differences evident amongst them. After due consideration, a modified generic method was used ensuring analytical suitability for use under local conditions. Control samples using sunflower (cooking) oil were analysed concurrently with the lipid extracts from biomass. It should be noted that since lipid extracts obtained in this study were in the region of 5-20% of biomass used, the sample sizes for esterification were limited by these quantities. High quality standards for chromatography were either prepared or diluted for optimizing analytical conditions used for quantifying both lipid and biodiesel samples (Table 4.3).

4.2.1.1 Direct transesterification of lipids

This procedure involved the addition of alcoholic catalysts to the lipid extract. Initial trials were conducted using five bases. They were sodium hydroxide (NaOH), potassium hydroxide (KOH), sodium carbonate (Na_2CO_3), sodium bicarbonate

($NaHCO_3$) and potassium carbonate (K_2CO_3). Sodium methoxide (CH_3ONa) was considered as a sixth option, but despite claims by Demirbas (2008), that the use of this catalyst provided a very rapid esterification method, the method was not used. The reason for this was that it would be extremely hazardous to prepare this catalyst if the method was considered for upscaling. For the five bases chosen, alcoholic solutions were prepared in concentrations of 0.5%, 1.0%, 1.5% and 2.0% of each base in methanol. However upon investigation, only NaOH and KOH were found to be totally soluble in methanol at room temperature at all the concentrations. The other bases were either not soluble, partially soluble or required heat for dissolution. Thus only NaOH and KOH were used for the esterification optimization experiment.

The transesterification procedure used was a modification of that reported by Addison (2012) who has successfully produced biodiesel from vegetable oils on a large scale. In this study, trials were conducted to establish the catalyst concentration that produced an optimum yield of biodiesel. Various concentrations of alkali catalysts in alcohol were used to esterify sunflower (cooking) oil during the optimization trials. This oil was used as small quantities of lipid extract did not enable simultaneous analysis of the numerous analyses conducted. The following procedure was adapted and modified from the works of several researchers (Addison, 2012; Anastopoulos *et al.*, 2009; Demirbas, 2008; Jain *et al.*, 2011; Lepage and Roy, 1986; Leung *et al.*, 2010; Miao *et al.*, 2009; Milinsk *et al.*, 2008; Schuchardta *et al.*, 1998).

The procedure was as follows:
For the optimization of the basic alcoholic catalyst concentration, an appropriate mass of NaOH was weighed into a small beaker and about 50 mL of methanol was added. This was stirred until the base dissolved completely. After allowing the mixture to cool it was transferred quantitatively into a 100 mL volumetric flask and the solution brought up to the calibration mark with methanol, stoppered and homogenized. An aliquot of 10 mL of the sunflower oil was transferred into a 150 mL beaker using a pipette. The oil was then placed on a hot plate and a stirrer bar

introduced into the beaker. The stirrer was set to rotate at approximately 400 rpm and the heat set to warm the oil to between 55ºC to 60ºC. The temperature was monitored with a thermometer. This was done with four beakers simultaneously, each placed on a hot plate. When the temperature was stable at approximately 55ºC ± 2ºC (approximately 10 min.), 2 mL of the alcoholic NaOH was added to the beaker, each concentration in a different beaker. With the temperature maintained as set, the mixture was stirred for two hours to ensure complete esterification. The hot plates and stirrers were then turned off and the solutions left overnight to allow the glycerol to settle. The next day an aliquot of the supernatant (biodiesel) was removed and transferred to a small vial for GC analysis. The determined optimum value was compared with the values obtained using the same concentration of KOH catalyst as this has also been suggested as a more popular catalyst (Farag *et al.*, 2012).

Further direct transesterification of samples was conducted using a method modified from Laurens et al, (2012) where scales for quantities were expanded. The analyses were performed on lipid extracts while sunflower oil was also tested as a control sample. Both acid and alkali catalysed methods were evaluated. The specifications for the reagents are shown in Table 4.1:

Table 4.1 List of reagents used for transesterification

REAGENT	GRADE	ASSAY (%)	DENSITY (g/mL)	SUPPLIER
HCl	AR	37.0	1.19	Sigma Aldrich
NaOH	AR	> 98.0	-	Sigma Aldrich
KOH	AR	≥ 86.0	-	Sigma Aldrich
Methanol	HPLC	99.9	0.792	Sigma Aldrich

All weighing was carried out on an analytical balance reading to four decimal places. All volumetric reagents of fixed aliquots were transferred using a pipette, micropipette or graduated measuring cylinder as required. All solutions were prepared under as sterile conditions as possible. All safety protocols were observed during the preparation of solutions.

The procedure was as follows:

1. For the acidic alcoholic catalyst, 5 mL of HCL was carefully added to approximately 50 mL of methanol in a beaker. After allowing the mixture to cool it was transferred quantitatively into a 100 mL volumetric flask and the solution brought up to the calibration mark with methanol, stoppered and homogenized.
2. For the base alcoholic catalyst, the appropriate mass of NaOH (or KOH) was weighed into a small beaker and approximately 50 mL of methanol was added. This stirred until the base was dissolved completely. After allowing the mixture to cool it was transferred quantitatively into a 100 mL volumetric flask and the solution brought up to the calibration mark with methanol, stoppered and homogenized.
3. A pre-determined mass (1.00 g) of the lipid extract was weighed accurately into a 150 mL beaker. The same procedure was repeated for two more beakers.
4. Into three other beakers, 1.00 g of sunflower oil was weighed and set aside to be used as a control samples.
5. 30 mL of methanolic HCl was added to one beaker containing the lipid extract and another beaker containing the sunflower oil.
6. 1 mL of the methanolic NaOH and 1 mL of the methanolic KOH was added separately to each of the other beakers containing lipid extracts and sunflower oil. A minimum alcohol to lipid ratio of 6:1 was used throughout to ensure complete transesterification as suggested by Anastopoulos et al (2009) and Leung et al (2010).
7. All beakers were transferred to separate hot plate stirrers. A stirrer bar was introduced to each beaker and stirring set at 400 rpm. The temperature controls were set to heat each beaker to between 55ºC and 60ºC. The samples were left to esterify for 2 hrs.
8. On completion, an aliquot of the supernatant ester from each sample was transferred to small vials for GC analysis.

4.2.1.2 In-situ transesterification using MAE

This procedure is a variation of the general direct procedure used for transesterification. The starting material was algal biomass. The method used for this transesterification is an adaptation of the acid catalysed method used by Laurens et al. Protocol has been changed to include the methods optimized by this study. The transesterification was conducted in-situ using a microwave oven. The following procedure was modified after studying the work of selected researchers (Ehimen *et al.*, 2010; Laurens *et al.*, 2012).

The procedure was as follows:

1. A pre-determined mass of 1.00 g of biomass was weighed accurately into each of three teflon vessels.
2. An equivalent mass (1.00 g) of sunflower oil was weighed into one teflon vessel for the control.
3. An aliquot of 30.00 ml of the binary mixture chloroform: ethanol, 1:1 was added to each vessel.
4. Using a measuring cylinder, 30 mL of 5% methanolic HCL was added to one of the vessels containing lipid extracts and the vessel containing the sunflower oil.
5. A micropipette was used to transfer 1.00 mL of 1.5 % methanolic NaOH and KOH separately to each of the remaining vessels with lipid extract.
6. An aliquot of 5 mL of the internal standard, methyl heptadecanoate (1mg/mL) was added to each of the vessels.
7. After the samples were prepared, the teflon vessels were sealed and transferred to the carousel and then placed in the microwave oven.
8. The above analyses were conducted in duplicate as only nine vessels were available.

9. The following table gives an indication of the procedure used for these transesterifications:

Table 4.2 Reagents added for transesterification for algal biomass lipids and sunflower oil (control sample)

	Algal biomass lipids			**Sunflower oil**
Reagent added	**1**	**2**	**3**	**4**
30 mL $CHCl_3$: EtOH, 1:1	✓	✓	✓	✓
30 mL HCl in MeOH	✓	✗	✗	✓
1 mL 1.5% NaOH	✗	✓	✗	✗
1 mL 1.5% KOH	✗	✗	✓	✗
Internal Standard	✓	✓	✓	✓

✓ = added

✗ = not added

10. The following parameters were used in a microwave program for the transesterification:

Power:	1600 W
Power %:	100
Initial heating ramp time:	5 min
Temperature of analysis:	100ºC
Hold time:	10 min.
Cooling time:	5 min.

11. After the transesterification was complete, the vessels were removed and all samples filtered using a Buchner funnel, flask and vacuum filtration to separate the biomass from the esterified mixture. The dark brown mixture was then decolourized using activated charcoal to produce a light brown biodiesel mixture.

12. The solvent was then evaporated on a water bath to about 20 mL. Thereafter 5 mL of hexane was added to the mixture to dissolve the FAME, stirred and left for one hour.
13. The collected samples were allowed to stand overnight. A small aliquot of the supernatant was siphoned off and transferred to a vial for gas chromatography.

It should be noted that trials were conducted using various methods before the methods above were adopted for transesterification of the sample extracts. These methods are not shown (Farag *et al.*, 2012; Laurens *et al.*, 2012; Lepage and Roy, 1986).

4.2.2 FTIR analysis

This method was has been used by various researchers as a rapid method for the determination of biodiesel blends, oils or lipids (Dean *et al.*, 2010; Higgins, 2012; Perston and Harris, 2009; Shimadzu-Corporation, 2013). Analyses conducted for this study made use of the Varian FTIR and Varian Resolution software (Australia). This method was examined for its feasibility as a rapid qualitative method for lipids extracted from biomass produced at our local raceway pond.

The procedure involved the following:

The attenuated total reflectance (ATR) cell was cleaned with ethanol and a background scan obtained on an empty cell. Thereafter, the reference extraction solvent was analyzed by placing 2 -3 drops of the solvent onto the zinc selenide cell of the ATR. A full scan was performed between wavenumbers 700 cm^{-1} and 4000 cm^{-1} at a resolution of 4 cm^{-1}. The cell was then cleaned with ethanol and 2-3 drops of the lipid extract was placed on the cell and a scan completed as before. The scans were then printed and evaluated. . It should be noted that all samples were analyzed at the different stages when the samples were available or prepared.

The experimental method was conducted as follows:

1. A blank/ background scan was performed on an empty ATR zinc selenide cell in the chosen wavelength range (Appendix C).

2. Scans were performed on all solvents used for the extraction of lipids. These scans were used as reference during the measurement of lipid extracts to nullify the effect of solvent absorption.
3. A thin film of crushed dry biomass was placed on the ATR cell. This was then placed in the sample compartment of the FTIR and scanned (Figure 4.1)
4. A control sample of sunflower (cooking) oil was analyzed by adding a drop of the oil to a cleaned ATR cell and a scan was completed in the range of measurement (Figure 4.3).
5. Lipid extracts for chloroform, ethanol, hexane (Appendix C), the FAME standard (Figure 4.4), a binary mixture comprising of chloroform: ethanol (1:1) lipid extract using MAE and its esterified sample were also analyzed in a similar manner (Figures 4.5 and 4.6).

4.2.3 Identification and quantitation of lipids by chromatography

Chromatography was used for the identification and quantification of the lipids present in the algal biomass. Eleven standards were chosen after carefully surveying the works of several researchers (Buchanan, 2008; Christie, 1990; David, 2005; Oveisi *et al.*, 2006; Ruppel and Huybrighs, 2008). The lipids selected were those identified by these researchers as algal lipids which favour the production of biodiesel. Table 4.3 gives a breakdown of the various properties of these lipids.

Each fatty acid standard was prepared separately at a concentration of 100 mg/L. A combined standard containing all the fatty acids was also prepared in the same concentration. The single standards were used to determine the retention times of each standard so that they could be identified in the mixed standard used for the external standard method of calibration. The data can be seen in Table 4.4 and the chromatogram in Figure 4.7.

Table 4.3 Common biodiesel fatty acids and their properties

No.	Common Name of Fatty Acid	IUPAC Name of Fatty Acid	Chemical formula	Assay %	Molar Mass (g/mol)	Melting point (range ^{o}C)	Boiling Point (^{o}C)	Solid/ Liquid	Saturated/ Unsaturated
1	Butyric acid	Butanoic acid	$C_4H_8O_2$	>99.5	88.11	-6 to -3	162	L	Satd
2		Undecanoic acid	$C_{11}H_{22}O_2$	>99	186.29	28 - 31	248 - 250	S	Satd
3	Lauric acid	Dodecanoic acid	$C_{12}H_{24}O_2$	>98	200.32	44 - 46	225	S	Satd
4		Tridecanoic acid	$C_{13}H_{26}O_2$	>99	214.34	41 - 42	236	S	Satd
5	Myristic acid	Tetradecanoic acid	$C_{14}H_{28}O_2$	>99	228.37	52 - 54	250	S	Satd
6	Palmitic acid	Hexadecanoic acid	$C_{16}H_{32}O_2$	>99	256.43	61- 62.5	271.5	S	Satd
7	Linolenic acid	methyl cis,cis,cis-9,12,15octatrienoic acid	$C_{18}H_{30}O_2$	>98.5	278.43	-11	230 - 232	L	Unsatd
8	Linoleic acid	9,12-octadecadienoic acid	$C_{18}H_{32}O_2$	>99	280.46	-5	229 - 230	L	Unsatd
9	Oleic acid	cis-9-octadecenoic acid	$C_{18}H_{34}O_2$	>99	282.47	13 -14	194 -195	L	Unsatd
10	Stearic acid	Octadecanoic acid	$C_{18}H_{36}O_2$	>99.5	284.48	67 - 72	361	S	Satd
11	Arachidonic acid	cis-5,8,11,14-eicosatetraenoic acid	$C_{20}H_{32}O_2$	>95	304.46	-49	169-171	L	Satd

The method for analysis was optimized after running trials and examining the results from various temperature programs for analysis. The optimized test conditions determined for the identification and quantification of lipids using a GC with an FID were as follows: A temperature program with an initial temperature of 200^{o}C, a hold time of 2 minutes, a ramp rate of 5^{o}C/minute and a final temperature of 240^{o}C held for 5 minutes was used. The Injector temperature was set at 220^{o}C and the Detector temperature was set at 250^{o}C. Nitrogen was used as a carrier gas with a split ratio of 1/100. The volume of sample injected was 1µL. The details of the instrument and the parameters used are in Appendix C.

The GC gas line pressures for oxygen, nitrogen and hydrogen were set as per the manufacturer's specifications. The conditions listed above were programmed on the GC and stabilized before analysis was performed. 1µL of the composite 11-component standard was first injected into the GC to establish the average standard

curve (Figure 4.7). Lipid extracts were filtered through a 0.45 µm filter, collected and then analyzed on the GC using a manual injection procedure. All standards and samples were analyzed in triplicate. Since the chloroform, ethanol and hexane extracts were identified from the extractions as the three solvents that produced the highest lipid quantities, they were analysed by chromatography to determine their fatty acid profiles. The lipid profiles for the binary mixtures using both SOX and MAE techniques were also obtained in the same manner. Table 4.5 gives a breakdown of the lipid profile and their quantities. Figures 4.8 to 4.12 show the chromatograms produced by these solvents.

4.2.4 Identification and quantitation of FAME (biodiesel) by chromatography

A second gas chromatograph was used for quantitative analysis of biodiesel (FAME) produced. 1µL of the composite 37-component standard was first injected into the GC to establish standard curves and optimize the instrument. An internal standard method was used to quantify the biodiesel. The optimized test conditions determined for the identification and quantification of FAME using a GC with an FID were as follows: A temperature program with an initial temperature of 60^{o}C, a hold time of 2 minutes, an initial ramp rate of 10^{o}C /minute up to 100^{o}C with no hold time, followed by a further ramp rate of 7^{o}C/minute to a final temperature of 240^{o}C held for 1 minute was used. The Injector temperature was set at 240^{o}C and the Detector temperature was set at 250^{o}C. Nitrogen was used as a carrier gas with a split ratio of 1/50. The volume of sample injected was 1µL. Heptadecanoic acid methyl ester was used as an internal standard. The details of the instrument and the parameters used are in Appendix C.

Gas pressures to the GC were set as per the manufacturer's specifications. The above conditions were programmed and stabilized on the GC before analysis commenced. 1µL of the composite 37-component standard was first injected into the GC to establish the standard curves. A minimum of ten runs was used to establish the average retention times shown by the optimized chromatogram in Figure 4.13. The data is included in Appendix C. The esterified samples were clarified and filtered

through a 0.45 µm filter, collected and then analyzed on the GC using a manual injection procedure. All standards and samples were analyzed in triplicate. The internal standard method was used for quantitation of the esters.

4.3 Results and Discussion

4.3.1 FTIR analysis

This method was chosen because it was cited as a reasonably fast method for determining the presence of fatty acids and biodiesel using suitable standards (Dean *et al.*, 2010; Miglio *et al.*, 2013; Perston and Harris, 2009; Shimadzu-Corporation, 2013). It cannot, however, replace gas chromatography which is more efficient at quantifying individual fatty acids and esters. Although this analysis was not part of the objective of this study it was performed as an adjunct to chromatography. It is for this reason that only five FTIR scans are shown here. Typical scans were conducted on the dried biomass, MAE lipid extract, ester of MAE lipid extract, a FAME standard and a control sample, sunflower oil, are shown below. A blank scan of the ATR cell is shown in Appendix C. This analysis was conducted to ascertain whether this method was suitable for determining the presence of fatty acids in the lipid extracts and esters in biodiesel. It should be noted that all solvents were used as reference.

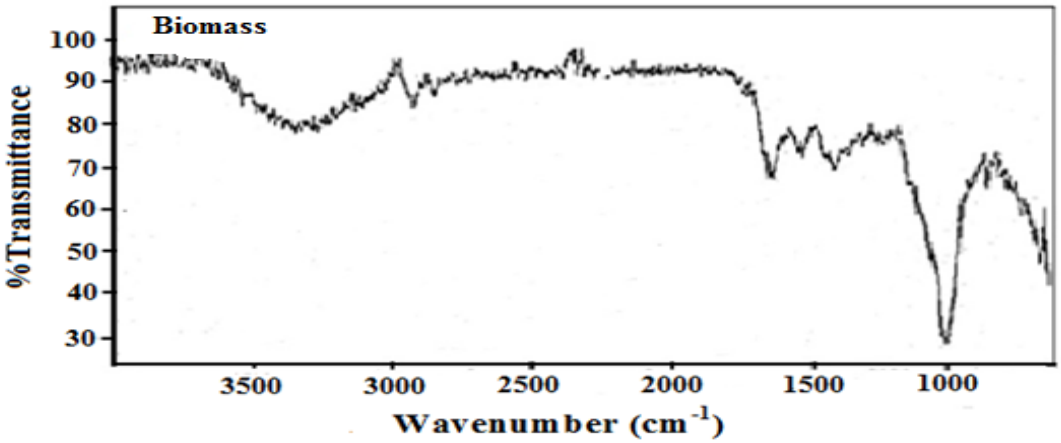


Figure 4.2 FTIR scan of dried algal biomass.

The scan in Figure 4.2 shows that a wide absorption band, characteristic of O-H stretching in carboxylic acids (in this case, fatty acid) is present in the region 3300–

2500 cm^{-1}. A further carbonyl stretch, C=O of a carboxylic acid appears as a lower intensity band in the region 1760-1690 cm^{-1}. A higher intensity band in this region can be observed for the control sample, sunflower oil (unsaturated) in Figure 4.3. The exact position of this band depends on whether the carboxylic acid is saturated or unsaturated, or has internal hydrogen bonding.

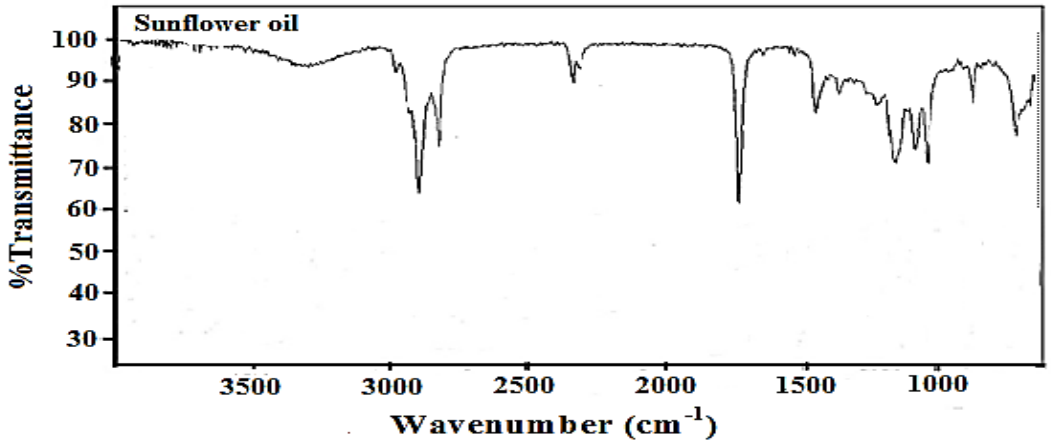


Figure 4.3 FTIR scan of sunflower oil (cooking oil).

