ASNAKE GUDISA EDE

PURIFICATION AND CHARACTERIZATION OF MICROALGAE LIPIDS: A VALUE-ADDITION TO BIODIESEL PRODUCTION

UNIVERSITY OF ALGARVE

Faculdade de Ciências e Técnologoa

2016

UNIVERSITY OF ALGARVE

PURIFICATION AND CHARACTERIZATION OF MICROALGAE LIPIDS: A VALUE-ADDITION TO BIODIESEL PRODUCTION

ASNAKE GUDISA EDE

Dissertação / Dissertation

Mestrado Erasmus Mundus em Inovação Química e Regulamentação
(Erasmus Mundus Master in Chemical Innovation and Regulation)

Trabalho efetuado sob a orientação de:
Work supervised by

Prof. Luísa Afonso Barreira (Supervisor)
Dr. Katkam N. Gangadhar (Co-supervisor)

2016

DECLARATION OF AUTHORSHIP AND COPYRIGHT

I declare that I am the author of this work, which is original. The work cites other authors and works, which are adequately referred in the text and are listed in the bibliography.

ASNAKE GUDISA EDE

ACKNOWLEDGEMENTS

I am unreservedly grateful to the European Commission for the scholarship funded as Erasmus Mundus Category ‘A’ student of Erasmus Mundus Joint Master Degree in Chemical Innovation and Regulation (EMM ChIR).

First & foremost, I offer my unfeigned and sincerest gratitude to my supervisors-Prof. Luísa Afonso Barreira and Dr. Katkam N. Gangadhar for their eternal support, professional guidance, constructive comments, patience and diligent help. I attribute the level of my Master Degree to them for this thesis, too, would not have been completed without their support.

My thanks in advance equally go to Hugo Pereira (Research scholar), Dr. Katkam N. Gangadhar (my co-supervisor) and Vera Gomes (CCMAR) who improved my laboratory skills from basic unit operations (extraction, biodiesel production…) to using column chromatography, HPLC-ELSD and GC-MS until I become self-ruling to activities closely related to my present thesis. Without these three professionals and my supervisor, my practical skills should not have been improved. My appreciations go to Prof. Joao Verela who provided me constructive comments and suggestion while I presented this thesis to Marine Biotechnology Group, Centre of Marine Science (CCMAR), University of Algarve. My sincere appreciations and thanks go to Tamara Filipa Santos (CCMAR) who supported me in translating my abstract from English version to Portuguese and organize laboratory meetings to present our work for Marine Technology Group, CCMAR, University of Algarve.

My deepest appreciation goes to our Programme coordinator -Prof. Isabel Cavaco and the directors -Prof. Daniel Sainz, Prof. Emilio Tagliavini and Prof. Teresa Fernandes for their commitment, determinations and charismatic leadership qualities for the continual success of the ChIR programme. I also thank all of my 2014/16 ChIR professors who taught me updated and recent sciences.

Last, but not least, I have no words to express the devotion of my wife, Aster Hailu Fufa, for her inspirations and enthusiasm. I emphatically say thank you to her Tender Loving Care and utterly dedicate this master to my kid-Koket Nahilet Asnake. My cheers also go to parents Tenangne Alebachew and Gudisa Ede and Getachew Gudisa for their unconditioned support to pursue my education as per my interest and ambition.

Above all, my thanks and praise go to Almighty for the many blessings He bestowed on me and for all things He has done in my life and for my ages. Thanks to His guidance, mercy, healings, unprecedented faithfulness, unfeigned love and protection.