Bands present in the range 1300-1000 cm-1 show characteristics of C-O stretching in esters. This may also be indicative of the presence of triglycerides in these samples.

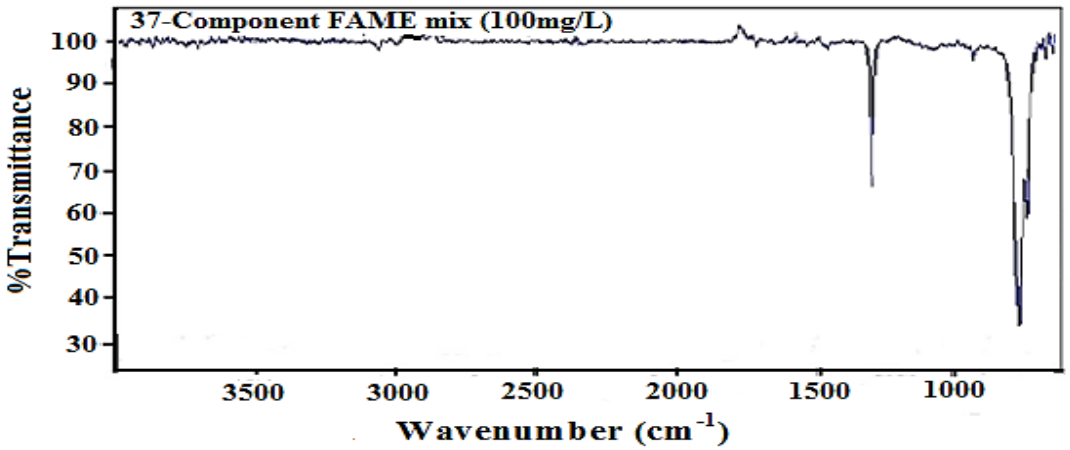


Figure 4.4 FTIR scan of 37-component FAME standard.

The above scan (Figure 4.4) of the FAME standard shows only a single band in the range 1300-1000 cm^{-1}. This is characteristic of C-O stretching in esters. The band in

the region of 730 cm^{-1} is indicative of C-H bending in long chain C-H bonds. The absence of the esters in their typical range of 1750-1730 cm^{-1} in the standard may be explained by the low concentration of the standard used. The 1000 mg/L standard which was also analyzed showed a similar profile indicating that a detection limit needs to be determined for this method. Similar scans can be observed for chloroform, ethanol and hexane extracts (Appendix C).

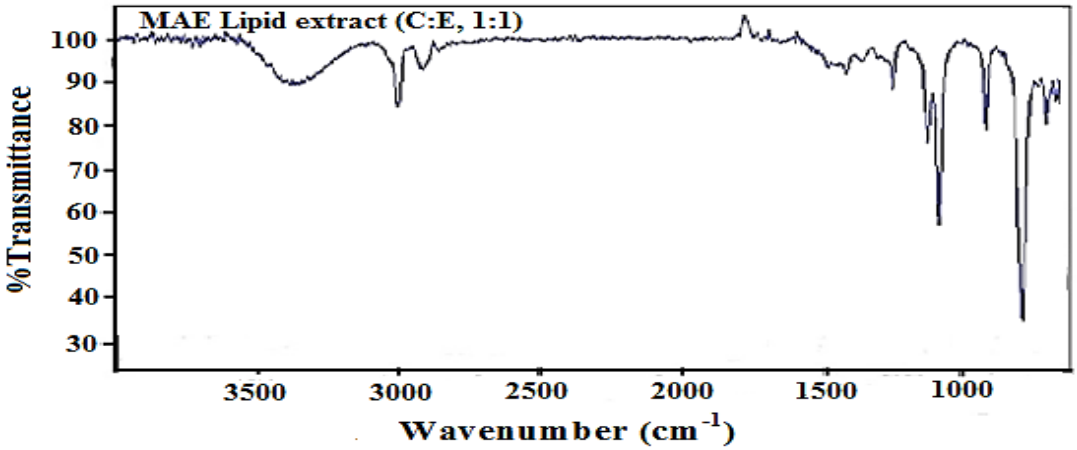


Figure 4.5 FTIR scan of MAE lipid extracted by the binary mixture chloroform: ethanol, 1:1.

The diagrams (Figure 4.5 and 4.6) show the scans of lipid extract using the MAE and an esterified sample of the same extract. The spectra of fatty acids (Figure 4.5) have strong absorptions between 3000-2800 cm^{-1}, and also in the fingerprint region of 1500-700 cm^{-1}. The absorptions observed in the region of 3000-2800 cm^{-1} are attributed to the asymmetric C-H stretching and symmetric C-H stretching of methyl and methylene groups. A characteristic broad band in the 3300-2500 cm^{-1} region, assigned to the hydrogen bonded O-H of most carboxylic acids, overlaps the C-H stretching region. Common bands for all short-chain fatty acids in the finger print region were observed between 1400-930 cm^{-1} indicating C-O-H in-plane bending, C-O stretching, and O-H out-of-plane bending, respectively (Koca *et al.*, 2007).The above spectra for algal biomass and its extract show similar characteristics to those found by Miglio et al, 2013.

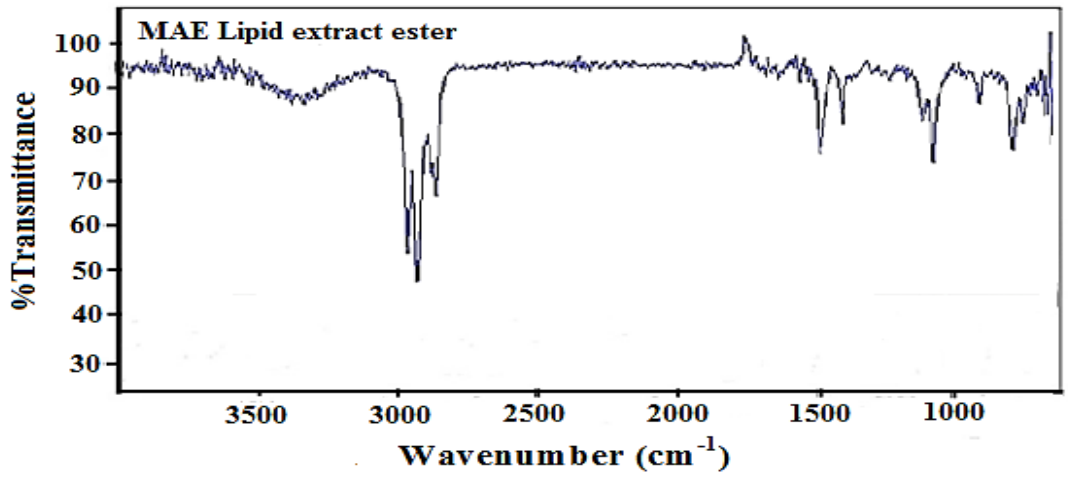


Figure 4.6 FTIR scan of MAE ester from lipid extracted by the binary mixture chloroform: ethanol, 1:1.

In Figure 4.6, the fingerprint region shows evidence of C-O stretching between 1300-1000 cm^{-1}. Strong absorption bands between 3000-2800 cm^{-1} can be seen. These are characteristic of C-H stretching of alkyl groups. The absence of the C=O stretch probably indicates the absence or very low concentrations of unsaturated esters. This, however, contradicted by the chromatographic method (see Table 4.5). Although many researchers ((Dean *et al.*, 2010; Perston and Harris, 2009; Seelenbinder and Higgins, 2011) have tested the feasibility of the FTIR method for the analysis of lipids and biodiesel blends, a detailed study needs to be conducted and validated before it can be used locally.

4.3.2 Chromatographic analysis

4.3.2.1 Lipids extracted

Table 4.4 shows the average retention time optimized for each FA. It also shows the relative quantities of each standard in the mixed standard (100 mg/L) prepared. Figure 4.7 is a chromatogram of the standards optimized during calibration of the GC using the external standard method.

Table 4.4 Retention times and areas obtained for the eleven FFA standards (100mg/L) analyzed by Gas Chromatography

#	Common name	IUPAC name	Time (min.)	Area (µV.min)	Area% (%)
1	Butyric acid	Butanoic acid	1.30	57.97	4.62
2	Undecylic acid	Undecanoic acid	3.42	118.44	9.433
3	Lauric acid	Dodecanoic acid	4.19	119.05	9.48
4	Tridecylic acid	Tridecanoic acid	5.09	122.61	9.77
5	Myristic acid	Tetradecanoic acid	6.13	117.21	9.34
6	Palmitic acid	Hexadecanoic acid	8.48	112.36	8.95
7	Stearic acid	Octadecanoic acid	10.43	119.8	9.55
8	Oleic acid	cis-9-octadecanoic acid	11.11	96.99	7.73
9	Linoleic acid	9,12-octadecadienoic acid	11.56	137.71	10.97
10	Linolenic acid	methylcis, cis,cis-9,12,15-octatrienoic acid	12.44	137.23	10.93
11	Arachidonic acid	cis, cis,cis-,8,11,14-eicosatetraenoic acid)	13.75	116.01	9.24
	Total			**1255.42**	**100.00**

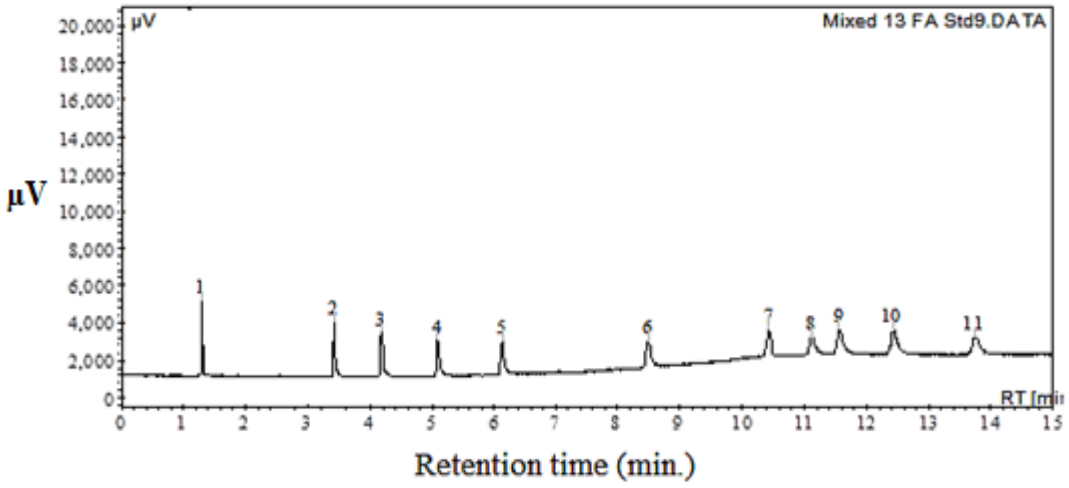


Figure 4.7 Chromatograph of eleven standards used for calibration of the gas chromatograph prior to analysis of extracts.

All chromatographic peaks were reintegrated after omitting the solvent peak. The fatty acid standards chosen identify with those that are suitable for the production of biodiesel (Karow, 2012; Knothe, 2007). The choice of column was made after

studying the choice of various researchers. The ZB-FFAP column used for this analysis has a high polarity. Changing the polarity of a polyester phase does not change the order of elution of components within a given chain-length group, but it can affect the elution order relative to components with other chain lengths. (Buchanan, 2012; Phukan *et al.*, 2011; Weyer *et al.*, 2009).

The quantities of lipids extracted (Table 4.5) and the separation profiles (Figure 4.8, 4.9 and 4.10) indicate that the fatty acids elute in decreasing polarity order and also with saturated fatty acids eluting before unsaturated fatty acids. The three chromatograms for chloroform, ethanol and hexane identify the lipids extracted by each of the solvents using the soxhlet method. Table 4.5a gives a list of the lipids extracted by each solvent.

Table 4.5a Chromatographic quantitation of FA extracted with chloroform, ethanol, hexane and a binary mixture

Lipid profiles obtained using GC analysis (HP5890A)							
No.	**Name**	**Abbreviation**	**Chloroform**	**Ethanol**	**Hexane**	**Chloroform: Ethanol (1:1)**	
						SOX	**MAE**
1	Butyric acid	C4:0	0.9	0.4	6.3	2.5	0.3
2	Undecanoic acid	C11:0	ND	0.1	ND	2.5	2.8
3	Lauric acid	C12:0	0.1	0.2	ND	4.0	0.5
4	Tridecanoic acid	C13:0	0.1	ND	ND	6.9	8.3
5	Myristic acid	C14:0	0.4	0.4	ND	3.8	0.8
6	Palmitic acid	C16:0	24.9	27.7	33.7	11.0	12.2
7	Stearic acid	C18:0	1.6	1.8	ND	9.1	8.0
8	Oleic acid	C18:1	1.4	1.3	0.4	13.7	12.1
9	Linoleic acid	C18:2	29.3	26.8	33.9	9.0	10.1
10	Linolenic acid	C18:3	9.6	10.6	12.3	17.2	22.5
11	Arachidonic acid	C20:4	12.1	12.3	10.8	0.7	1.2
	Sub Totals	**(Identified Lipids)**	**80.4**	**81.6**	**97.5**	**80.4**	**78.8**
	Sub Totals	**(Unidentified Lipids)**	**19.6**	**18.4**	**2.5**	**19.6**	**21.2**
	Total		**100**	**100**	**100**	**100**	**100**
	ND = not detected						

The following chromatograms show the extraction profiles of the single solvents and binary solvent used in the extraction of lipids.

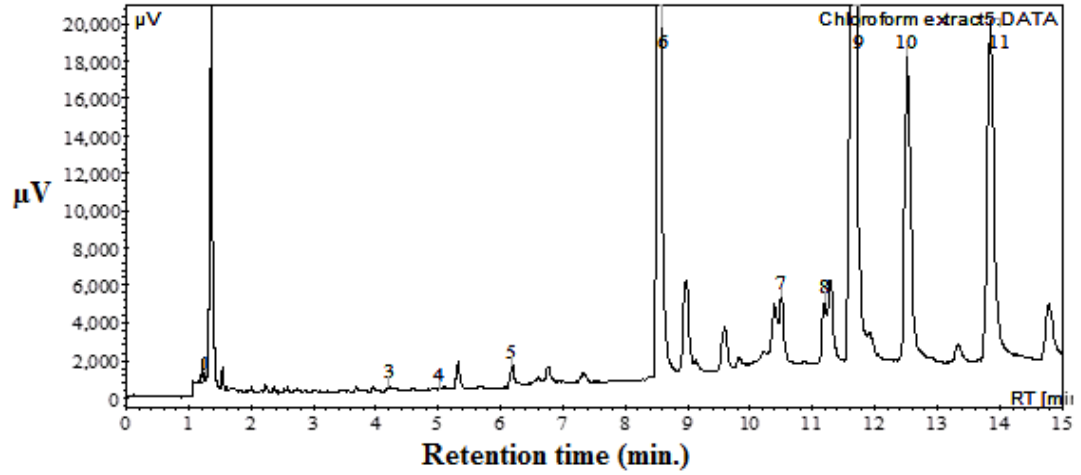


Figure 4.8 Chromatogram of chloroform lipid extract by SOX.

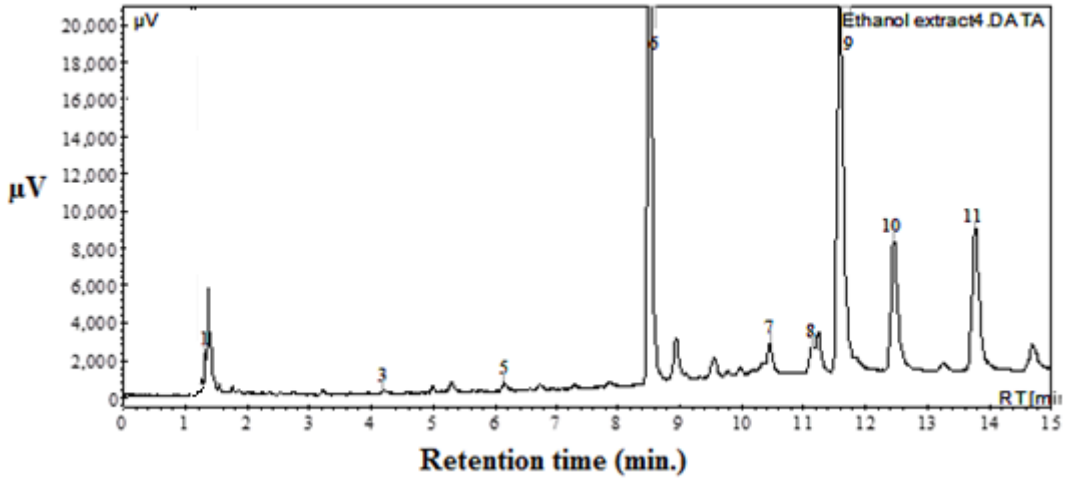


Figure 4.9 Chromatogram of ethanol lipid extract by SOX.

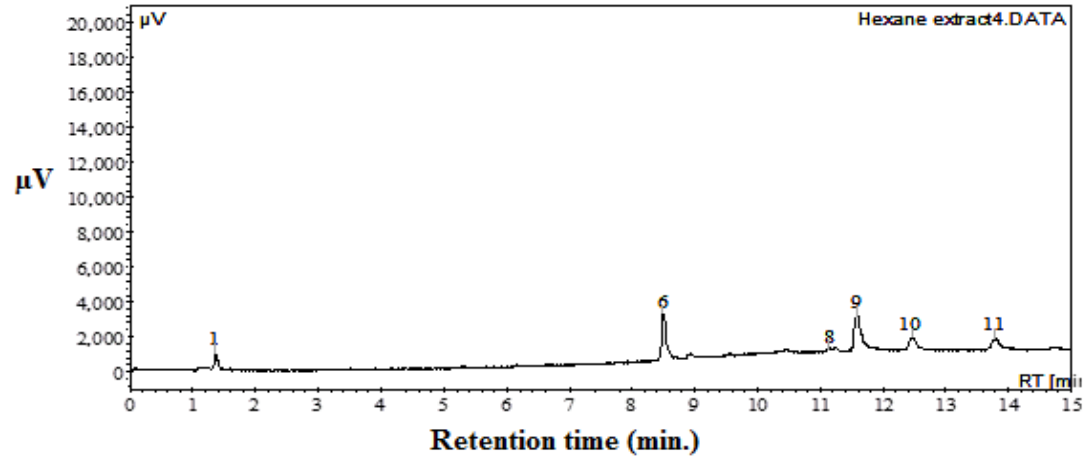


Figure 4.10 Chromatogram of hexane lipid extract by SOX.

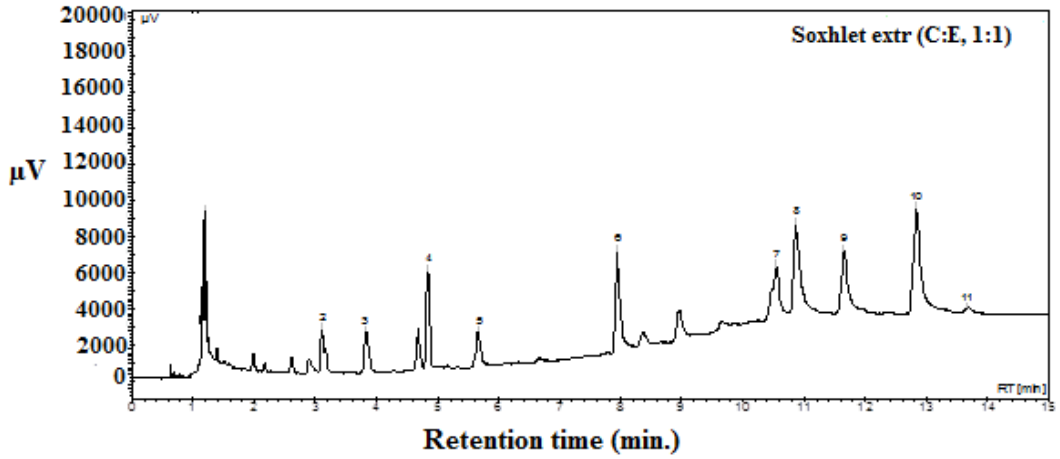


Figure 4.11 Chromatogram of lipid extracted using Chloroform: Ethanol (1:1) by the SOX method.

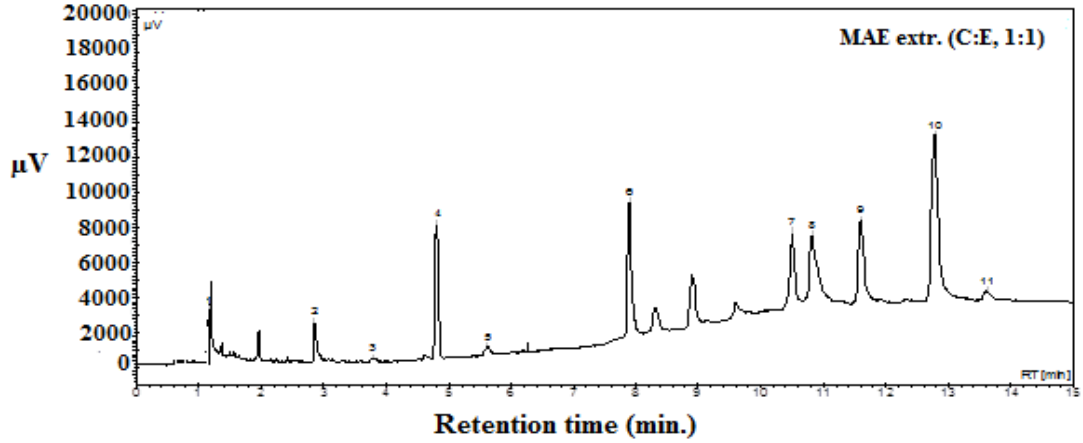


Figure 4.12 Chromatogram of lipid extracted using Chloroform: Ethanol (1:1) by the MAE method.

The chromatograms, Figures 4.8 to 4.12, illustrate the number and types of lipids extracted by the each solvent hence showing the efficiency of the solvent in extracting the range of lipids found in the biomass analyzed. The chromatograms are integrated to the same scale. Chloroform performs marginally better as an extraction solvent when combined with ethanol than as an individual solvent. The extraction profiles for the three single solvents and the binary mixture for soxhlet and microwave extraction appear to be very similar. On closer examination, it can be seen that although hexane appears to produce the largest percentage of identifiable lipids, it is the only solvent that shows no presence of five of the saturated fatty acids listed as essential for biodiesel production. However, approximately one-third of its

extract is made up of palmitic acid (SFA) probably indicating that this fatty acid makes up a large component of the non-polar fatty acids found in the extract. This, therefore, would make hexane unsuitable as a solvent for extraction when used alone despite it being used or preferred by many researchers (Lee *et al.*, 2010; Ryckebosch *et al.*, 2011; Samorì *et al.*, 2010). Paula and Roberto (2011). These researchers cite hexane as a preferred solvent for extraction of lipids in food products, but its toxic value may limit its use. However, in the context of producing biodiesel, it may not have as great a negative influence as it does in food. Chloroform and ethanol could be used effectively to extract lipids as single solvents despite having a relatively low percentage (1.5%) of monounsaturated fatty acids when compare to these solvents being used in combination (~12-14% MUFA, Table 4.5b). The binary solvent mixture is also capable of extracting the entire range of saturated and unsaturated fatty acids (C_4 to C_{20}) shown on Table 4.5a.

Table 4.5b Quantities obtained for the FA extracts for chloroform, ethanol, hexane and the binary mixture analysed by gas chromatography

	Saturated and Unsaturated lipid extracts				
				Chloroform: Ethanol (1:1)	
	Chloroform	**Ethanol**	**Hexane**	**SOX (C:E 1:1)**	**MAE (C:E 1:1)**
SFA	28.0	30.6	40.0	39.8	32.9
MUFA	1.4	1.3	0.4	13.7	12.1
PUFA	51.0	49.7	57.0	26.9	33.8
Total Unsaturated FA	52.4	51.0	57.4	40.6	45.9

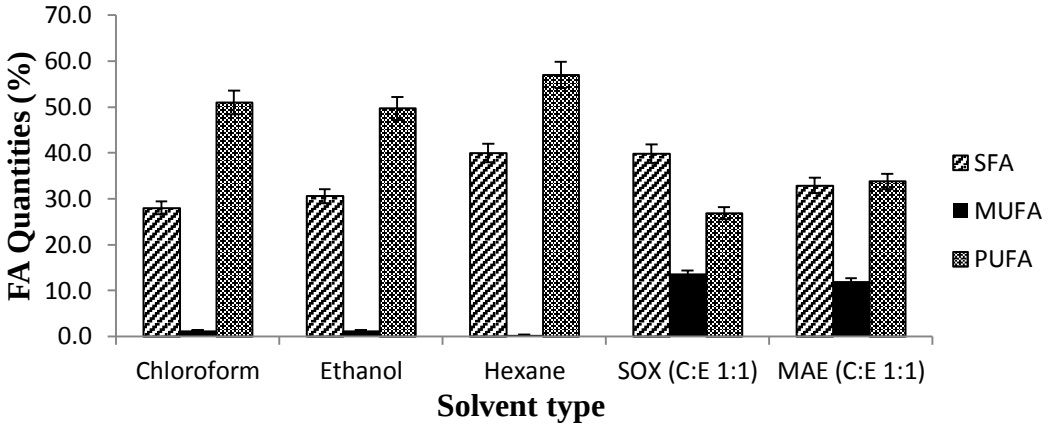


Figure 4.13 Graph showing SFA, MUFA and PUFA extracted from algal biomass by three solvents and binary solvents using two extraction methods.

There are three main types of fatty acids present in triglyceride, viz., saturated (SFA, Cn: 0), monounsaturated (MUFA, $C_{n:1}$) and polyunsaturated (PUFA, $C_{n:2,3}$). The percentages or quantities may differ depending on the type of oil or oil source (Koria and Nithya, 2012). The quality of fuel (biodiesel) is reflected by the composition of these fatty acids. The presence of low levels of SFA and PUFA and high levels of MUFA exhibit properties of high quality biodiesel (Knothe, 2007). Based on this premise, extracts of lipids by single solvents would not produce as high a quality of biodiesel as those extracts produced by binary solvents. Table 4.5b shows a higher percentage of MUFA (>12%) produced by the binary mixture, chloroform:ethanol (1:1), as compared to single solvents, chloroform, ethanol and hexane (<1.5%). It has become apparent that a balance between the ratio of saturated and unsaturated fatty acids has to be maintained for the oil or lipid to be used as feedstock for biodiesel production (Sharma *et al.*, 2008). Based on the results shown in Figure 4.13, this would be best satisfied by binary solvent extraction using either the SOX or MAE method.

4.3.2.2 Transesterification by catalysis with varied NaOH concentrations

The purpose of this experiment was to determine which esterification methods would be feasible for the production of biodiesel from the algal lipids extracted previously. Although many transesterification procedures are available, they have not been tested specifically for the type of samples used here (Farag *et al.*, 2012; Laurens *et al.*, 2012; Leca *et al.*, 2010; Lepage and Roy, 1986; Patil *et al.*, 2011; Schuchardta *et al.*, 1998; Vyas *et al.*, 2010). It is a fact that many esterification procedures for lipids abound in literature. It was for this reason that several esterification trials were conducted on various methods used in literature, before a decision was taken to optimize the methods carried out in this study. Since base esterifications were conducted at various concentrations, KOH and NaOH were selected after the preliminary study of a range of bases (see Material and Methods, section 4.2.1.1).
The concentrations chosen were in the range 0.5% to 2.0% m/v.

Table 4.6 Quantities of SFA, MUFA and PUFA obtained with varied concentrations of NaOH as catalyst

Optimization of base esterification (NaOH 0.5-2.0%)				
	NaOH concentrations			
Concentrations	**0.5%**	**1.0%**	**1.5%**	**2.0%**
SFA	0.2	2.2	7.9	15.3
MUFA	9.4	1	10.6	3.4
PUFA	0	17.5	31.5	20.5

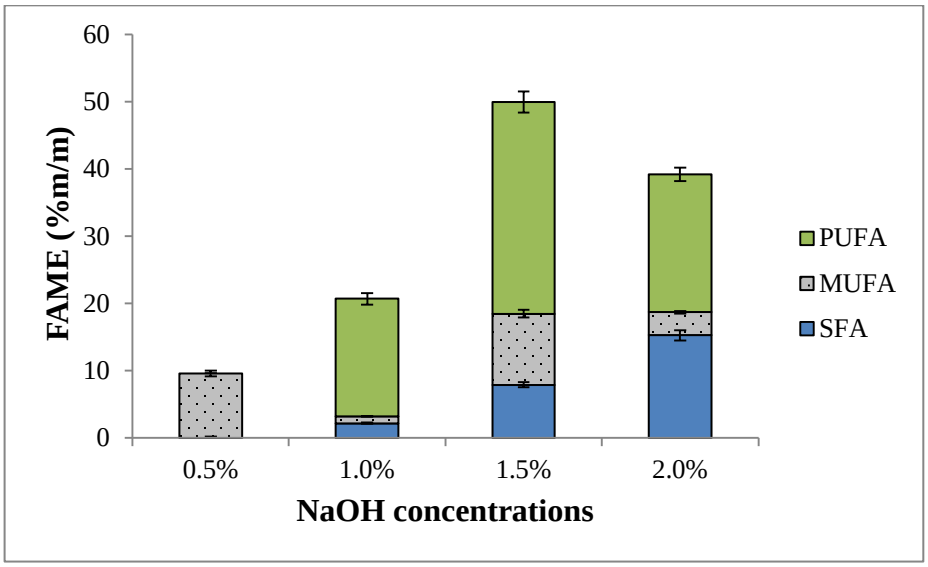


Figure 4.14 Optimization of esterification method using various concentrations of NaOH as base catalyst.

Chromatography was only conducted on the entire range of samples for NaOH and its optimum concentration was compared with the same concentration for KOH (Appendix C). Table 4.6 lists the quantities of saturated and unsaturated fatty acid methyl esters determined using the chosen concentrations of NaOH catalyst in alcohol (methanol). Figure 4.14 shows that the 1.5% NaOH catalyst concentration produced the largest quantity (optimum) of saturated and unsaturated fatty acid methyl esters from the esterification process, ≥50.0%.

4.3.2.3 Comparison of NaOH versus KOH catalysis

In a separate experiment conducted using a new batch of algal biomass, the optimum NaOH catalysed esterification was compared with 1.5% KOH as catalyst (see Chromatograms C10 and C 11 in Appendix C). Ruppel and Huybrighs (2008), state that within EN14214, method EN14103 specified that that the FAME and linolenic acid methyl ester content is used to profile vegetable oil or animal oil feedstock used in biodiesel production call for calibration of all FAME components By relative response to a single compound, methyl heptadecanoate. Therefore the results for this comparison were expressed as a mass fraction in percent using methyl heptadecanoate (C_{17}). The specifications recommended for FAME and linolenic acid are shown on Table 4.7 (Ruppel and Huybrighs, 2008).

The following formulae were used in the calculation of FAME concentration:

$$\mathbf{C} = \frac{\mathbf{\Sigma A - A_{IS}}}{\mathbf{A_{IS}}} \times \frac{\mathbf{C_{IS} \times V_{IS}}}{\boldsymbol{m}} \times \mathbf{100\%}$$

Where,

$\mathbf{\Sigma A}$ = total peak area $\mathbf{C_{14} - C_{24}}$

$\mathbf{A_{IS}}$ = internal standard (C_{17}) peak area

$\mathbf{C_{IS}}$ = concentration of internal standard solution, in mg/mL

$\mathbf{V_{IS}}$ = volume of the internal standard solution used, in mL

$\boldsymbol{m}$ = mass of the sample, in mg

Similarly linolenic acid methyl ester is also expressed as a mass fraction in percent as follows (Ruppel and Huybrighs, 2008):

$$\mathbf{L} = \frac{\mathbf{A_L}}{\mathbf{\Sigma A - A_{IS}}} \times \mathbf{100\%}$$

Where,

$\mathbf{\Sigma A}$ = total peak area $\mathbf{C_{14} - C_{24}}$

$\mathbf{A_{IS}}$ = internal standard (C_{17}) peak area

$\mathbf{A_L}$ = linolenic acid methyl ester peak area.

The values in Table 4.7 were computed from the chromatograms in Appendix C (Figure C10 and C11) and based on 5 mL of 1 mg/mL internal standard.

Table 4.7 Values obtained for FAME and linolenic acid when the 1.5% m/v NaOH and KOH catalysed ester were compared

Comparison of 1.5%NaOH ester versus 1.5%KOH ester			
	1.5% NaOH	**1.5% KOH**	**Specifications**
Total FAME (% m/v)	12.8	85.0	>90%
Linolenic acid methyl ester (% m/v)	3.2	3.1	>1% <15%

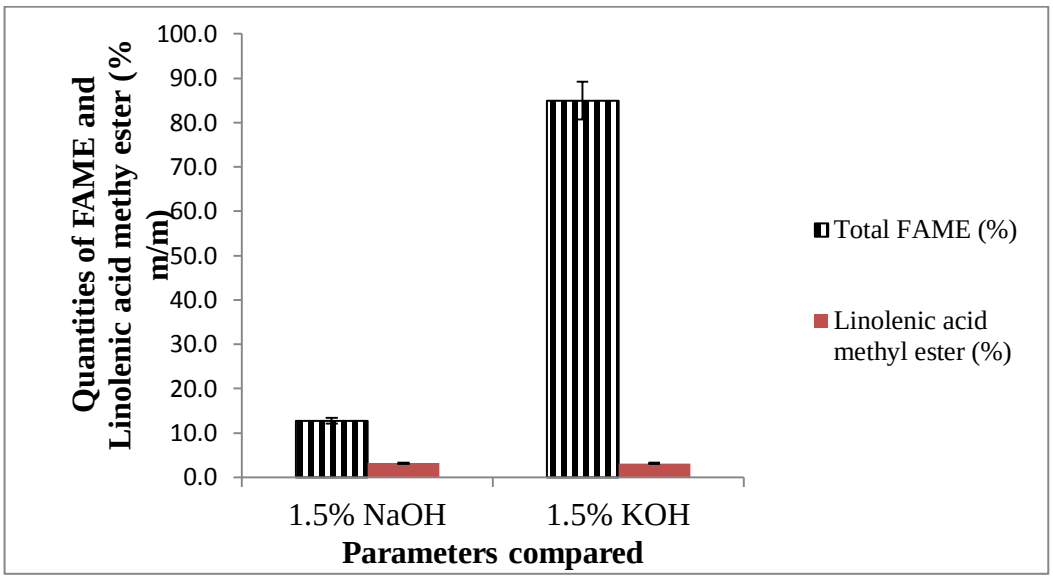


Figure 4.15 Graph comparing FAME and linolenic acid methyl ester quantities in algal biomass for NaOH versus KOH.

From Figure 4.15, it can be seen that the KOH catalyst produced more than 70% of the FAME (biodiesel) produced by the NaOH catalyst. Both catalysts produced almost the same amount of linolenic acid ester. Although the KOH ester did not meet the minimum specification for biodiesel, it was only 5% less than the 90% required. This shows that KOH is a better catalyst than NaOH as suggested by Vyas et al, (2010).

4.3.2.4 Direct transesterification versus in-situ transesterification using acid and base catalysis.

The following chromatogram is a separation of the 37-component standard used for FAME analysis.

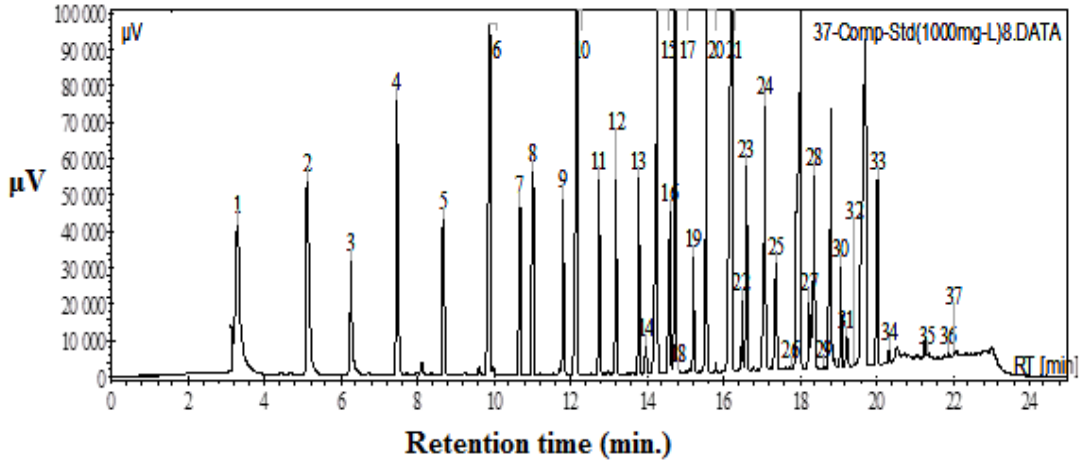


Figure 4.16 Chromatogram of 37-component standard for FAME.

The chromatograms (Figures 4.17 to 4.20) illustrate the separation profiles for direct and in-situ transesterification of lipids extracted from algal biomass. The analyses were conducted using an acid (HCl) catalyst and two base (NaOH and KOH) catalysts as stated previously. The sunflower (cooking) oil was used as a control since it has been used successfully to produce biodiesel. The chromatograms for the base transesterifications are shown in Appendix C. These control samples were not used in the in-situ process for biodiesel production. The primary purpose of these experiments was to determine, firstly, whether the direct or in-situ method was more suited to fast and efficient biodiesel production; and secondly, to determine whether acid or base was better at producing good quality and yield of biodiesel. The type of FAME (biodiesel) produced is determined primarily by the ratios of saturated and unsaturated methyl or ethyl esters produced. Therefore, apart from determining the quantity of saturated and unsaturated fatty acid esters present, it is essential to determine the degree of unsaturation (DU) for each product. Researchers Wang et al, (2012), Ramos et al, (2009), and Bowen, (2010), indicate that this property (i.e. DU)

is essential in determining the quality of biodiesel. Furthermore, Bowen, (2010), reported that unsaturated fats are excellent for cold weather biodiesel production and use. In the South African context, this may not present as serious a problem as colder continental climates, as we generally have mild winters. However, he also states that a higher saturated fat content is likely to increase the gel point of the fuel, while a large unsaturated fat content may present problems with oxidation stability. Wang, (2012), found that the oxidation stability (OS) decreased linearly with DU while the iodine value (IV), increases linearly with DU. The above observations will be discussed further in the next chapter.

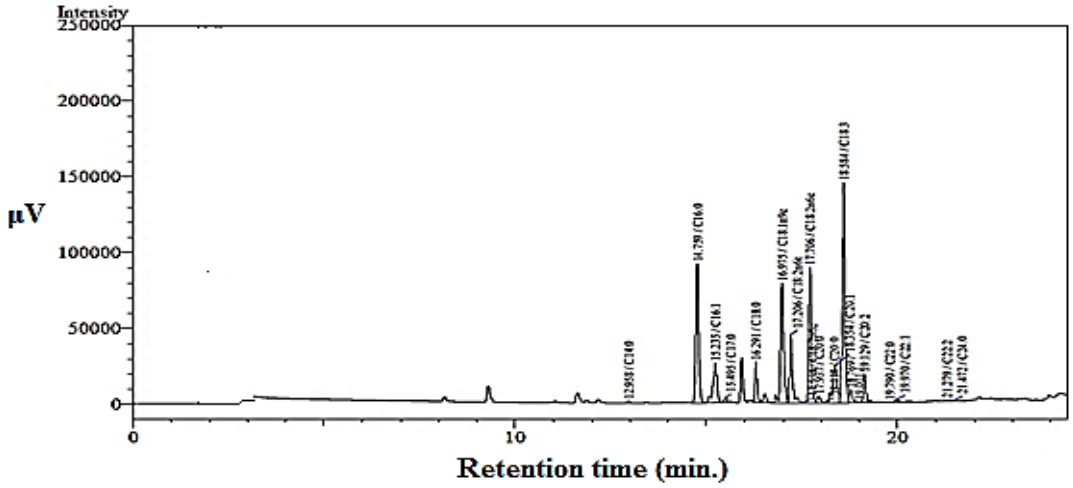


Figure 4.17a *Direct* transesterification with 5% alcoholic HCl as catalyst for algal lipid.