ABSTRACT

The aim of the present work was to obtain a neutral lipids fraction of microalgae free of polar lipids (i.e., phospholipids and glycolipids) to produce biodiesel and, at the same time, get phospholipids and glycolipids rich fractions as value added products under a biorefinery approach. To achieve this objective, ethanolic extracts of the wet biomass (70.0%, w/w) of two microalgal strains (*Nannochloropsis oculata* and *Phaeodactylum tricornutum*) were prepared and partitioned into three fractions, namely hexane, colloidal and water fractions, using a binary solvent mixture (hexane + water), employing a new process called as Liquid Tri-phase System (LTPS). HPLC-ELSD was used to analyse the lipid classes and GC-MS to assess the fatty acid profiles. *N. oculata* had an ethanolic extract yield of 38.2% (w/w, dry weight) and *P. tricornutum* of 30.3%. Lipid class distribution (% w/w, total lipids) of *N. oculata* was 45.1, 22.1 and 32.8 for neutral lipids, glycolipids and phospholipids, respectively; and for *P. tricornutum* it was 23.2, 49.1 and 27.7, respectively. When the LTPS process was applied, lipid distribution was: hexane (27.4%), colloidal (53.4%) and water (19.2%) for *N. oculata*; and 30.6, 55.4, and 14.0% for *P. tricornutum.* The colloidal phases in both strains contain more than 23.0% glycolipids and 68.0% phospholipids. HPLC-ELSD confirmed that the neutral lipids fraction was obtained free of glycolipids and phospholipids. The fatty acids composition showed that the neutral lipids fraction or hexane phase of the LTPS is suitable for biodiesel production as it contains more than 83.0% of saturated and monounsaturated fatty acids, which are less prone to oxidation compared to polyunsaturated fatty acids (PUFA). Additionally, a colloidal phase fraction rich in glycolipids, that can be converted into biosurfactants, and phospholipids that can be processed as liposomes, aquaculture feed or infant formula additives as it is enriched in eicosapentaenoic acid (EPA) or other long chain poly unsaturated fatty acids, was obtained.

Key words: Microalgae, wet biomass extraction, liquid tri-phase system, lipid classes, biodiesel, bio-refinery

RESUMO

Factores associados aos combustíveis fósseis como os fortes impactos ambientais, incerteza de aprovisionamento energético e ainda necessidade de reduzir o consumo de energia, têm levado a uma procura por substitutos viáveis das energias não-renováveis. Os biocombustíveis devido às semelhanças com os combustíveis convencionais, apresentam-se como potenciais candidatos para a substituição do diesel derivado do petróleo por fontes renováveis.

Os biocombustíveis designados de primeira geração foram produzidos a partir de produtos agrícolas, contudo a concorrência com a industria alimentar aliada à necessidade de terra arável e água potável instituíram fortes barreiras aos seu sucesso.

A segunda geração de biocombustíveis usou como matéria-prima resíduos agrícolas nomeadamente culturas lignocelulosicas em vez dos produtos alimentares. No entanto, a tecnologia utilizada não se apresentou comercialmente rentável para uma exploração comercial. No processo de optimização, quatro tecnologias distintas foram usadas para a produção de combustíveis líquidos tendo por base lignoceluloses. O primeiro sistema baseia-se no processo de gaseificação para a produção de gás de síntese, seguida por Fischer-Tropsch ou síntese de metanol para produzir alcanos ou metanol respectivamente. A segunda tecnologia empregou pirólise e liquefacção termoquímica de bio-óleos para produzir alcanos. Estes compostos além de apresentarem um preço elevado, a modernização do processo requer altas pressões de Azoto (H_2). O terceiro método baseou-se na hidrólise da lignocelulose para a produção de monómeros de açúcar, os quais foram ainda convertidos em etanol e/ou hidrocarbonetos aromáticos por meio de fermentação e/ou desidratação. Porém, o processo de gaseificação necessita de ser levada a cabo a uma temperatura de 1000° C, o que representa um elevado gasto energético e monetário. O quarto método inclui o hidro-tratamento e desoxigenação de óleos vegetais. Os métodos descritos apresentam-se como uma tecnologia complexa e de elevado custo de manutenção, sendo fundamental aprofundar os estudos relacionados com as conversões térmicas da biomassa para biocombustíveis, bem como a implementação dos métodos descritos em larga escala.

Estes factores levaram ao aparecimento da terceira geração de biocombustíveis, produzidos a partir de microalgas. Esta fonte, é considerada promissora, visto que detém uma elevada produtividade lipídica ao longo de todo o ano, não necessitando de terra arável nem água potável e não entrando em competição direta com a indústria alimentar. Contudo a sua utilização é condicionada pela falta de tecnologias para aplicação em escala industrial e no elevado preço de processamento, sendo a desidratação da biomassa o passo de maior consumo energético e o que acarreta os maiores custos de todo o processo.