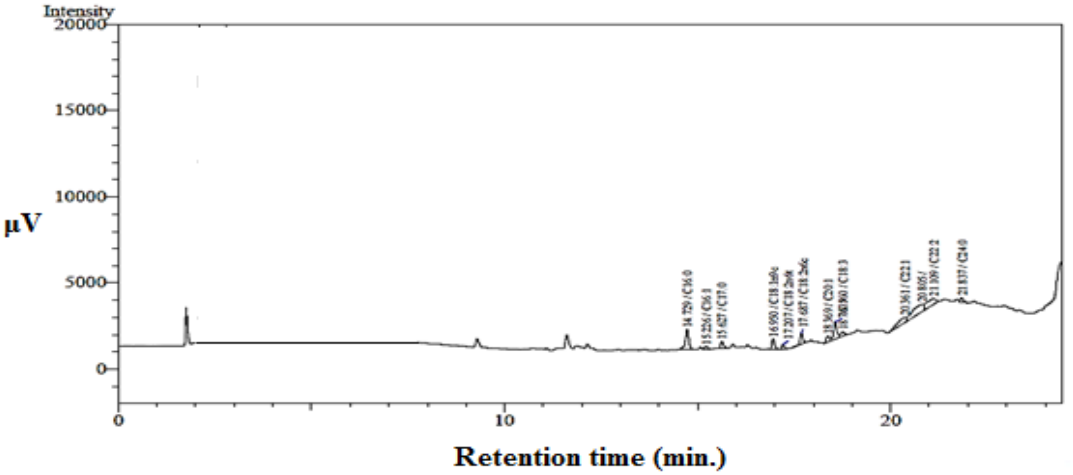


Figure 4.17b In situ transesterification with 5% alcoholic HCl as catalyst for algal lipid.

Figure 4.17a and b show that a better separation profile is obtained from the direct esterification process indicating that better separation of the various esters is taking place during the acid esterification process.

For Figures 4.18a and b below, the direct esterification process one again shows slightly better separation providing better identification of the saturated and unsaturated esters.

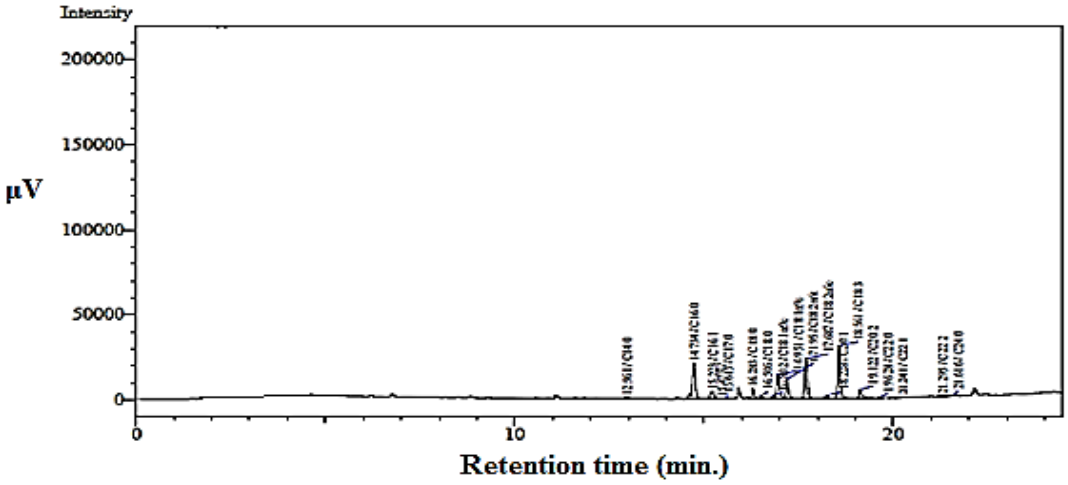


Figure 4.18a Direct transesterification with 1.0% alcoholic NaOH as catalyst for algal lipid.

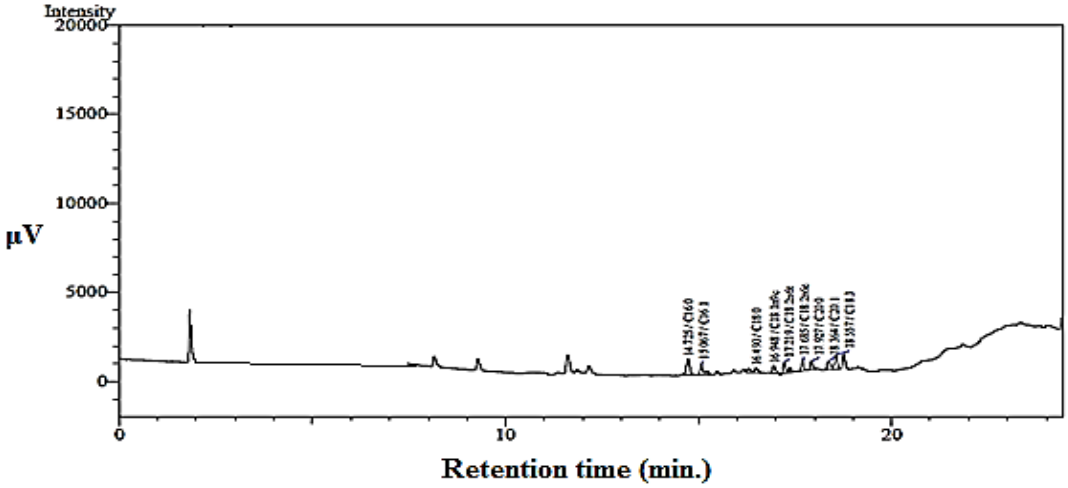


Figure 4.18b In situ transesterification with 1.0% alcoholic NaOH as catalyst for algal lipid.

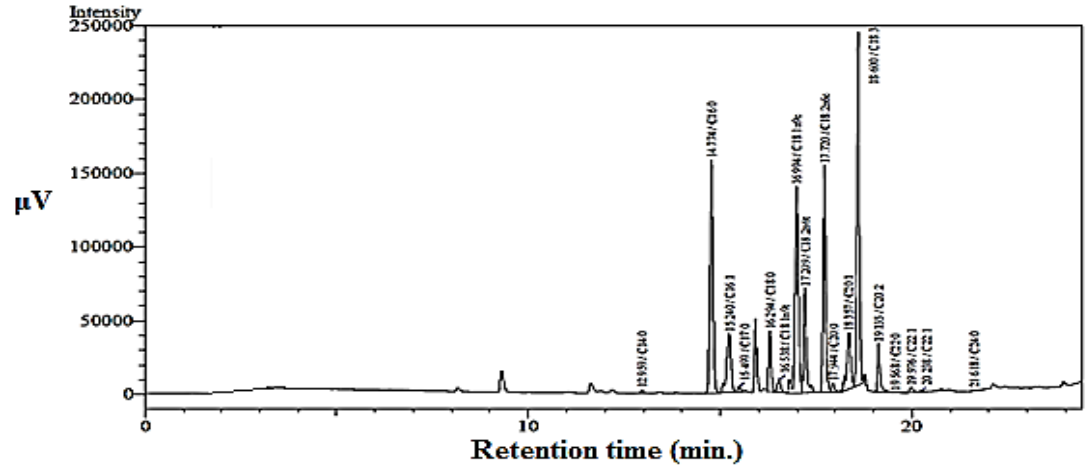


Figure 4.19a ***Direct*** **transesterification with 1.5% alcoholic KOH as catalyst for algal lipid.**

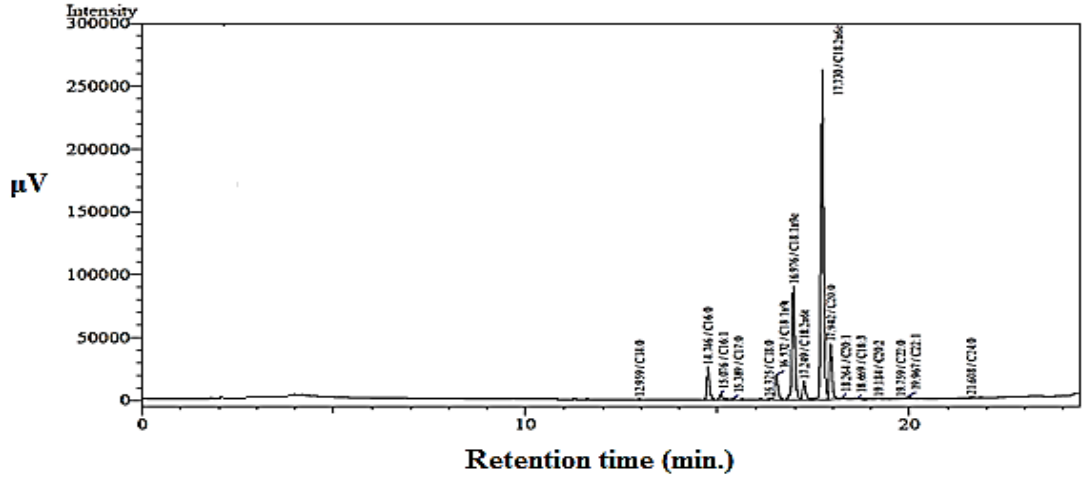


Figure 4.19b In situ transesterification with 1.5% alcoholic KOH as catalyst for algal lipid.

The KOH profiles also show that the direct method is also marginally better at producing the defined esters required for biodiesel.

The control samples (Figure 4.20a and b) show similar separation profiles. This is also probably due to the sample having been a refined oil with none of the "impurities' evident in biomass.

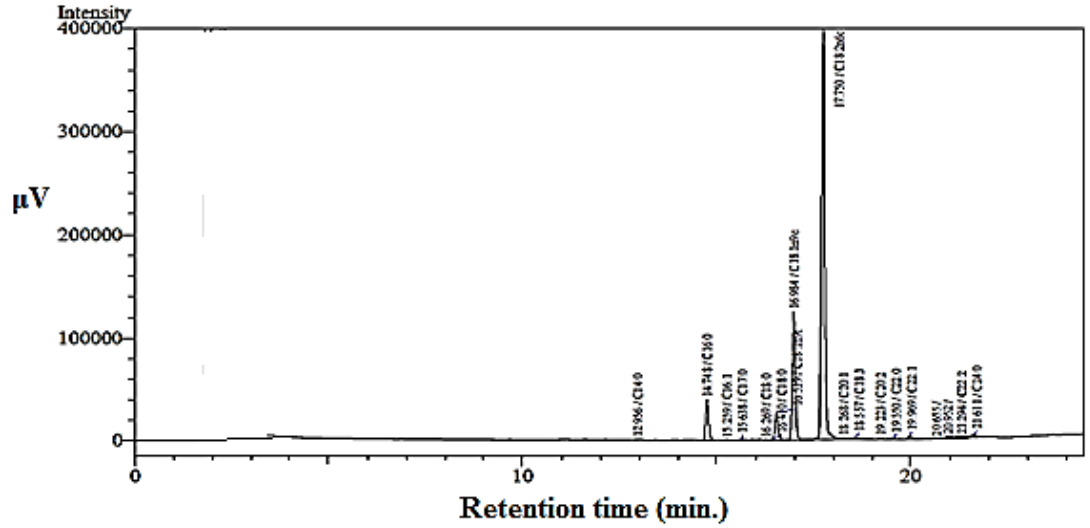


Figure 4.20a Direct transesterification with 5% alcoholic HCl as catalyst for Sunflower oil control sample.

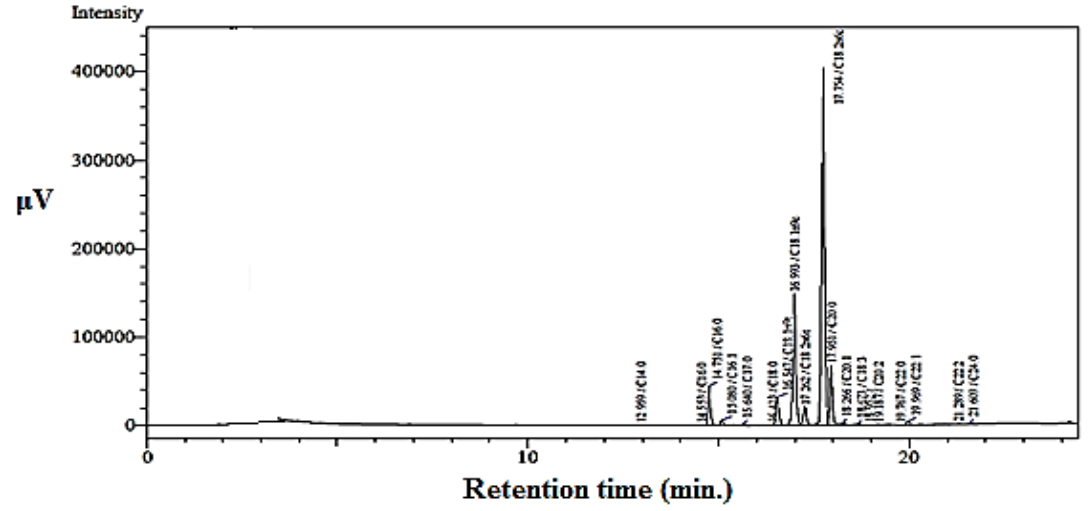


Figure 4.20b In-situ transesterification with 5% alcoholic HCl as catalyst for Sunflower oil control sample.

Table 4.8 shows the values calculated for SFA, MUFA, PUFA, TUFA and DU. The NaOH catalysis produced marginally lower quantities of TUFA (72.5%) by the direct transesterification method and the sunflower oil ester produced by this method was also marginally lower (88.2%) than those produced by the KOH and acid catalysis (92.2% and 99.7% respectively). The in-situ method was applied primarily to the lipid extracts. It can be seen from Table 4.8 that the KOH and acid catalysed esters were, once again, lower than that produced by NaOH, indicating some consistency

with the method used. The KOH catalysed method for the lipid extract produced the highest quantity of TUFA (83.7%) while the acid catalysis produced the lowest (34.7%). The degree of unsaturation (DU) is relatively consistent with the direct esterification method, but fairly irregular with the in-situ method. The Table 4.8 shows that the DU by the direct method (123.0%) is almost three times the value of the in-situ method (46.4%) for the acid catalysis, but for the KOH catalysis it is higher (141.0%) than the direct extraction value (125.9%). Demirbas and Demirbas (2011) state that a high proportion of saturated and monounsaturated fatty acids is considered optimal for fuel quality such that fuel polymerization during combustion would be substantially less than what would occur with polyunsaturated fatty acid derived fuel. A tally of the saturated and monounsaturated quantities obtained (Table 4.8) show that the acid and KOH catalysed esters meet this criteria (SFA + MUFA > PUFA) while that for NaOH does not (SFA + MUFA < PUFA). It can also be seen that the acid catalysed ester produces far less SFA and MUFA (32.5 % m/m) when compared to PUFA (66.9% m/m).

Table 4.8 Calculated SFA, MUFA, PUFA, TUFA and DU values obtained from the chromatograms for separation of esters

Direct versus In-situ transesterification.										
	SFA (% m/m)		MUFA (% m/m)		PUFA (% m/m)		TUFA (% m/m)		DU	
Esterification protocol	Direct	In-situ	Direct	In-situ	Direct	In-situ	Direct	In-situ	Direct	In-situ
Lipid extract/Biomass + Acid catalyst	21.8	17.0	29.0	23.0	47.0	11.7	76.0	34.7	123.0	46.4
Lipid extract/Biomass + NaOH catalyst	27.0	35.4	19.5	29.8	53.0	34.8	72.5	64.6	125.5	99.4
Lipid extract/Biomass + KOH catalyst	21.7	16.2	30.9	26.4	47.5	57.3	78.4	83.7	125.9	141.0
S.O. + acid catalyst	7.2	14.0	25.3	27.7	66.9	58.1	92.2	85.8	159.1	143.9
S.O. + NaOH catalyst	11.8	–	0.2	–	88.0	–	88.2	–	176.2	–
S.O. + KOH catalyst	0.3	–	69.8	–	29.9	–	99.7	–	129.6	–

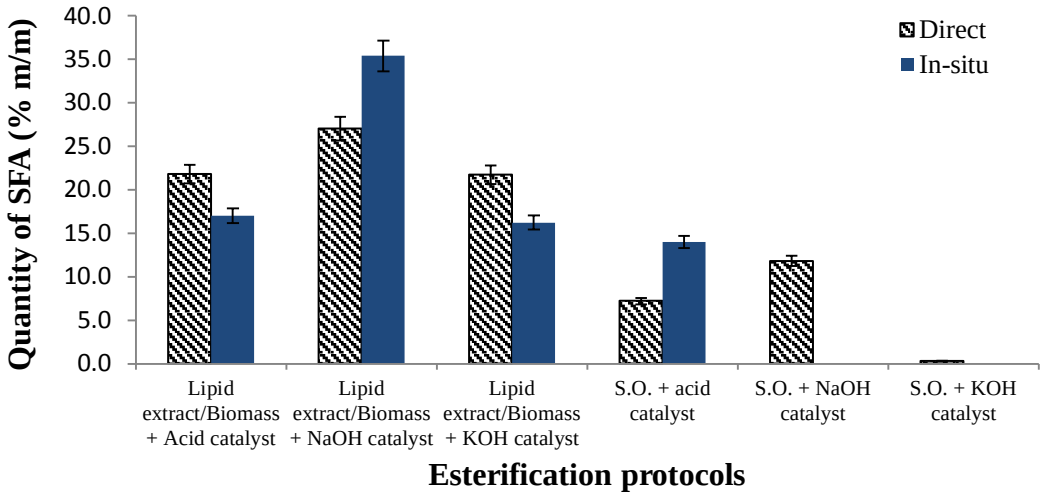


Figure 4.21a SFA quantities comparing direct versus in-situ transesterification.

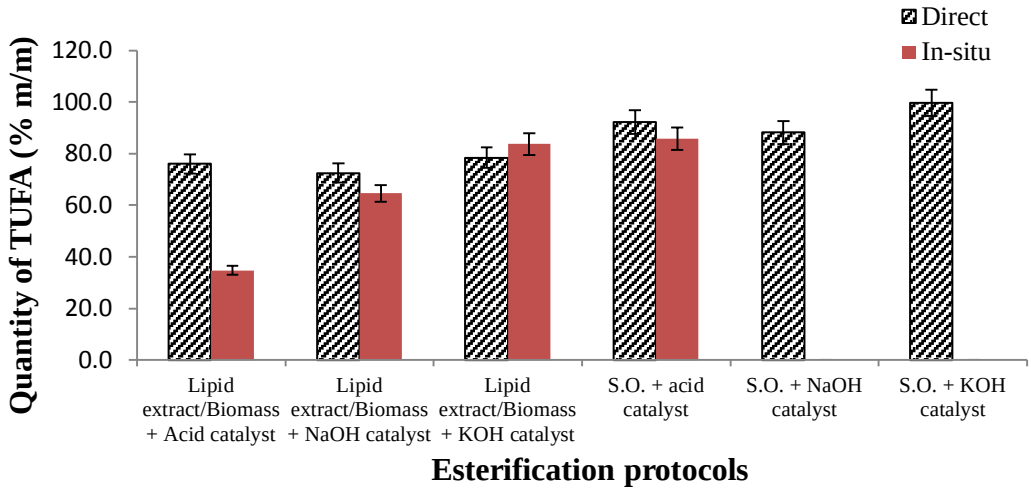


Figure 4.21b TUFA quantities comparing direct versus in-situ transesterification

Figure 4.21a compares saturated fatty acid methyl esters obtained using the direct and in-situ transesterification methods. The NaOH catalysis produced higher quantities of SFA's for both the algal lipid ester and the control (sunflower oil) sample by the direct esterification method. The KOH and acid (HCl) catalysis produced virtually the same quantities of SFA (21.7% and 21.8% respectively) by the direst transesterification method. Similarly, in Figure 4.21b, the KOH and acid catalysis again produce similar quantities of TUFA (78.4% and 76.0% respectively).

This study has found that the in-situ method requires too many interventions, e.g. filtration, purification, clarification and sometimes even more filtration to produce the final product. Although it may be promoted as a one step process by some researchers who find merit with the procedure, it has been found to be very labour intensive. It is also far from being a very cost effective method for the production of biodiesel especially when consideration is made for upscaling. Based on the results produced by the experiments conducted, the acid and base esterification methods may be used with confidence. However, KOH still stands as a popular choice as it produces the best overall quality of biodiesel by the three catalysts (Farag *et al.*, 2012).

4.4 Conclusions

- The FTIR method needs to be evaluated further for it to be considered for both qualitative and quantitative analysis. Although it will not replace gas chromatography as a more efficient method for quantitation of individual esters, it does have the advantage of being a reasonably fast method for analysis.
- The binary solvents produced a better range of saturated and unsaturated fatty acids when compared to single solvents. The implication is that a better quality of biodiesel will therefore be produced.
- The base, KOH, proved to be better as a catalyst than NaOH. This was probably due to NaOH favouring the saponification of the ester to a greater extent.
- Although the three catalysts used were reasonably effective in producing biodiesel, KOH proved to be the best at producing a quality product (FAME) with favourable ratios of saturated, monounsaturated and polyunsaturated esters.
- The chromatography method was effective in separating biodiesel components with a high degree of efficiency.

CHAPTER 5

CHEMICAL AND PHYSICAL CHARACTERIZATION OF BIODIESEL

5.1 Introduction

Biodiesel quality has to be satisfactory to make a viable product for marketability. It is thus essential to perform tests to check if the biodiesel produced from algal biomass can meet specifications for diesel fuels. A large range of tests may be performed on the biodiesel produced. For logistical reasons only selected analyses were performed to determine the quality of biodiesel produced. However, commercial scale biodiesel production will necessitate complete characterization. Chemical and physical characterization was conducted using the analyses listed on Table 5.1. The results obtained were compared to those reported by regulatory bodies indicating standard specifications for biodiesel (Demirbas, 2009).

Lab-scale tests have to be conducted thoroughly for the production of biodiesel before any attempts can be made at upscaling to industrial level. The following is a background of some of the tests performed.

The acid value gives an indication of the breakdown of triacylglycerol's into free fatty acids. This may have an adverse effect on the quality of lipids. High acid values have been shown to increase fuelling system deposits and may increase the potential for corrosion. For the producer it may indicate poorly refined product or poor process control when converting the feedstock oils into biodiesel. In the engine, the fuel can display a strong solvency effect on rubber seals and hoses, deposits and potential filter clogging which may lead to a drop in fuel delivery pressure and a loss of power.

Iodine value (*IV*) is directly proportional to the degree of unsaturation (number of double bonds) and inversely proportional to the melting point of lipids. An increase

in *IV* indicates high susceptibility of lipid to oxidative rancidity due to high degree of unsaturation.

One of the most commonly used methods for determining the iodine value of lipids is "Hanus method". The lipid to be analyzed is weighed and dissolved in a suitable organic solvent, to which a known excess of iodine bromide (IBr) is added. Some of the IBr reacts with the double bonds in the unsaturated lipids, while the rest remains:

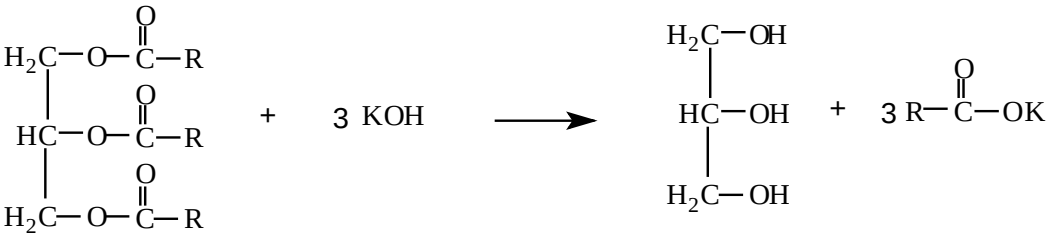
$$R\text{-}CH{=}CH\text{-}R + IBr_{excess} \rightarrow R\text{-}CHI\text{-}CHBr\text{-}R + IBr_{remaining}$$

The amount of IBr that has reacted is determined by measuring the amount of IBr remaining after the reaction has gone to completion ($IBr_{reacted} = IBr_{excess} - IBr_{remaining}$). The amount of IBr remaining is determined by adding excess potassium iodide to the solution to liberate iodine, and then titrating with a sodium thiosulfate ($Na_2S_2O_3$) solution in the presence of starch to determine the concentration of iodine released:

$$IBr_{remaining} + 2KI \rightarrow KBr + KI + I_2$$

$$I_2 + \text{starch} + 2Na_2S_2O_3\ (\text{blue}) \rightarrow 2NaI + \text{starch} + Na_2S_4O_6\ (\text{colourless})$$

The saponification value is the number of mg of potassium hydroxide required to neutralize the free acids and to saponify the esters in 1 g of the substance. The saponification number is a measure of the average molecular weight of the triacylglycerol's in a sample. Saponification is the process of breaking down a neutral fat into glycerol and fatty acids by treatment with alkali. The smaller the saponification number the larger the average molecular weight of the triacylglycerol's present i.e. Saponification value is inversely proportional to the mean molecular weight of fatty acids (or chain length).



Oxidation stability is a test for the shelf life of the product. It determines how long a product can be stored before the fuel starts to chemically react with oxygen molecules to form peroxides. These peroxides then undergo a complex series of chemical reactions resulting in polymerization of the base fuel. All fuels have a shelf life after which they start to degenerate. Fuels should generally be able to be stored for six months before this process starts, and both mineral diesel and biodiesel can have anti-oxidants added to them to improve the stability of the fuel. Smaller producers do not purchase and blend these anti-oxidants, but it may not always be necessary if the fuel is to be consumed shortly after production. The process is accelerated if the product is stored for long periods in hot conditions, or in vessels that may contain rust. Long-term storage should remove the air/ fuel interface by using nitrogen blanketing and airtight tanks. In an engine, oxidation can result in the formation of sludge like deposits in the fuel filter and fuel delivery system. This forms hard deposits and lacquers on parts that are present in the combustion process.

Cetane number is an index used to determine a fuels ignition time delay. Biodiesel cetane numbers depend upon the feedstock used for its production, and are superior to those of mineral or fossil fuel diesel. These diesels often require a cetane booster additive at refinery level. This chapter is an evaluation of the biodiesel produced in the laboratory. The methods for characterization of the biodiesel were chosen randomly. The theoretical aspects base their evaluation on the quantities of saturated and unsaturated fatty acids produced from the analytical work conducted. The values obtained were compared with specifications obtained from South African National Standards (SANS 1935: 2004), European National Standards (EN 14214) and American Standards for Testing Materials (ASTM 6751).

5.2 Materials and Methods

Methods used for both physical and chemical characterization of biodiesel produced were adapted from ASTM methods as indicated below. Thus it was not necessary to develop any new methods since these methods have been tried and tested previously. However, this is the first instance of the use of these methods to characterize biodiesel produced from the algal species studied. Analyses requiring a control or blank were conducted concurrently with sample analyses as required by the tests shown below. The acid value, iodine value and saponification value tests were performed using the potentiometric titration method. The instrument used was the TIM855 Titration Manager manufactured by Radiometer Analytical, France. All tests were performed using AR grade reagents or HPLC grade for solvents. All analyses were conducted in triplicate unless stated otherwise.

The tests used for biodiesel characterization were as follows:

5.2.1 Acid value (*AV*)

The acid value (*AV) is* the number that expresses, in milligrams the quantity of potassium hydroxide required to neutralize the free acids present in 1 g of the substance. This test was performed as per methods described in Appendix A (*Acid Value*, 2006; *Analytical methods to measure the constants of fats and oils*, 2013). The test was performed using isopropyl alcohol, standardized 0.1N KOH (potassium hydroxide) and phenolphthalein indicator. A mass of sample between 2.0 and 2.2 g was weighed accurately into the titration vessel. Exactly 100 mL of isopropanol was added to the sample which was dissolved completely. The sample was heated on a water bath for 10 minutes, then cooled and titrated with the standardized KOH after adding indicator.

5.2.2 Iodine value (*IV*)

The iodine value (*IV*) gives a measure of the average *degree of unsaturation* of a lipid: the higher the iodine value, the greater the number of C=C double bonds. By definition the iodine value is expressed as the grams of iodine absorbed per 100g of

lipid. This test was performed as per methods extracted (see Appendix A, p.) (*Iodine Value*, 2006; *Analytical methods to measure the constants of fats and oils*, 2013). The reagents used for this analysis were: Hanus iodine, 15% potassium iodide, 1% starch indicator, chloroform and standardized 0.1 N sodium thiosulphate. The sample mass was between 0.3 and 0.4 g. this was weighed accurately into a titration vessel. Chloroform and Hanus iodine were added volumetrically, the flask was then covered and shaken continuously for 30 minutes. The potassium iodide solution was then added and flask was shaken. Thereafter 100 mL of deionized water was added using a measuring cylinder and the solution was titrated in the normal way, adding starch solution after a yellow solution is observed. After the addition of starch, the titration was continued until the blue colour formed, disappears. The endpoint values were recorded and calculations completed.

5.2.3 Saponification value (*SV*)

The saponification value is the number of mg of potassium hydroxide required to neutralize the free acids and to saponify the esters in 1 g of the substance. This test was performed as per methods extracted (see Appendix A, p.) (*Analytical methods to measure the constants of fats and oils*, 2013). The reagents used for this analysis were: standardized 0.5 N alcoholic KOH, 0.5 N HCl and phenolphthalein indicator. The procedure involved accurately weighing 2 g of sample and adding exactly 25.00 mL of the standardized KOH into the flask. This was then refluxed at approximately 100ºC for one hour with occasional stirring. The titration was the carried out on the hot solution with the standardized HCl after adding indicator. Titration values were recorded and calculations completed.

5.2.4 Density

This test was performed by weighing an accurate volume of biodiesel and measuring its mass on an analytical balance at 20ºC. The density was calculated from the equation:

$\rho = M/V$ where ρ = density, M = mass and V = volume.

Density correction was carried out using the following formula:

$$\rho_{(15)} = \rho_{(T)} + 0.723\,(T-15)$$

where,

$\rho_{(15)}$ = density of biodiesel at 15ºC

$\rho_{(T)}$ = density at any other temperature in the range of 20ºC to 60ºC

T = any temperature in the range of 20ºC to 60ºC

5.2.5 Oxidation stability (*OS*)

Theoretical values of the OS test was conducted as per Wang et al, (2012), based on the Degree of unsaturation (DU) obtained for samples analysed. This was compared with specifications to determine the fuel quality and stability.

5.2.6 Cetane number (*CN*)

Cetane number is related to ignition delay time and combustion quality. The theoretical value is determined using the DU obtained for samples analysed and formula determined by Wang et al, (2012). This was compared to the specification for quality of fuel produced.

5.3 Results and Discussion

Table 5.1 shows all the results obtained for the characterization tests as well as the local and international specifications for biodiesel relative to these tests. It should be noted that the specifications are general for biodiesel produced from various plant-based sources and are not unique to microalgal sources. However, biodiesel specifications should follow a universal standard regardless of the source of the raw material. The reason for this is that biodiesel application and usage is generally limited to certain industries, one of which is the automotive industry. Hence, biodiesel has primarily been targeted to replace fossil fuel diesel which has similar specifications.

Table 5.1 Characterization test results obtained for algal biodiesel compared to local and international specifications and predicted values for biodiesel (Gopinath *et al.*, 2009; Wang *et al.*, 2012)

Characterization tests performed for Algal biodiesel												
		SPECIFICATIONS			BIODIESEL PRODUCED							
					Measured		Predicted					
							Wang et al, 2012		Gopinath et al, 2009		SANS1935:2004	
Tests	Units	SANS 1935:2004	EN14 214	ASTM 6751	ALE	SO	ALE	SO	ALE	SO	ALE	SO
Acid value (AV)	mg KOH/g	< 0.5	< 0.5	< 0.5	0.94	1.43	–	–	–	–	–	–
Iodine value (IV)	g I_2/100g of FAME	≤ 140	< 120	–	109	114	109	131	108	127	123	136
Saponification value (SV)	mg KOH/g	185-198	–	–	191	195	–	–	207	198	–	–
Density (ρ)	Kg/m^3	860-900	860-900	860-900	876	–	–	–	–	–	–	–
Oxidation stability (OS)	hours	≥ 6	≥ 6	≥ 3	–	–	13	14	–	–	–	–
Cetane number (CN)	Index unit	≥ 51	≥ 51	≥ 47	–	–	50	46	–	–	–	–

where,

ALE = algal lipid extract
SO = sunflower oil

Table 5.1 includes analyses performed on biodiesel produced at lab-scale from algal biomass with biodiesel produced from sunflower as the control sample. Measured values are compared with values predicted using formulae determined by Wang et al, (2012), and Gopinath et al, (2009).

The acid value, expressed as milligrams of potassium hydroxide per gram of sample, is a measure of acidic substance in the oil. It is used as a guide in the quality control as well as monitoring oil degradation during storage (Fan *et al.*, 2009).
It is also a measure of the acidic groupings in a product. The acid value may be overestimated if other acid components are present in the system, *e.g.* amino acids or acid phosphates. From the results shown in Table 5.1, the measured acid value for biodiesel is almost double that of the specification while the control sample is about three times the specification. This would indicate that this product may pose a slight threat for automotive use and may be slightly more corrosive than desired. Some form of intervention, e.g. neutralization or washing of the product may be required to reduce its acidity and bring it more in line with the specification.

The iodine value is a basic measure of the number of double bonds in an oil or biodiesel. This is of importance to the producer to choose feedstock. The higher an iodine value the lower the cetane number. This reduces engine performance, but also makes the fuel more prone to oxidative stability damage. Table 5.1 shows that both the measured and predicted values are within specifications if the SANS maximum of 140 g I_2/100g of FAME is considered. However the control sample is slightly higher in terms of the EN14214 specification. This would imply that a higher degree of unsaturation is present in the control sample whereas the ALE biodiesel is relatively better. A further correlation may be drawn between the iodine value and the degree of unsaturation (DU). As the iodine value is a measure of total unsaturation of a fatty material, a linear increase with DU may be expected. The more unsaturation is present in the oil, the higher the iodine value (Knothe, 2002). A comparison of the iodine values obtained with the DU in Table 4.8 shows that SO which has a higher degree of unsaturation than the ALE tested also has a higher iodine value.

The saponification value, saponification number or Koettstorfer number represents the number of milligrams of potassium hydroxide required to saponify 1g of fat.

It is a measure of the average molecular weight of all the fatty acids present. As most of the mass of a fat/triester is in the 3 fatty acids, it allows for comparison of the average fatty acid chain length. The long chain fatty acids found in fats have a low saponification value because they have a relatively fewer number of carboxylic functional groups per unit mass of the fat as compared to short chain fatty acids. If more moles of base are required to saponify N grams of fat then there are more moles of the fat and the chain lengths are relatively small (SABS, 2004). The determined value for both sample (ALE) and control (SO) fall within the specifications, but the Gopinath et al (2009) prediction is slightly above specification for the control sample. It is, however less than 5% outside the maximum specification and hence statistically acceptable.

Density is the measurement of mass per unit volume at a set temperature. This test is more of interest to the biodiesel producer as it can indicate incomplete reactions. By implication a higher or lower density would indicate that the esterification was either incomplete or more saturated fatty acid esters were present. Density is an important biodiesel parameter, with impact on fuel quality. Density of fuel as an influence on injection systems, pumps, and injectors which are expected to deliver an amount of fuel precisely adjusted to provide proper combustion. Density data are also required for the purpose of designing reactors, distillation units and separation process, storage tanks, and process piping (Pratas *et al.*, 2011). The measured value for prepared biodiesel was well within the specification. This, therefore, is indicative of a good quality biodiesel.

Oxidation stability is one of the major issues affecting the use of biodiesel because of its content of polyunsaturated methyl esters. It is generally very difficult to meet this limit for biodiesel fuels derived from many common raw materials, unless antioxidants are added to the biodiesel (Knothe, 2006). Stability of fatty compounds is influenced by factors such as presence of air, heat, traces of metal, peroxides, light, or structural features of the compounds themselves, mainly the presence of double bonds. Oxidation stability generally determines the shelf life of a product. One of

the main criteria for the quality of biodiesel is the storage stability. Vegetable oil derivatives especially tend to deteriorate owing to hydrolytic and oxidative reactions. Their degree of unsaturation makes them susceptible to thermal and/or oxidative polymerization, which may lead to the formation of insoluble products that cause problems within the fuel system, especially in the injection pump (Meher *et al.*, 2004). The analysis shows that these values are greater than the minimum specification. The predicted values thus show that this would not be a problem with the biodiesel produced.

Cetane number is measured by matching against the blends of two reference fuels, namely, n-cetane and a-methylnaphthalene. Biodiesel cetane number depends on the feedstock used for its production. The longer the fatty acid carbon chains and the more saturated the molecules, the higher the cetane number (Bajpai and Tyagi, 2006). A cetane that is too low will cause hard starting, rough and unsteady idling, engine clatter and increased exhaust smoke. Fuels with a high cetane number will burn before there has been a mix between the fuel and air, and will result in smoking. The predicted values shown on Table 5.1 are within 98% of the specifications. This indicates that the product is of an acceptable standard. Cetane number is widely used as diesel fuel quality parameter related to the ignition delay time and combustion quality. The higher the cetane number the better its ignition properties. An adequate cetane number is required for good engine performance. High cetane numbers help ensure good cold start properties and minimize the formation of white smoke (Pratas *et al.*, 2011).

A further comparison was made with the sample (ALE) and control (SO) with three selected oils used to produce biodiesel by Ramos et al (2009).

Table 5.2 Measured and calculated values of selected tests compared with oils tested by Ramos et al. (2009)

		Specification	Measured/Calculated				
Tests	**Units**	**SANS 1935:2004**	**ALE**	**SO**	**Palm oil**	**Olive oil**	**Soybean oil**
Acid value (AV)	**mg KOH/g**	< 0.5	0.94	1.43	0.12	0.13	0.14
Iodine value (IV)	**g I_2/100g of FAME**	≤ 140	109	114	57	84	128
Oxidation stability (OS)	**hours**	≥ 6	13	14	4.0	3.3	1.3
Cetane number (CN)	**Index unit**	≥ 51	50	46	61	57	49

(Ramos *et al.*, 2009)

The acid values of the palm, olive and soybean oils were below the specified minimum and indicate that very little problems relating to corrosion will be experienced with fuels produced. However, as stated previously, the ALE and So tested in our laboratory have slightly higher acid values and some corrective action may be required to improve the biodiesel quality in this respect. Iodine values for palm and olive oils reveal a lower degree of lower degree of unsaturation than our laboratory samples, but all are within the SANS 1935:2004 specification. The lower DU for the palm and olive oils may also compromise the biodiesel quality to a small extent. The oxidation stability for ALE and SO are virtually the same as soybean oil and all oils meet the local standard. The cetane number for biodiesel should be a minimum of 5. According to this, biodiesels of soybean and sunflower oil were out of specification. This would imply that a higher degree of unsaturation of the oils did may have been the cause of these samples not meeting the specification for the cetane number. Low cetane numbers have been associated with more highly unsaturated components such as the esters of linoleic (C18:2) and linolenic (C18:3) acids. This is evident in Table 4.5a where both linoleic and linolenic acids are on average relatively high for the ALE analysed in this study.

5.4 Conclusion

- The results for acid value show that some intervention may be necessary to improve the quality of biodiesel produced so that it meets all specifications.
- The iodine values obtained show that the ALE is producing a reasonable quantity of unsaturated fatty acids and hence the specifications for biodiesel are met.
- The saponification values obtained show that the carboxylic functional groups per unit mass of fat is acceptable for biodiesel production.
- The OS value for the tested samples was above the minimum specification. Since there is no indication of any maximum specification, it is assumed that the values obtained are acceptable, although they are twice the minimum specified.
- The cetane numbers are slightly below specification but within 98% of the norm for biodiesel. This factor is not expected to present any major problems with the biodiesel produced under local conditions.
- The comparison drawn with the three other oils shows that there is a reasonable correlation with the ALE and SO. The only factor that may be of some concern is the acid value where some corrective action may be required.