Com base nas limitações apresentadas para os biocombustíveis de terceira geração, este trabalho tem como principal objectivo obter a partir de biomassa de microalgas uma fração lipídica, livre tanto de fosfolipídos como de glicolípidos, para posterior produção de biodiesel. As fracções obtidas e ricas em fosfolípidos ou glicolípidos serão ainda analisadas pela sua possível aplicação na área biomédica ou alimentar sobre um esquema de bio-refinaria.

Para este trabalho o etanol foi usado para extrair lípidos neutros dos polares da biomassa húmida de microalga (70.0% w/w), e com isto, possibilitar o uso exclusivo de lípidos neutros para a produção do biodiesel, com vista a aumentar tanto a qualidade como o rendimento do processo. Os lípidos polares obtidos são ainda considerados produtos de valor acrescentado, classificados como ácidos gordos de cadeia longa poli-insaturados e altamente propensos a instabilidade oxidativa.

Como resultado, os extractos brutos de lípidos foram divididos em três classes, por cromatografia em coluna de sílica-gel em sistema trifásico. Com o uso de clorofórmio, acetona e metanol foi possível separar lipídios neutros (NLs), glicolípidos (GLs) e fosfolípidos (PLs) para cada tipo de solvente, respectivamente. No entanto estes solventes orgânicos, classificados como cancerígenos não podem ser utilizados na industria alimentar. Foi assim, aplicado um sistema de fase binária, utilizando hexano e água com o objectivo de obter as três principais classes de lípidos. Três fases foram observadas depois da mistura, a fase superior de hexano, a fase coloidal e a fase aquosa inferior. A fracção coloidal foi deixada no papel de filtro enquanto que a fase correspondente ao hexano e água foi filtrada, recolhida e posteriormente separada num funil de separação. Os perfis lipídicos foram analisados por HPLC-ELSD enquanto a composição de ácidos gordos foi determinada por cromatográfica gasosa (GC-MS).

A espécie *N. oculata* apresentou o maior rendimento para o extracto etanólico com 38.2% (w/w, peso seco), seguido pela *P. tricornutum* com 30.3%. A distribuição de lípidos por classe (%w/w, lípidos totais) de *N. oculata* foi de 45.1, 22.1 e 32.8 e para *P. tricornutum* de 23.2, 49.1 e 27.7 para lípidos neutros, glicolípidos e fosfolípidos, respectivamente. De modo semelhante, a distribuição de lípidos no sistema-Tri em fase líquida foram hexano (27.4%), coloidal (53.4%) e água (19.2%) para *N. oculata*; e 30.6, 55.4, e 14.0% para *P. tricornutum*. A distribuição da classe coloidal em % (w/w, coloidal total) apresentou resultados de *N. oculata* (NLs = 2.7 (5.1%), GLs = 12.5 (23.4%), PLs = 38.2 (71.5%); e para *P. tricornutum* (NLs = 1.6 (3%), GLs = 15.9 (28.7%), PLs = 37.9 (68.3%). Por HPLC-ELSD foram confirmadas as diferentes classes de lípidos e a separação dos NLs tanto de GLs como de PLs.

Triacilglicerol (TAG), diacilglicerol (DAG), monoacilglicerol (MAG), GLs e PLs foram detectados nos extractos brutos de microalgas e separados em 40 minutos, por um sistema quaternário de hexano, acetato de etilo, água e isopropanol. O elemento considerado menos polar, TAG, foi aluído primeiro, seguido de DAG, FA e MAG, GLs e PLs. TAG apresentou-se como o lípido dominante tanto no extracto em bruto como na fracção de NLs.

A composição de ácidos gordos para as duas fases (hexano e coloidais) mostrou que os lípidos neutros apresentam-se como adequados para a produção de biodiesel, com mais de 85.0% de ácidos gordos saturados e monoinsaturados que são menos propensos a instabilidade oxidativa.

Os glicolípidos podem ser convertidos para biossurfactantes enquanto que fosfolípidos podem ser processados para lipossomas como sistema de entrega de drogas ou alimentos em aquacultura. A fracção coloidal enriquecida em ácido eicosapentaenóico (EPA) pode ser optimizada e usada no tratamento da aterosclerose, cancro, e doenças neuro-degenerativas.