CHAPTER 6

CONCLUSIONS and RECOMMENDATIONS

6.1 CONCLUSIONS

The overall results show that the fuel being produced in the laboratory is of an acceptable quality. More comprehensive analysis incorporating other characterization tests will need to be performed before any decisions are taken with regard to using the methods for large scale application. This is just one step towards the production of biodiesel as a replacement for fossil fuel.

- The chlorella and scenedesmus species that were studied have proved to be strains of algae that can produce lipids of satisfactory quantity and standard for the production of biodiesel.

- Of the four extraction methods examined, the soxhlet and microwave assisted extraction methods were almost equally efficient at extraction of lipids from algae. The MAE was determined to be better than the soxhlet method as it was equally efficient at extraction of lipids using lower volumes of solvent in less time. This was shown by the MAE method yielding an average of 10.0% lipids for chloroform, ethanol and hexane after 30 mL of solvent was used in an extraction time of 10 minutes, while the soxhlet method yielded 10.36% lipids using an extraction volume of 100 mL of solvent with an extraction time of 3 hours.

- Chloroform, ethanol and hexane were more efficient than the other solvents used for extraction. This was shown by these three solvents producing lipid quantities between 10% to 11% while all the other solvents produced lipid quantities between 2 and 10 %. The best extraction efficiency was achieved

by the binary solvent mixture made up of chloroform and ethanol in a 1:1 ratio. Under the conditions optimized, this solvent ratio yielded a lipid content of 11.76%. One of the objectives of this study was to determine extraction technologies that would be fast and efficient at a reasonable cost. The methods chosen and optimized are probably the most efficient, but the actual cost of production of biodiesel need to be determined.

- A comparison of the oven-drying, sun-drying and freeze-drying method for microalgae was conducted. The oven-drying method proved to be the most practical method for use at reasonable cost. It also produced an average of 1% greater yield of lipids than the freeze-drying method over a three month period.

- The RSM method for optimization of parameters for extraction of lipids compared well with the OFAT method and results were within the 95% confidence interval.

- Non-solvent methods for extraction e.g. hydrothermal methods have been suggested by other researchers, but the method demands a high input of energy and may thus prove to be too expensive.

- Physical methods like the expeller press will not be feasible for extraction of the type of biomass produced. Pelletizing of the algae may assist in making this method feasible, but the cost of pelletizing the biomass would not warrant further exploration of this method for extraction.

- The quantities of chlorophyll were determined for accurate quantitation of lipids. Other plant pigments e.g. carotenoids, which are considered as “contaminants” form part of the lipid extract and need to be determined to enable easier purification of the biodiesel product. Although the contribution of these “contaminants” is less than 1% of the total lipid mass they contribute

to the darkening of biodiesel significantly. It has, however, not influenced the process of conversion of the lipid extract to biodiesel.

- Ageing experiments showed that it is possible to store dried microalgal biomass in a moisture-free atmosphere for at least three months without the algae losing its efficiency for producing biodiesel.

- The transesterification protocols examined for conversion to biodiesel has shown that the 1.5% alcoholic KOH catalyst was able to produce 85.0% FAME as opposed to the 1.5% alcoholic NaOH which produced only 12.8% FAME (biodiesel). The KOH catalyst also produced biodiesel with a ratio of saturates to unsaturates conducive to a good fuel product. The direct esterification method in this study proved to be better than the in-situ method for biodiesel production. The in-situ method was also more labour intensive.

- It was found that the FTIR method was more suited to qualitative analysis than quantitative analysis while chromatography proved to be a fast and efficient method for both qualitative and quantitative determination of biodiesel.

- The characterization tests show that the quality of biodiesel produced was satisfactory. It also shows that the methods used in this study were feasible for the production of biodiesel and met with local and international specifications. Since theoretically predicted values matched measured values it may be safely assumed that the oxidation numbers and cetane numbers are likely to be close to or the same as measured values. The complete range of characterization tests need to be performed before up-scaling is attempted.

6.2 RECOMMENDATIONS

The following recommendations are suggested when considering further investigation:

- Microalgal cultivation protocols need to be monitored to identify optimum lipid production time and yields. Harvesting of algae should be done as soon as maximum lipids yields are achieved. This has to be determined.
- The quantitation of all contaminants (i.e. other compounds excluding lipids) should be conducted to verify if any of these hinder the production of biodiesel.
- Experiments need to be conducted on techniques which are not solvent dependent to examine their feasibility for biodiesel production.
- Other acid and base transesterification techniques should be investigated to determine their suitability for biodiesel production. Further optimizing of transesterification techniques may be required to produce exact ratios of SFA, MUFA and PUFA for high quality biodiesel.
- The FTIR method may be researched further to determine its qualitative and quantitative abilities for samples used in this study.
- The GC-MS method should be examined for its quantitation abilities for biodiesel.
- The full range of specified tests should be examined to ensure the viability of biodiesel produced for upscaling.

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APPENDICES

APPENDIX A: METHODS

Soxhlet extraction

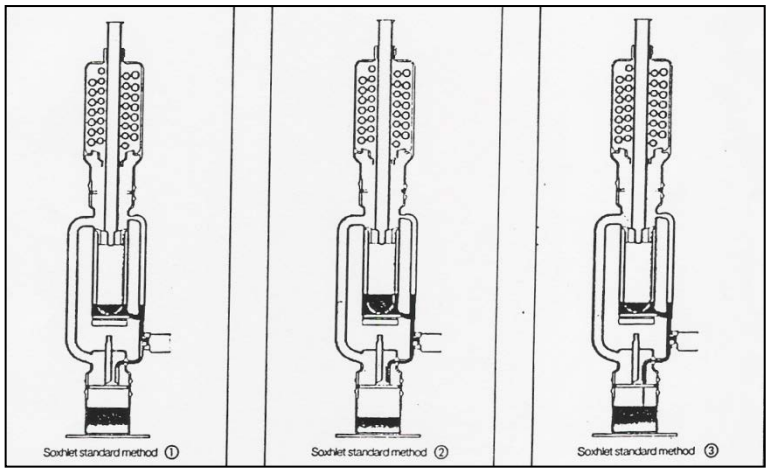

Figure A1: Procedure for Soxhlet standard

1. Soxhlet standard

The solvent evaporates, rises up to the condenser and condenses. The glass valve is closed.
The solvent level increases up to the optical sensor. The sample is extracted.
The optical sensor detects the solvent level and opens the glass valve. The solvent with the extract flows back into the solvent cup.

If the preprogrammed number of cycles is reached and/or if the extraction time is up, the system goes to the next step.

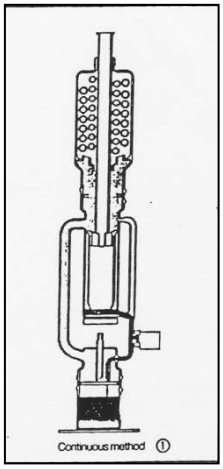

Figure A2: Procedure for the continuous method

4. Continuous

The solvent evaporates, rises up to the condenser and condenses. The glass valve is-open, the optical sensor is inactive.When the programmed extraction time is up, the system goes to the next step.

Soxhlet standard Mode - Programming Example

Program description:
A standard extraction according to Soxhlet is carried out. The solvent level is detected by the optical sensor and this releases a magnetic valve which then allows solvent to flow.

Key	Display	Description
SELECT		
	The **Mode** display blinks	Select the Soxhlet Standard mode using the ▲/▼ keys
⏩	Display **STEP 1** blinks	(STEP 1 = Extraction phase)
⏩	Display **HEATING** blinks	Select the heating level (1-20) using the ▲/▼ keys Select Level 9 for naphtha 40/60
⏩	Display **CYCLES** blinks	Select the number of cycles using the ▲/▼ keys If the number of cycles = 0 is entered, then the only stop criteria used is the extraction time.
⏩	Display **H: MIN** blinks	Select the extraction time (0 bis 99:99 h) using the ▲/▼ keys. If no time limit is entered, then the only stop criteria used is the number of cycles. If both parameters are entered (time and number of cycles), then both criteria must be fulfilled before the device goes to the next step.
⏩	Display **STEP 2** blinks	(STEP 2 = Rinse phase)
⏩	Display **HEATING** blinks	Select the heating level (1-20) using the▲/▼ keys Select Level 9 for naphtha 40/60 If the extracted product is to be dried in an inert gas, the inert gas supply must be activated by pressing the Inert Gas key. The inert gas valve is automatically opened up during the appropriate step.
⏩	Display **H: MIN** blinks	Select the rinse time (0 to 99:99 h) using the ▲/▼ keys. The only stop criteria used for **STEP 2** is the rinse time.
⏩	Display **STEP 3** blinks	(STEP 3 = Drying phase)
⏩	Display **HEATING** blinks	Select the heating level (1-20) using the ▲/▼ keys Select Level 4 for naphtha 40/60
⏩	Display **H: MIN** blinks	Select the drying time (0 bis 99:99 h) using the ▲/▼ keys. The only stop criteria used for **STEP 3** is the drying time.
SELECT		
	No display blinks.	The program you have entered is stored as program 0.

Chapter 5

Analytical methods to measure the constants of fats and oils

1. Acid value (Acid Number)

Significance

Acid value is the measure of hydrolytic rancidity. In general, it gives an indication about edibility of the lipid.

- Edible oil contain > 1%
- Pharmaceutical oil must not have any acidity.

$$AV = \frac{\text{ml of KOH x N x 56}}{\text{Weight of Sample}} = \text{mg of KOH}$$

N = Normality of KOH

% Free Fatty Acid (FFA) = AV x 0.503

Material

- Fat or Oil
- Absolute ethanol alcohol
- Phenolphthalein
- 0.1 N KOH

Procedure

1. Place 5.0 g of fat or oil in a dried conical flask.
2. Add 25 ml of absolute ethanol alcohol and add (2-3) drops of phenolphthalein
3. Heat with shaking in water bath (65%) for 10 minutes ,then cool Titrate the solution against 0.1 N KOH until pink color appears (end point).
4. Record your observations.
5. Calculate the acid value (AV) and free fatty acid (%FFA) using above laws.

2. Saponification Number

Saponification Value of Fats and Oils

Fat or oil	Saponification Value
Milk fat	210-233
Coconut oil	250-264
Cotton seed oil	189-198
Soybean oil	189-195
lard	190-202
Butter fat and vegetable fats	~ 220 – 250

Material

- Fat or Oil
- 0.5 N alcoholic potassium hydroxide (alcoholic KOH) (prepared by dissolving 30 g potassium hydroxide in 20 mL of water and make the final volume to 1 L using 95 % ethanol. Leave the solution to stand for 24 h before decanting and filtering the solution.
- 0.5 N Hydrochloric acid
- Phenolphthalein.

Procedure

1. Weigh approximately 2 g of the fat or oil into a 250 mL conical flask.
2. Add 25 mL of alcoholic potassium hydroxide solution (0.5 N).
3. Attach a reflux condenser and heat the flask contents on a boiling water bath for 1 hour with occasional shaking.
4. While the solution is still hot , add 3 drops of phenolphthalein indicator and titrate the excess potassium hydroxide with the 0.5 N hydrochloric acid (Vml of hydrochloric acid at end point represents S).
5. Do same above procedure but without sample (Vml of hydrochloric acid at end point represents B).
6. calculate the saponification number by using the following law:

$$SP\# = \frac{56.1(B - S) \times N \text{ of HCl}}{\text{Gram of Sample}}$$

B: ml of HCl required by Blank.

S: ml of HCl required by Sample.

3. Ester Value

The ester value is defined as the mg of KOH required to react with glycerin (glycerol / or glycerin) after saponify one gram of fat. It is calculated from the saponification
Value *(SV)* and the acid Value (AV):

Ester Value (EV)	**=**	**Saponification Value (SV) – Acid Value (AV)**
% *glycerin*	**=**	***Ester Value* × 0.054664**

4. Iodine Value (I.V)

TYPICAL IODINE NUMBERS	
Coconut oil	8 - 10
Butter	25 - 40
Beef tallow	30 - 45
Palm oil	37 - 54
Lard	45 - 70
Olive oil	75 - 95
Peanut oil	85 - 100
Cottonseed oil	100 - 117
Corn oil	115 - 130
Fish oils	120 - 180
Soybean oil	125 - 140
Safflower oil	130 - 140
Sunflower oil	130 - 145
Linseed oil	170 - 205

Material

- Oil or fat
- Hanus solution (it's prepared by dissolving 18.2 g of iodine in 1L of glacial acetic acid and then add 3 ml of bromine water for increasing the halogen content.
- 15% potassium iodide solution
- 1% starch solution
- 0.1 N Sodium thiosulfate solution.

Procedure

1. Weigh approximately 0.25 g of the fat or oil into a 250 mL conical flask.
2. Add 10 ml of chloroform.
3. Add 30 ml of Hanus solution and close the flask completely by Para film, then leave the solution for 30 minutes with shaking continuously.
4. Add 10 ml of 15% potassium iodide solution and then shake.
5. Add 100 ml of distilled water (DW).
6. Titrate the iodine solution against 0.1 N Sodium thiosulfate solution till yellow color formed , then add 2-3 drops of starch solution where blue solution formed and then continue with titration till the blue color is disappeared (Volume (ml) of $Na_2S_2O_3$ at end point represents S)
7. Do same above procedure but without sample (Volume (ml) of $Na_2S_2O_3$ at end point represents B).
8. Calculate the iodine number by using the following law:

$$\text{Iodine Value} = \frac{(B - S) \times N \text{ of } Na_2S_2O_3 \times 0.127\text{g/meq} \times 100}{\text{Weight of Sample (g)}}$$

B: V ml of $Na_2S_2O_3$ volume for blank
S: V ml of $Na_2S_2O_3$ volume for sample

Lubrizol

LUBRIZOL TEST PROCEDURE

TP-AATM-109-01
Edition: August 12, 2006
Previous Edition: May 1, 2000

Acid Value

DEFINITION

Acid Value is expressed as milligrams of potassium hydroxide required to neutralize the free acids in one gram of the material.
Acidity (as defined in USP) is expressed as the number of milliliters of 0.1N alkali required to neutralize the free acids in 10 grams of the substance.
NOTE: Use part I for all samples plus in-process samples that also require Hydroxyl Value. For in-process samples not needing Hydroxyl Value, use Part II which provides a fast estimation of the Acid Value.

EQUIPMENT REQUIRED

1. Erlenmeyer flasks - 125 ml.
2. Dispensing devices capable of dispensing 25 ml (± 1%) or 50 ml (± 1%).
3. Analytical balance capable of weighing to the nearest 0.1mg.
4. Hot plate, Magnetic stirrer, stirring bars.
5. Dispensing device capable of dispensing 1 ml.
6. Burette, in compliance with one of the following:
   - 10 ml graduated in 0.05 ml divisions with a tolerance of ±0.04ml
   - 25 ml graduated in 0.10 ml divisions with a tolerance of ±0.05ml
   - 50 ml graduated in 0.10 ml divisions with a tolerance of ±0.07ml

REAGENTS

1. Neutralized ethanol or IPA (see below*) - to 1 liter of Reagent Ethanol or LC Grade IPA add 1 ml 1% Phenolphthalein Indicator. Titrate with 0.1N Methanolic Potassium Hydroxide to a faint pink color.
2. 0.1N Methanolic Potassium Hydroxide - commercially available.
   NOTE: The normality of this base is accurate to 3 significant figures while we only report to 2 significant figures. The base is used up to about 1 month. Past standardizations have shown that the titer does not change significantly.
3. 0.5N Methanolic KOH solution - commercially available (can be standardized by method AATM 1002).
4. 1% Phenolphthalein in I.P.A. - commercially available.
5. Anhydrous Ether - reagent grade*.

PROCEDURE - Part I

1. Accurately weigh the specified amount of sample into a tared 125 ml Erlenmeyer flask and record weight to the nearest 0.1 mg.

   Determine sample size as follows:

Anticipated Acid Value	Sample Size	Normality KOH
Less than 1.0	4 (±0.1)g	0.1N
1 - 25	2 (±0.1)g	0.1N
25 - 50	1 (±0.1)g	0.1N
50 - 150	3 (±0.1)g	0.5N
Greater than 150	2.5 (±0.1)g	0.5N

2. Using a dispensing device, add 50 ml neutralized IPA and a stirring bar and mix to dissipate, warming on a heat source if necessary. Allow sample to cool.
   *For products insoluble in IPA after warming, substitute the following: Add 25 ml neutralized ethanol. Warm, stirring to dissolve. Cool and add 25 ml anhydrous ether.
3. Prepare a reagent blank containing 50 ml neutralized IPA, or 25 ml each neutralized ethanol and ether if used. NOTE: When using 0.5N KOH no blank is needed.
4. Using a dispensing device, add approximately 1 ml phenolphthalein.
5. Fill burette and titrate using Potassium Hydroxide solution to a faint pink color which remains for 30 seconds or more.
6. Read the burette as accurately as possible to two decimal places.

CALCULATIONS for Part I

$$\text{Acid Value} = \frac{(\text{ml sample} - \text{ml blank}) \times \text{N KOH} \times 56.1}{\text{g sample}}$$

Using 0.5N KOH:

$$\frac{\text{ml sample} \times \text{N KOH} \times 56.1}{\text{g sample}}$$

PRECISION:

In those cases where the ml of 0.1N KOH used is less than 1.

Combining two sets of data, we obtain:

- Average of 13 determinations = 0.46
- Standard Deviation = 0.0375
- 95% Confidence Limit = 0.46 ± 0.02

In those cases where the ml of KOH used is greater than 1.

Example using a Wool Grease:

- Average of 12 determinations = 7.72
- Standard Deviation = 0.110
- 95% Confidence Limit 7.722 ± 0.070

PROCEDURE - Part II

1. Weigh 2.8 g of filtered (if necessary) sample into a tared 125 ml Erlenmeyer flask.
2. Using a dispensing device, add 50 ml neutralized IPA and a stirring bar and mix to dissipate, warming on a heat source if necessary. Allow sample to cool.
   - For products insoluble in IPA after warming, substitute the following: Add 25 ml neutralized ethanol. Warm, stirring to dissolve. Cool and add 25 ml anhydrous ether.
3. Using a dispensing device, add approximately 1 ml phenolphthalein.
4. Fill burette and titrate using Potassium Hydroxide solution to a faint pink color which remains for 30 seconds or more.
5. Read the burette as accurately as possible to 2 decimal places.

CALCULATIONS for Part II

Acid Value = ml for sample x 2

Lubrizol

LUBRIZOL TEST PROCEDURE

TP-AATM-112-01
Edition: October 16, 2006
Previous Edition: December 7, 1998

Iodine Value

DESCRIPTION OF METHOD

The iodine value method is reproducible only if the exact conditions of the test are carefully followed. Any changes in strength of reagent, sample size or reaction time may produce varying results.

The exact specified weight of the sample is accurately weighed into a glass stoppered iodine flask, and dissolved in chloroform. The measured volume of Hanus reagent is accurately added and after thorough mixing, is placed in the dark for exactly one hour. A corresponding reagent blank is simultaneously prepared.

At the end of the specified time, the reaction is stopped by adding potassium iodide and diluting with water to prevent loss of the free iodine. The amount of iodine present is determined by titrating with sodium thiosulfate using starch indicator. The difference between a reagent blank titration and the titration of the test sample represents the amount of iodine absorbed by the sample. The iodine value is calculated as grams of iodine per 100 grams of sample.

Hanus reagent is a solution of iodobromide in concentrated acetic acid. The iodine combines with double bonds slowly under these conditions. A large excess of the halogens must be present to complete the reaction. At the end of the reaction the unconsumed iodine should be greater than 60% of the total. If the sample titration is less than 60% of the blank titration, take a smaller sample and repeat the analysis.

APPARATUS

1. Iodine flasks, 250 ml. glass stoppered
2. 50 ml. Burette with a tolerance of ±0.07 ml
3. 25 ml Class A volumetric pipette with a tolerance of ±0.07 ml or appropriate dispenser
4. Dispensing device capable of dispensing 10 ml (±1%)
5. Dispensing device capable of dispensing 20 ml (±1%)
6. Graduated cylinder capable of containing 100 ml
7. Analytical balance capable of weighing to the nearest 0.1mg
8. Dispensing device capable of dispensing 3 ml

REAGENTS AND SOLUTIONS

1. Chloroform, A.R.
2. Hanus Solution, A.O.A.C.
3. Potassium Iodide - 10% solution
4. Stabilized starch solution - commercially prepared
5. Sodium thiosulfate, 0.1N - commercially prepared or AATM 1004

PROCEDURE

1. Accurately weigh the sample into a tared 250 ml iodine flask. Determine the sample size from the following table. Accurately record weight to the nearest mg.

Anticipated Iodine Value	Sample Size
0 - 30	0.8 ± .01 g
30 - 50	0.5 ± .01 g
50 - 100	0.25 ± .01 g
100 - 150	0.16 ± .01 g

2. Using the dispensing device, add 10 ml chloroform. Dissolve by swirling (NOTE: 50% aqueous products will not dissolve completely until Hanus solution is added).
3. Accurately add 25.0 ml Hanus solution and stopper immediately. Swirl to mix thoroughly and place in the dark.
4. Simultaneously prepare a corresponding reagent blank containing 10 ml chloroform and 25 ml Hanus solution. Stopper immediately and place the flask in the dark with the test sample.
5. After 60 minutes, using the dispensing device, add 20 ml 10% potassium iodide.
6. Add by graduated cylinder, 100 ml water rinsing the neck and side walls of the flask during addition of the water.
7. Immediately titrate the solution using 0.1N sodium thiosulfate until the aqueous layer's color begins to lighten.
8. Using the dispensing device, add approximately 3 ml starch solution and continue titrating until the blue color of the aqueous layer begins to disappear.
9. Towards the end of the titration, at intervals, stopper the flask and shake vigorously to extract any iodine remaining in the chloroform layer.
10. When the end point appears to have been reached, again stopper and shake vigorously, allow the layers to separate and add two drops of starch solution to verify that no blue color is formed in the aqueous layer.
11. Read the burette accurately to two decimal places. Record.

CALCULATIONS

$$\text{Iodine Value} = \frac{(\text{ml blank} - \text{ml sample}) \times \text{N sodium thiosulfate} \times 12.69}{\text{g sample}}$$

PRECISION

Product	Average	Std Dev	Coeff of Var	df
Acetulan	7.42	0.107	1.448	26

APPENDIX B: DATA

Typical data captured during extraction of lipids.

K Ramluckan: Biodiesel

Microwave Extraction of Algal lipids using Chloroform and Ethanol mixtures

VARIATION of SOLVENT RATIOS (BINARY)

Fixed Parameters:	
Stage	0
Power Max. (W)	1600
Power %	100
Stirrer speed (s)	0

No.	f_x										
		Date	**30-03-2011**								
		Program	**Shan100DEGBIN**								
		Solvent volume	**30 mL**								
		Solvent	**Chloroform : Ethanol**			**Chloroform : Ethanol**			**Chloroform : Ethanol**		
		RATIO	**1:1**			**1:2**			**1:3**		
		Mass	**1 g**								
		Temperature	**100°C**								
		Time	**10min.- optimized**								
		Sample No	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
1		Mass of sample	1.0009	1.0046	1.0167	1.0033	1.0131	1.0193	1.0238	1.0113	1.0257
		Flask number	25	26	27	7	8	9	10	11	12
2		Flask + Lipid mass	94.0978	111.5468	105.2663	104.7710	103.2244	101.3365	99.6743	98.0758	107.7987
3		Flask mass	94.0311	111.4771	105.2054	104.7285	103.1875	101.2996	99.6153	98.0202	107.7390
4	2 - 3	Lipid mass	0.0667	0.0697	0.0609	0.0425	0.0369	0.0369	0.0590	0.0556	0.0597
5	4/1*100	**% Lipids**	6.66	6.94	5.99	4.24	3.64	3.62	5.76	5.50	5.82
6		**Average % Lipids**		**6.53**			**3.83**			**5.69**	
7		**Std. dev.**		**0.49**			**0.35**			**0.17**	

No.	f_x										
		Date	**6-04-2011**								
		Program	**Shan100DEGBIN**								
		Solvent volume	**30 mL**								
		Solvent	**Chloroform : Ethanol**			**Chloroform : Ethanol**					
		RATIO	**3:1**			**2:1**					
		Mass	**1 g**								
		Temperature	**100°C**								
		Time	**10min.- optimized**								
		Sample No	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
1		Mass of sample	1.0249	1.0061	1.0259	1.0114	1.0092	1.0054			
		Flask number	7	8	9	13	14	15			
2		Flask + Lipid mass	103.0844	105.7769	109.8706	112.4101	109.5633	113.5118			
3		Flask mass	103.0324	105.7339	109.8269	112.3572	109.5141	113.4453			
4	2 - 3	Lipid mass	0.0520	0.0430	0.0437	0.0529	0.0492	0.0665			
5	4/1*100	**% Lipids**	5.07	4.27	4.26	5.23	4.88	6.61			
6		**Average % Lipids**		**4.54**			**5.57**				
7		**Std. dev.**		**0.47**			**0.92**				

APPENDIX C:

FTIR and CHROMATOGRAPHY

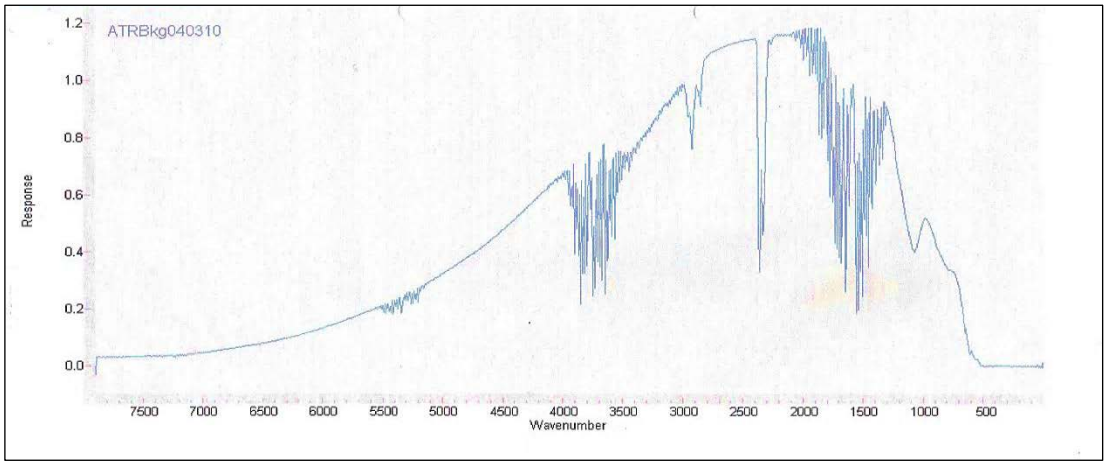

Figure C1 Scan of ATR background – blank.

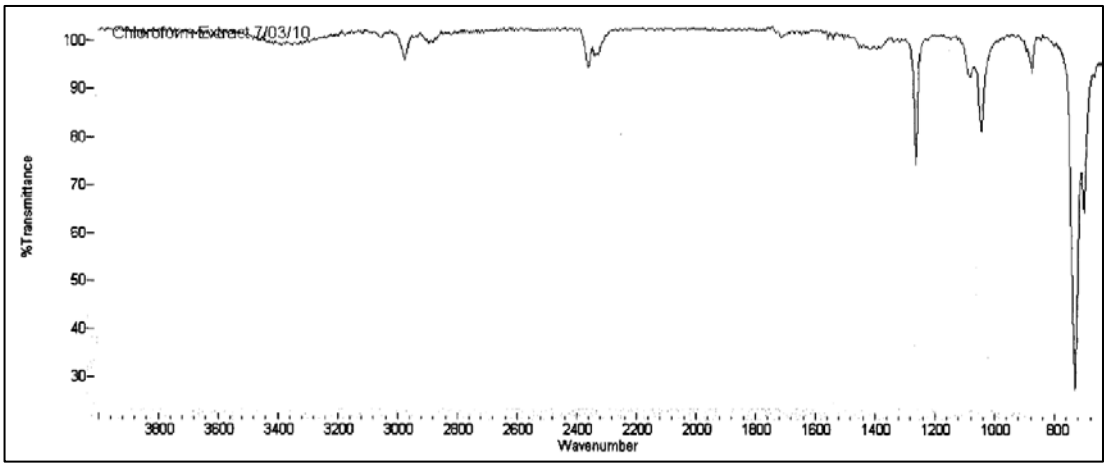

Figure C2 Scan of lipid extract by chloroform.

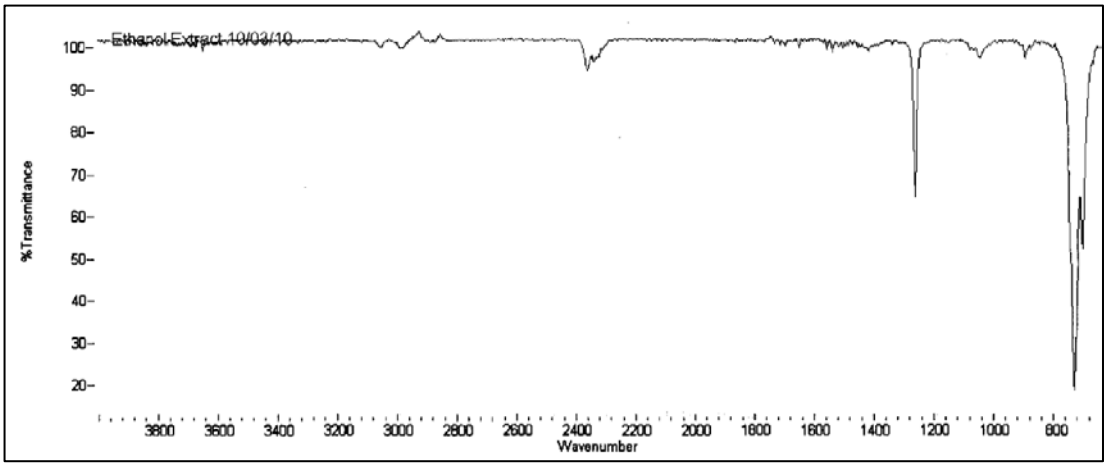

Figure C3 Scan of lipid extract by ethanol.

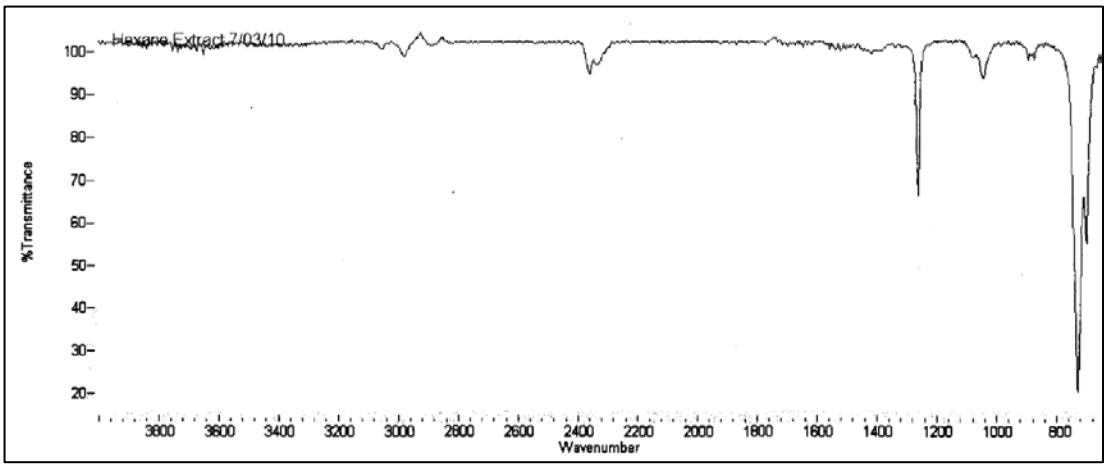

Figure C4 Scan of lipid extract by hexane.

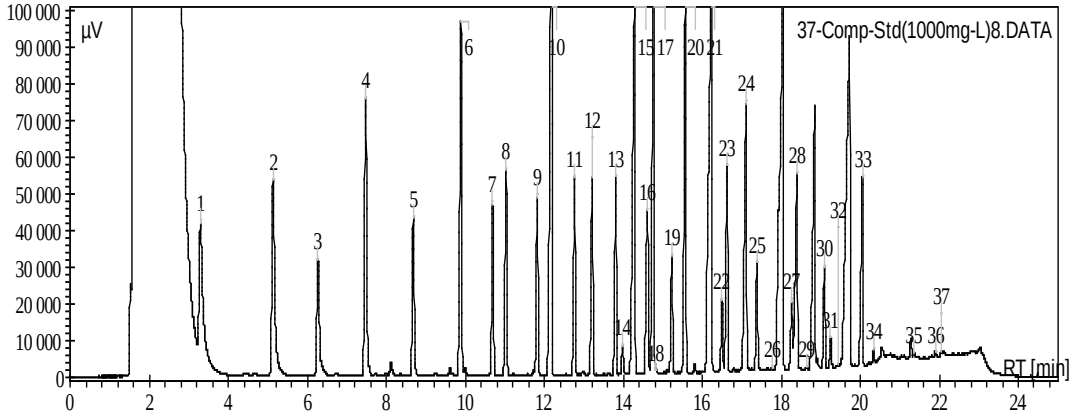

Figure C5 FAME standard chromatogram under optimized conditions.

#	Name	Time [Min]	Area [µV.Min]	Area % [%]
1	1	3.30	3470.6	2.691
2	2	5.13	4567.3	3.542
3	3	6.27	2392.2	1.855
4	4	7.48	4812.8	3.732
5	UNKNOWN	8.00	33.3	0.026
6	UNKNOWN	8.12	215.7	0.167
7	5	8.68	2499.5	1.938
8	UNKNOWN	9.23	25.0	0.019
9	UNKNOWN	9.61	96.4	0.075
10	6	9.89	5307.1	4.115
11	UNKNOWN	9.98	109.0	0.085
12	7	10.69	2394.0	1.856
13	8	11.03	2906.5	2.254
14	UNKNOWN	11.72	66.2	0.051
15	9	11.82	2454.5	1.903
16	10	12.19	9654.0	7.486
17	11	12.76	2717.5	2.107
18	UNKNOWN	12.97	49.7	0.039
19	12	13.21	2812.6	2.181
20	13	13.81	2656.4	2.060
21	14	13.98	397.2	0.308
22	15	14.28	7329.4	5.683
23	16	14.60	2865.9	2.222
24	17	14.76	6933.3	5.376
25	18	14.82	56.2	0.044
26	UNKNOWN	15.04	36.7	0.028
27	UNKNOWN	15.12	63.2	0.049
28	19	15.22	1632.6	1.266
29	20	15.56	5033.4	3.903
30	UNKNOWN	15.80	100.5	0.078
31	21	16.22	9676.6	7.503
32	22	16.49	987.5	0.766
33	23	16.62	2813.6	2.182

34	UNKNOWN	16.79	41.1	0.032
35	24	17.11	4513.2	3.500
36	25	17.38	1446.3	1.122
37	26	17.76	32.9	0.025
38	UNKNOWN	18.04	10380.6	8.049
39	27	18.26	1000.6	0.776
40	28	18.39	3361.2	2.606
41	29	18.63	28.9	0.022
42	UNKNOWN	18.84	4890.2	3.792
43	UNKNOWN	18.90	151.0	0.117
44	30	19.09	1356.7	1.052
45	31	19.24	400.8	0.311
46	32	19.45	25.7	0.020
47	UNKNOWN	19.73	9985.2	7.743
48	33	20.05	2921.1	2.265
49	34	20.33	156.2	0.121
50	UNKNOWN	20.54	255.7	0.198
51	UNKNOWN	21.04	34.1	0.026
52	UNKNOWN	21.10	36.9	0.029
53	UNKNOWN	21.28	279.8	0.217
54	35	21.35	67.4	0.052
55	36	21.89	81.0	0.063
56	37	22.05	49.5	0.038
57	UNKNOWN	22.33	25.7	0.020
58	UNKNOWN	22.76	10.7	0.008
59	UNKNOWN	23.03	254.2	0.197
60	UNKNOWN	24.32	3.3	0.003
61	UNKNOWN	24.47	4.5	0.003
	Total		128960.9	100.000

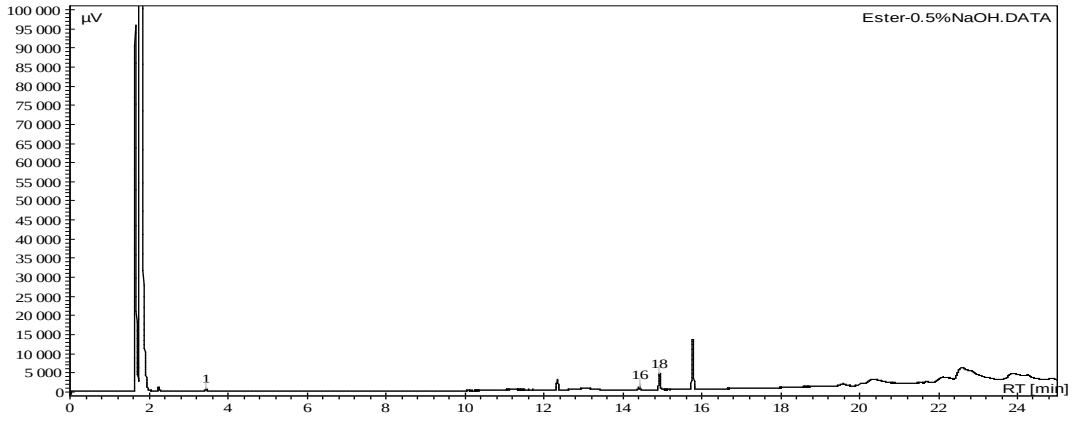

Figure C6 Chromatogram of 0.5% NaOH catalyzed ester for algal lipid

#	Name	Time [Min]	Area [µV.Min]	Area % [%]
1	UNKNOWN	0.04	18.4	0.037
2	UNKNOWN	1.65	3411.6	6.899
3	UNKNOWN	1.77	44814.4	90.626
4	UNKNOWN	2.24	42.9	0.087
5	1	3.45	37.7	0.076
6	UNKNOWN	12.34	125.4	0.254
7	16	14.41	37.2	0.075
8	18	14.93	192.5	0.389
9	UNKNOWN	15.77	594.8	1.203
10	UNKNOWN	22.54	174.6	0.353
	Total		49449.6	100.000

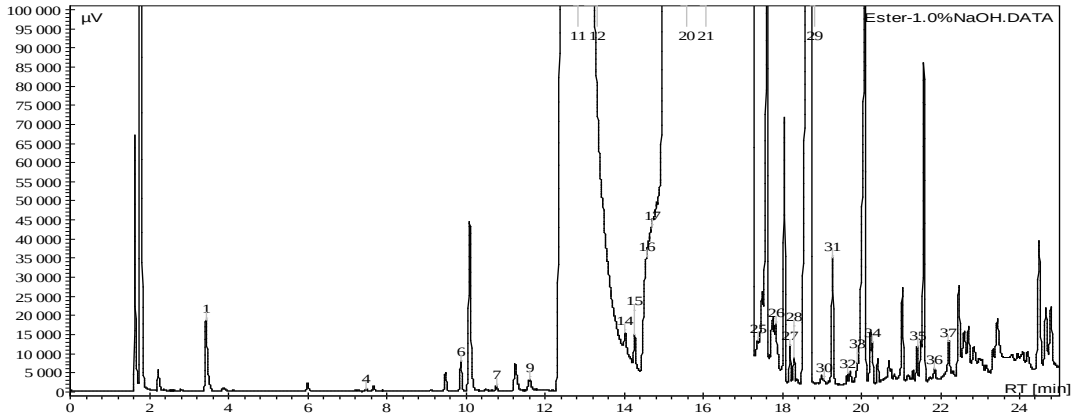

Figure C7 Chromatogram of 1.0% NaOH catalyzed ester for algal lipid

#	Name	Time [Min]	Area [µV.Min]	Area % [%]
1	UNKNOWN	0.02	2.5	0.001
2	UNKNOWN	1.63	2093.1	0.774
3	UNKNOWN	1.75	22287.0	8.246
4	UNKNOWN	2.22	256.3	0.095
5	1	3.43	1290.6	0.478
6	UNKNOWN	3.86	40.1	0.015
7	UNKNOWN	6.00	116.4	0.043
8	4	7.48	33.6	0.012
9	UNKNOWN	7.67	77.0	0.028
10	UNKNOWN	9.48	273.1	0.101
11	6	9.87	432.6	0.160
12	UNKNOWN	10.09	2913.1	1.078
13	7	10.76	65.0	0.024
14	UNKNOWN	11.24	564.7	0.209
15	9	11.60	244.5	0.090
16	UNKNOWN	12.69	131639.7	48.707
17	11	12.72	1227.2	0.454
18	UNKNOWN	12.76	1823.9	0.675
19	UNKNOWN	12.81	1266.5	0.469
20	UNKNOWN	12.87	224.7	0.083
21	12	12.99	378.6	0.140
22	14	14.02	236.9	0.088
23	15	14.26	395.3	0.146
24	16	14.56	2141.2	0.792
25	17	14.69	98.6	0.036
26	UNKNOWN	15.42	700.9	0.259
27	20	15.45	97.5	0.036
28	UNKNOWN	15.72	308.2	0.114
29	21	15.94	12020.3	4.448
30	UNKNOWN	17.26	6839.2	2.531
31	25	17.38	298.9	0.111
32	UNKNOWN	17.49	1272.4	0.471
33	UNKNOWN	17.61	7659.8	2.834
34	UNKNOWN	17.66	81.8	0.030
35	UNKNOWN	17.76	1161.9	0.430
36	26	17.83	1057.4	0.391