Palavras-chave: Microalgas, extração lipídica, fração lipídica, biodiesel, bio-refinaria

TABLE OF CONTENTS

FIGURE INDEX

TABLE INDEX

SCHEME INDEX

ANNEX INDEX

LIST OF ABBREVIATIONS AND ACRONYMS

AA	-	Arachidonic acid
BC	-	β-carotene
ChIR	-	Chemical innovation and regulation
CHL	-	Chlorophyll
DAG	-	Diacylglycerol
DGDG	-	Digalactosyl diacylglycerol
DHA	-	Docosahexaenoic acid
ELSD	-	Evaporative light scattering detector
EPA	-	Eicosapentaenoic acid
FAMEs	-	Fatty acid methyl esters
FAs	-	Fatty acids
FFAs	-	Free fatty acids
GC-MS	-	Gas chromatography – mass spectrometry
GHGs	-	Greenhouse gas
GLs	-	Glycolipids
HC	-	Hydrocarbon
HPLC	-	High performance liquid chromatography
LC-PUFAs	-	Long chain polyunsaturated fatty acids
LTPS	-	Liquid tri-phase system
MAG	-	Monocylglycerol
MGDG	-	Monogalactosyl diacylglycerol

MUFAs	-	Monounsaturated fatty acids
NLs	-	Neutral lipids
PA	-	Phosphatidyic acid
PC	-	Phosphatidylcholine
PE	-	Phosphatidylethanolamine
PG	-	Phosphatidylglycerol
PI	-	phosphatidylinositol
PLs	-	Phospholipids
PS	-	Phosphatidylserine
PUFAs	-	Polyunsaturated fatty acids
SFAs	-	Saturated fatty acids
TAG	-	Triacylglycerol
TFAs	-	Total fatty acids
TLC	-	Thin layer chromatography
TLs	-	Total lipids
US DOE	-	United State Department of Energy
US EIA	-	United State Energy Information Administration

1. INTRODUCTION

1.1. General Background

Fossil fuels are the world's primary energy consumption in the transportation sector (Houghton et al., 2001), which are finite and, at current usage rates, are expected to be depleted in this century, and thus not renewable in the short period (Akansu, Dulger, Kahraman, & Veziroğlu, 2004). They are the largest contributor for greenhouse gas emissions (GHGs);and in 2006 were associated with the emissions of 29 Giga tonnes of CO_2 (Raupach et al., 2007). It is estimated that natural processes remove only about 12 Giga tonnes, therefore, compatible mitigation strategies are required to neutralise the excess CO_2 (Raupach, et al., 2007). The 1997 Kyoto Protocol called for a 5.2% reduction in GHGs emissions worldwide (Houghton, et al., 2001).

Besides, the persistent hike in global prices of crude oil affects trade balance of oil importing countries (Bol, Bhattacharya, & Mishra, 2004). Subsequently, government regulations and full or partial tax exemption were established to promote the utilization of alternative fuels to fossil fuels (Ralf Gubler, 2007). In this regard, the EU has set a target that 10% of the total transportation fuels will be of biofuels by 2020 (Pacini, Silveira, & Da Silva Filho, 2013). Prediction shows coal, gas and oil will be the main used energy sources until 2050; but the energy derived from biomass and other renewables are expected to increase dramatically (Fig.1.1) (Shell, 2008).

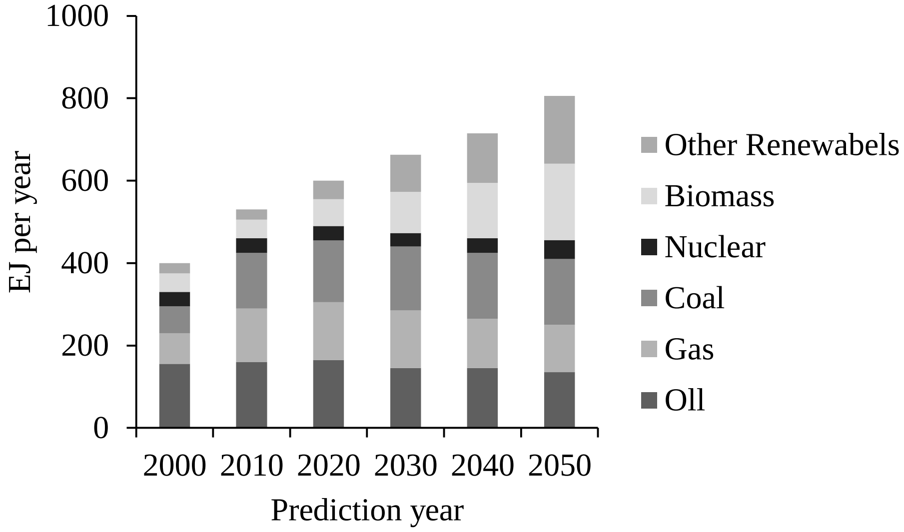


Fig. 1.1. Predicted world energy consumption by Shell (Shell, 2008)

Therefore, the aforementioned issues were considered to be the important trigger for many initiatives to search for alternative sources of energy (Azam, Waris, & Nahar, 2005). Amongst these renewable energy sources, biofuels for instance biodiesel, bioethanol, biomethane, biohydrogen and biobutanol received attention of which, biodiesel is a potential candidate for a petro-diesel substitute. Biodiesel, is a mixture of fatty acid alkyl (methyl, ethyl, or propyl) esters, which are mainly generated from vegetable oil or animal fat. Its production increased at a faster rate across the world, at an average rate of 32% per year, between 2000 and 2005 (R Gubler, 2006). Due to the similarities of physico-chemical properties with petroleum-based diesel, biodiesel can be used in conventional diesel engines without significant modifications (Venendaal, Jørgensen, & Foster, 1997). However, biodiesel is claimed to be instable towards oxidation and has high viscosity. But, these properties depend on feedstock types and biomass to biofuel conversion procedures.

Energy density, weight, and storage size are important characteristics for transportation fuels (US DOE,2007; US EIA,2013). For example, hydrogen presents the highest specific energy (Fig.1.2). However, vehicles must carry their fuel supply so, to determine which fuel is best for motor vehicles, energy density of the fuel is important.

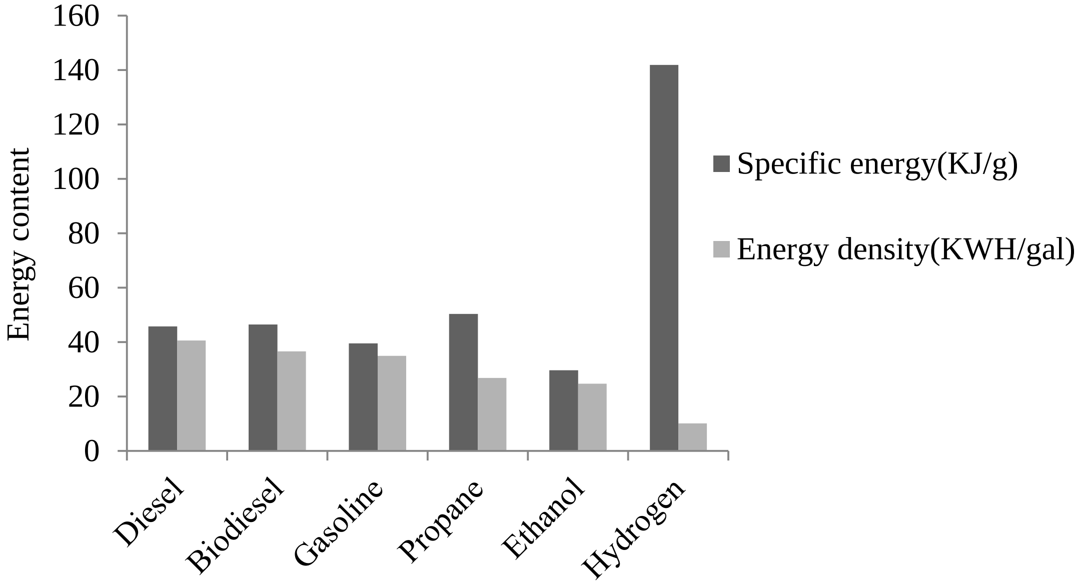


Fig. 1.2. Energy density of various transportation fuels (US DOE,2007; US EIA,2013)

Hydrogen, because it is so light, requires 15.9 times the container volume to provide the same energy as diesel (US DOE,2007; US EIA,2013). Hence, its energy density is so small that using hydrogen as transportation fuel for heavy-duty trucks is expensive and impractical. Biodiesel and diesel are similar with respect to energy density (US DOE,2007; US EIA,2013).