37	UNKNOWN	18.05	2882.4		1.066
38	27	18.19	394.5		0.146
39	28	18.30	356.7		0.132
40	UNKNOWN	18.37	51.9		0.019
41	29	18.72	32441.2	12.003	
42	UNKNOWN	18.98	105.8		0.039
43	30	19.05	62.2		0.023
44	UNKNOWN	19.17	35.8		0.013
45	31	19.27	1613.5		0.597
46	32	19.64	121.4		0.045
47	UNKNOWN	19.71	180.6		0.067
48	UNKNOWN	19.79	33.1		0.012
49	33	19.90	304.2		0.113
50	UNKNOWN	20.09	9727.2		3.599
51	UNKNOWN	20.22	665.3		0.246
52	34	20.27	417.6		0.155
53	UNKNOWN	20.41	305.5		0.113
54	UNKNOWN	20.61	183.8		0.068
55	UNKNOWN	20.69	333.1		0.123
56	UNKNOWN	20.74	171.7		0.064
57	UNKNOWN	20.83	146.8		0.054
58	UNKNOWN	21.03	1399.5		0.518
59	UNKNOWN	21.19	151.5		0.056
60	UNKNOWN	21.25	34.6		0.013
61	UNKNOWN	21.31	145.3		0.054
62	35	21.40	419.7		0.155
63	UNKNOWN	21.50	503.4		0.186
64	UNKNOWN	21.58	3829.0		1.417
65	UNKNOWN	21.73	62.9		0.023
66	36	21.84	202.9		0.075
67	37	22.20	713.4		0.264
68	UNKNOWN	22.31	86.4		0.032
69	UNKNOWN	22.46	1464.7		0.542
70	UNKNOWN	22.60	935.4		0.346
71	UNKNOWN	22.70	707.0		0.262
72	UNKNOWN	22.83	379.0		0.140
73	UNKNOWN	22.96	44.2		0.016
74	UNKNOWN	23.15	74.3		0.027
75	UNKNOWN	23.32	291.0		0.108
76	UNKNOWN	23.43	1172.5		0.434
77	UNKNOWN	23.80	81.0		0.030
78	UNKNOWN	23.94	131.2		0.049
79	UNKNOWN	24.08	311.4		0.115
80	UNKNOWN	24.19	306.4		0.113
81	UNKNOWN	24.48	2337.0		0.865
82	UNKNOWN	24.66	1106.5		0.409
83	UNKNOWN	24.78	1166.4		0.432
84	UNKNOWN	24.88	56.9		0.021
	Total		270268.0		100.000

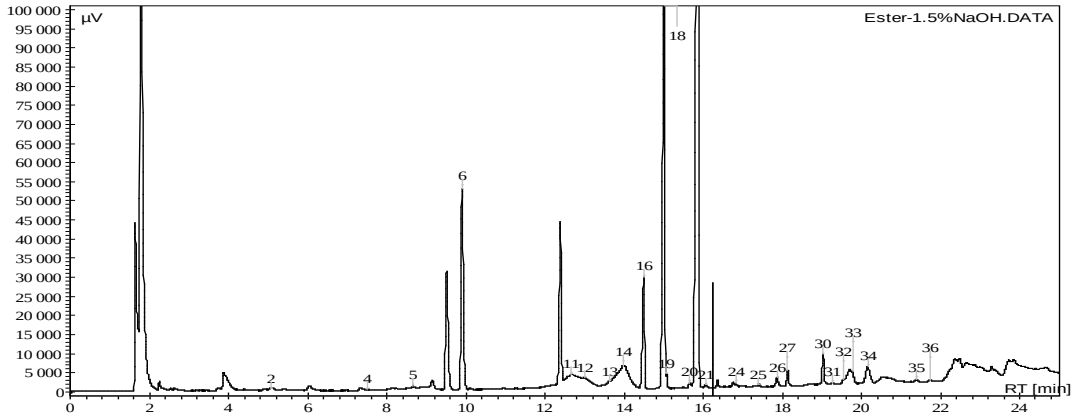

Figure C8 Chromatogram of 1.5% NaOH catalyzed ester for algal lipid

#	Name	Time [Min]	Area [µV.Min]	Area % [%]
1	UNKNOWN	0.03	10.6	0.014
2	UNKNOWN	1.64	2862.8	3.880
3	UNKNOWN	1.78	23372.1	31.674
4	UNKNOWN	2.25	89.3	0.121
5	UNKNOWN	2.53	15.7	0.021
6	UNKNOWN	2.62	11.8	0.016
7	UNKNOWN	3.75	34.3	0.046
8	UNKNOWN	3.88	125.4	0.170
9	UNKNOWN	4.87	10.2	0.014
10	2	5.07	23.8	0.032
11	UNKNOWN	6.03	63.7	0.086
12	UNKNOWN	7.33	72.9	0.099
13	4	7.51	3.9	0.005
14	5	8.65	27.0	0.037
15	UNKNOWN	9.15	156.4	0.212
16	UNKNOWN	9.51	2201.7	2.984
17	6	9.90	3504.8	4.750
18	UNKNOWN	10.11	25.7	0.035
19	UNKNOWN	12.38	2357.1	3.194
20	11	12.65	64.3	0.087
21	12	13.00	21.8	0.030
22	UNKNOWN	13.43	9.5	0.013
23	13	13.61	37.8	0.051
24	14	13.98	1923.9	2.607
25	16	14.49	1768.7	2.397
26	18	15.01	7560.3	10.246
27	19	15.07	130.1	0.176
28	20	15.66	102.9	0.139
29	21	15.88	21860.9	29.626
30	UNKNOWN	16.06	48.8	0.066
31	UNKNOWN	16.24	247.5	0.335
32	UNKNOWN	16.25	1047.6	1.420
33	UNKNOWN	16.76	53.3	0.072
34	24	16.83	39.4	0.053
35	25	17.40	23.8	0.032
36	26	17.86	143.2	0.194

37	27	18.13	198.9	0.270
38	30	19.03	418.3	0.567
39	31	19.27	18.3	0.025
40	32	19.55	87.0	0.118
41	UNKNOWN	19.70	507.2	0.687
42	33	19.77	188.5	0.255
43	34	20.16	569.1	0.771
44	UNKNOWN	20.51	82.7	0.112
45	35	21.38	24.3	0.033
46	36	21.73	42.1	0.057
47	UNKNOWN	22.35	611.4	0.829
48	UNKNOWN	22.46	271.9	0.368
49	UNKNOWN	22.64	55.7	0.076
50	UNKNOWN	23.29	59.7	0.081
51	UNKNOWN	23.72	398.4	0.540
52	UNKNOWN	23.85	202.8	0.275
	Total		73789.6	100.000

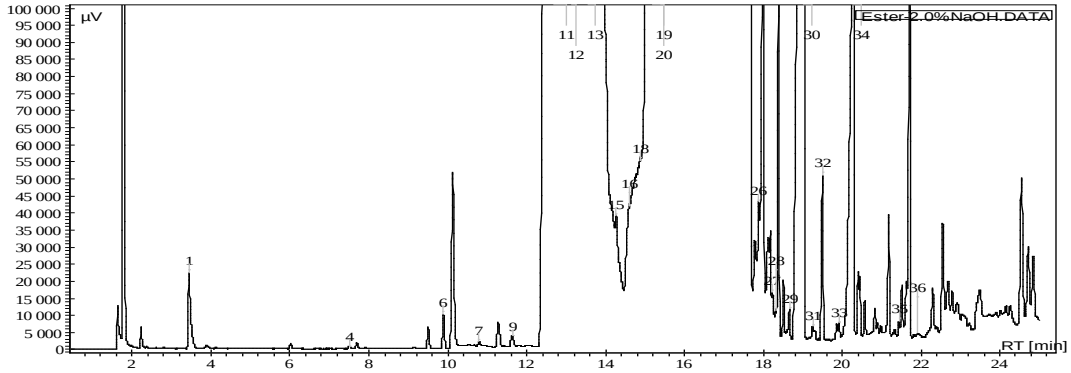

Figure C9 Chromatogram of 2.0% NaOH catalyzed ester for algal lipid

#	Name	Time [Min]	Area [µV.Min]	Area % [%]
1	UNKNOWN	1.63	688.3	0.183
2	UNKNOWN	1.76	41476.8	11.021
3	UNKNOWN	1.90	43.1	0.011
4	UNKNOWN	1.98	16.2	0.004
5	UNKNOWN	2.22	243.5	0.065
6	UNKNOWN	2.36	17.5	0.005
7	UNKNOWN	2.80	16.5	0.004
8	1	3.44	1582.9	0.421
9	UNKNOWN	3.88	36.6	0.010
10	UNKNOWN	6.01	78.7	0.021
11	4	7.51	51.0	0.014
12	UNKNOWN	7.69	100.3	0.027
13	UNKNOWN	9.50	337.9	0.090
14	6	9.89	549.3	0.146
15	UNKNOWN	10.13	3451.0	0.917
16	7	10.79	72.6	0.019
17	UNKNOWN	11.28	536.9	0.143
18	9	11.63	251.3	0.067
19	UNKNOWN	12.67	104524.7	27.773
20	11	12.70	9500.2	2.524
21	UNKNOWN	12.81	4932.1	1.310
22	UNKNOWN	12.87	3227.9	0.858
23	UNKNOWN	12.93	2151.4	0.572
24	UNKNOWN	12.98	4562.4	1.212
25	UNKNOWN	13.08	5147.4	1.368
26	12	13.13	1500.5	0.399
27	UNKNOWN	13.20	735.8	0.196
28	UNKNOWN	13.23	389.8	0.104
29	UNKNOWN	13.29	123.5	0.033
30	UNKNOWN	13.35	58.8	0.016
31	UNKNOWN	13.38	147.0	0.039
32	UNKNOWN	13.41	114.7	0.030
33	UNKNOWN	13.44	238.0	0.063
34	UNKNOWN	13.50	168.1	0.045
35	13	13.57	65.9	0.018
36	15	14.27	316.4	0.084
37	16	14.59	2862.8	0.761
38	UNKNOWN	14.78	361.5	0.096
39	18	14.87	136.0	0.036

40	19	15.18	43952.8	11.679
41	20	15.42	15061.9	4.002
42	UNKNOWN	15.70	244.0	0.065
43	UNKNOWN	17.70	9587.3	2.547
44	UNKNOWN	17.78	954.9	0.254
45	26	17.89	1621.6	0.431
46	UNKNOWN	18.01	11498.0	3.055
47	UNKNOWN	18.14	1648.3	0.438
48	UNKNOWN	18.18	963.2	0.256
49	27	18.23	453.9	0.121
50	28	18.32	192.9	0.051
51	UNKNOWN	18.39	3909.8	1.039
52	UNKNOWN	18.51	626.6	0.166
53	UNKNOWN	18.59	58.1	0.015
54	29	18.66	383.1	0.102
55	30	19.04	50553.4	13.432
56	UNKNOWN	19.17	36.7	0.010
57	31	19.25	217.1	0.058
58	UNKNOWN	19.31	123.1	0.033
59	UNKNOWN	19.40	12.4	0.003
60	32	19.51	2393.8	0.636
61	UNKNOWN	19.87	188.8	0.050
62	33	19.92	213.4	0.057
63	34	20.29	15370.3	4.084
64	UNKNOWN	20.41	820.6	0.218
65	UNKNOWN	20.45	633.0	0.168
66	UNKNOWN	20.58	413.4	0.110
67	UNKNOWN	20.83	318.9	0.085
68	UNKNOWN	20.90	136.9	0.036
69	UNKNOWN	20.98	141.6	0.038
70	UNKNOWN	21.18	1713.6	0.455
71	UNKNOWN	21.29	39.6	0.011
72	UNKNOWN	21.34	99.0	0.026
73	35	21.44	187.5	0.050
74	UNKNOWN	21.52	752.6	0.200
75	UNKNOWN	21.63	821.5	0.218
76	UNKNOWN	21.71	5455.6	1.450
77	UNKNOWN	21.82	33.7	0.009
78	36	21.90	14.1	0.004
79	UNKNOWN	22.30	972.7	0.258
80	UNKNOWN	22.40	104.3	0.028
81	UNKNOWN	22.55	1967.1	0.523
82	UNKNOWN	22.69	1176.0	0.312
83	UNKNOWN	22.79	649.2	0.172
84	UNKNOWN	22.92	551.0	0.146
85	UNKNOWN	23.05	67.0	0.018
86	UNKNOWN	23.23	55.0	0.015
87	UNKNOWN	23.49	1254.6	0.333
88	UNKNOWN	23.91	83.2	0.022
89	UNKNOWN	24.02	151.2	0.040
90	UNKNOWN	24.16	401.2	0.107
91	UNKNOWN	24.27	420.1	0.112
92	UNKNOWN	24.55	3046.0	0.809
93	UNKNOWN	24.72	1525.0	0.405
94	UNKNOWN	24.85	1268.5	0.337
	Total		376354.4	100.000

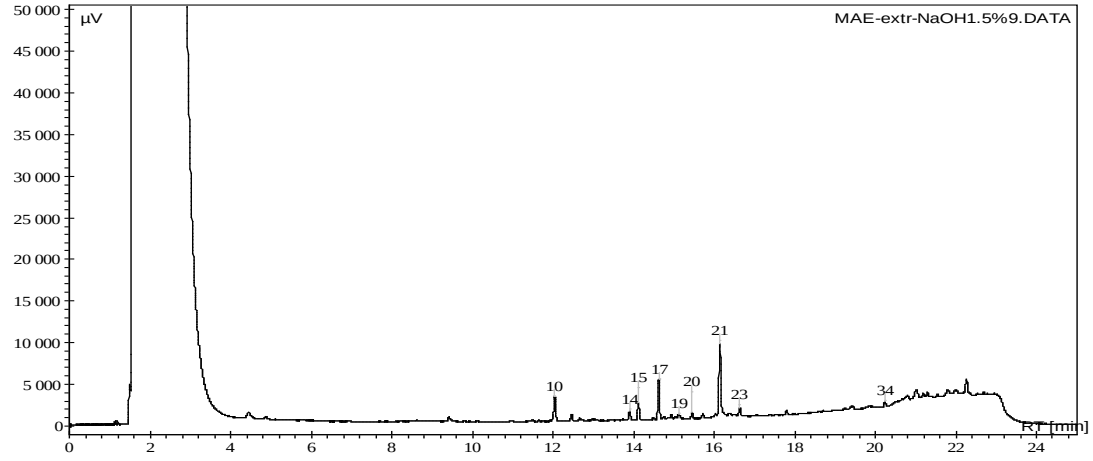

Figure C10 Chromatogram of 1.5% NaOH catalyzed ester for comparison With 1.5% KOH catalyzed ester

#	Name	Time [Min]	Area [µV.Min]	Area % [%]
1	UNKNOWN	1.49	64.1	4.6
2	UNKNOWN	9.41	24.8	1.8
3	10	12.04	151.9	11.0
4	UNKNOWN	12.46	39.9	2.9
5	14	13.90	52.3	3.8
6	15	14.12	96.8	7.0
7	17	14.62	222.1	16.0
8	19	15.11	17.9	1.3
9	20	15.45	31.7	2.3
10	21	16.14	446.7	32.2
11	23	16.63	42.4	3.1
12	34	20.23	27.1	2.0
13	UNKNOWN	21.01	76.8	5.5
14	UNKNOWN	22.26	92.0	6.6
	Total		1386.5	100.0

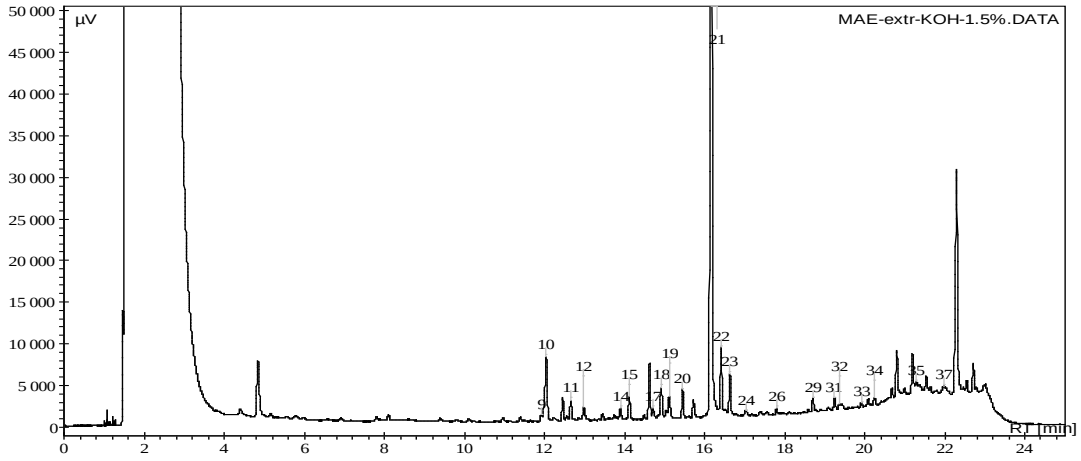

Figure C11 Chromatogram of 1.5% KOH catalyzed ester for comparison With 1.5% NaOH catalyzed ester

#	Name	Time [Min]	Area [µV.Min]	Area % [%]
1	UNKNOWN	1.07	18.4	0.2
2	UNKNOWN	1.13	9.2	0.1
3	UNKNOWN	1.21	7.0	0.1
4	UNKNOWN	1.25	5.0	0.0
5	UNKNOWN	4.40	37.6	0.3
6	UNKNOWN	4.84	465.3	4.1
7	UNKNOWN	8.10	33.1	0.3
8	9	11.92	42.1	0.4
9	10	12.04	520.9	4.6
10	UNKNOWN	12.45	133.4	1.2
11	UNKNOWN	12.56	23.6	0.2
12	11	12.65	109.0	1.0
13	12	12.98	71.8	0.6
14	UNKNOWN	13.45	37.4	0.3
15	14	13.89	65.6	0.6
16	15	14.12	128.5	1.1
17	UNKNOWN	14.62	314.9	2.8
18	17	14.71	64.0	0.6
19	18	14.92	177.7	1.6
20	UNKNOWN	15.03	39.5	0.4
21	19	15.11	146.1	1.3
22	20	15.45	161.0	1.4
23	UNKNOWN	15.72	101.8	0.9
24	21	16.17	4802.7	42.8
25	22	16.41	341.4	3.0
26	23	16.63	215.6	1.9
27	24	17.03	39.7	0.4
28	26	17.80	27.3	0.2
29	29	18.70	72.4	0.6
30	31	19.24	72.9	0.6
31	32	19.37	17.7	0.2
32	33	19.92	26.2	0.2
33	UNKNOWN	20.09	32.9	0.3
34	34	20.24	47.9	0.4
35	UNKNOWN	20.68	50.4	0.4

36	UNKNOWN	20.80	263.8	2.4
37	UNKNOWN	20.99	29.7	0.3
38	UNKNOWN	21.19	214.3	1.9
39	35	21.29	39.7	0.4
40	UNKNOWN	21.54	79.4	0.7
41	37	21.98	37.6	0.3
42	UNKNOWN	22.29	1742.2	15.5
43	UNKNOWN	22.55	73.1	0.7
44	UNKNOWN	22.71	171.1	1.5
45	UNKNOWN	22.78	23.4	0.2
46	UNKNOWN	23.01	85.9	0.8
	Total		11220.1	100.0