In general, biofuels have the following socio-economic and environmental advantages over fossil fuels:

- In most case they are carbon-neutral (Daroch, Geng, & Wang, 2013).
- Biofuel industry creates huge jobs. For example, if oil industry creates jobs for one person, coal creates 4 and ethanol creates 152 (De Castro, 2007).
- Emissions from biofuels are less toxic (Daroch, et al., 2013).
- Biofuel improves trade balance. In Brazil, for instance, the replacement of gasoline by bio-ethanol saved US$ 1.8 billion/year (Dufey, Vermeulen, & Vorley, 2007).
- It is also renewable (vegetable oils, animal fats and waste cooking oils, etc.) (Agarwal & Das, 2001; Kalligeros et al., 2003).

Hence, consideration of these advantages leads to evolution of biofuel generations from different renewable resources like vegetable-based oils, sugar cane, corn, lignocellulosic and microalgal biomass (Fig.1.3).

Food crops Lignocelluloses Microalgae

Fig. 1.3. Biomass sources for biofuel production

Sources of Feedstocks

First Generation

The first generation biofuels mainly biodiesel have been mainly generated from food and oil crops .But, competition with food and fibre production for arable land, fresh water and fertiliser requirement are barriers to their success (Group & Management, 2009; Koh & Wilcove, 2008).

Second Generation

The second generation biofuels are from lignocelluloses to produce particularly alkanes, gasoline and diesel fuels from the whole plant matter like agricultural residues rather than food crops (Koh & Wilcove, 2008; Moore, 2008). However, technology for conversion has not reached the scale required for commercial exploitation (Koh & Wilcove, 2008).

Third Generation

Third generation biofuels are produced from microalgal biomass. Algae are primitive organism lacking roots, stems and leaves, but have chlorophyll ‘a’ as their primary photosynthetic pigment (Nisbett, Peng, Choi, & Norenzayan, 2001). Prokaryotic cells (*cyanobacteria*) lack membrane-bound organelles (plastids, mitochondria, nuclei, Golgi bodies, and flagella) and are more akin to bacteria than algae. Eukaryotic cells, which comprise of many different types of common algae, do have these organelles that control the functions of the cell, allowing it to survive and reproduce. The most important classes of Eukaryotes are: green algae (*Chlorophyta*), red algae (*Rhodophyt*a) and diatoms (*Bacillariophyta*) (Khan, Hussain, Prasad, & Banerjee, 2009).

Algae can grow autotrophic- or heterotrophically; the former require only inorganic compounds such as CO_2, salts and a light energy source for growth, while the latter require an external source of organic compounds, as well as nutrients, as an energy source. Some photosynthetic algae are mixotrophic, i.e. they have the ability to both perform photosynthesis and acquire exogenous organic nutrients (Brennan & Owende, 2010). For autotrophic processes, photosynthesis is a key component, whereby microalgae convert solar radiation and CO_2 absorbed by chloroplasts into adenosine triphosphate (ATP) the usable energy currency at cellular level, which is used in respiration to produce energy to support growth (Zilinskas & Zilinskas, 1974).

Microalgae Biomass Production

To optimize microalgal biomass production raceways open ponds and closed photobioreactor have been developed (Fig.1.4). Raceways and open ponds are usually built in concrete, but compacted earth lined ponds with white plastic have also been used. Closed photobioreactor systems include the tubular, flat panel, and column (Brennan & Owende, 2010).

Open systems are relatively cheaper but have poor biomass productivity. Closed systems yield high biomass but are expensive. These area needs further work to search for an ideal method of biomass production for industrial scale application.

Fig.1.4. Photo of open system - raceway (left) and closed system-tubular photobioreactor (right)

Biomass Harvesting

The harvesting of microalgal biomass which generally requires one or more solid–liquid separation steps is a challenging phase of the algal biomass production process, and usually accounts for 20–30% of the total costs of production (Brennan & Owende, 2010). Filtration, flocculation (Fig.1.5) and centrifugations are the most commonly used harvesting technologies.

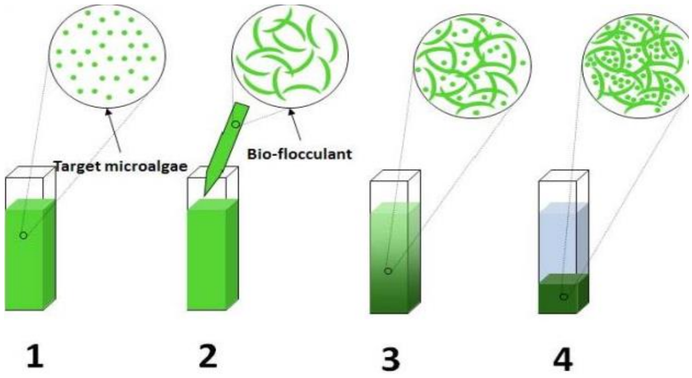


Fig. 1.5. Harvesting of microalgae biomass by flocculation

Lipid Content

While many microalgae strain naturally have high lipid content (ca. 20–50% dry weight), it is possible to increase the concentration and modulate the lipid profile by optimising the growth determining factors.

For instance, *Nannochloropsis* strain was shown to have 68.5% lipid content after nitrogen deprivation (Bondioli et al., 2012). Lipid content of *N. oculata* increased from 30.8% to 50.4% upon 2% CO_2 aeration (Chiu et al., 2009).

Lipid production increased by 14.9% with increasing temperature from 20°C to 25°C (Converti, Casazza, Ortiz, Perego, & Del Borghi, 2009). UV-A application increases saturated FAs to PUFAs ratio (Forján et al., 2011). Similarly, *P. tricornutum* TAG levels increased from 69 to 75% in nitrogen limitation (Hu et al., 2008). The decrease from 25°C to 10°C for 12h leads to highest yields of PUFAs and EPA in *P. tricornutum* (Jiang & Gao, 2004).

Advantages of using Microalgae

Microalgae biomass is considered a better feedstock for biofuel and/or other value added products because microalgae

- Oil productivity exceeds the yield of the best oilseed crops. For instance, biodiesel yield is of 12,000 Lha^{-1} for microalgae compared with 1190 Lha^{-1} for rapeseed (Schenk et al., 2008);
- Needs less water than terrestrial crops (Dismukes, Carrieri, Bennette, Ananyev, & Posewitz, 2008);
- Can be cultivated in brackish water or sea water and on non-arable land (Searchinger et al., 2008);
- Have a rapid growth potential , the exponential growth rates can double their biomass in periods as short as 3.5 h (Metting Jr, 1996);
- Biomass production can fixate high amount of CO_2 (1 kg of dry algal biomass /1.83 kg of CO_2) (Chisti, 2007);
- Nutrients for cultivation(especially nitrogen and phosphorous) can be obtained from wastewater, therefore, apart from providing growth medium, there is a dual potential for treatment of wastewaters (Cantrell, Ducey, Ro, & Hunt, 2008);
- Cultivation does not require herbicides or pesticides (Rodolfi et al., 2009);
- Produce valuable co-products such as proteins , β-carotene, and residual biomass after oil extraction (Brennan & Owende, 2010; Spolaore, Joannis-Cassan, Duran, & Isambert, 2006);
- Oil yield may be enhanced under nitrogen deficiency or high light intensity (Qin, 2005).

Bio-refinery

Among the factors that affect the cost of biofuels from microalgae is biomass to biofuel conversion technologies (Brennan & Owende, 2010).One approach to make it an economically competitive feedstock is to apply the bio-refinery concept (Fig.1.6).

The bio-refinery concept embraces a whole crop approach of biomass conversion pathways leading to a whole portfolio of valuable products, drawing direct similarities to today's petroleum refineries, in which multiple fuels, basic chemicals, intermediate products and sophisticated products are produced from petroleum (Cherubini, 2010; Morais & Bogel-Lukasik, 2013).

The most important energy products that can be produced in biorefineries are:

- Gaseous biofuels (biogas, syngas hydrogen,and biomethane)
- Solid biofuels (biochar)
- Liquid biofuels (bioethanol, biodiesel, Fischer-Tropsch fuels, and bio-oil)

Some of material products are

- Biochemicals (fine chemicals and pharmaceuticals, building blocks, and bulk chemicals)
- Organic acids (succinic, lactic, itaconic, fumaric and other sugar derivatives)

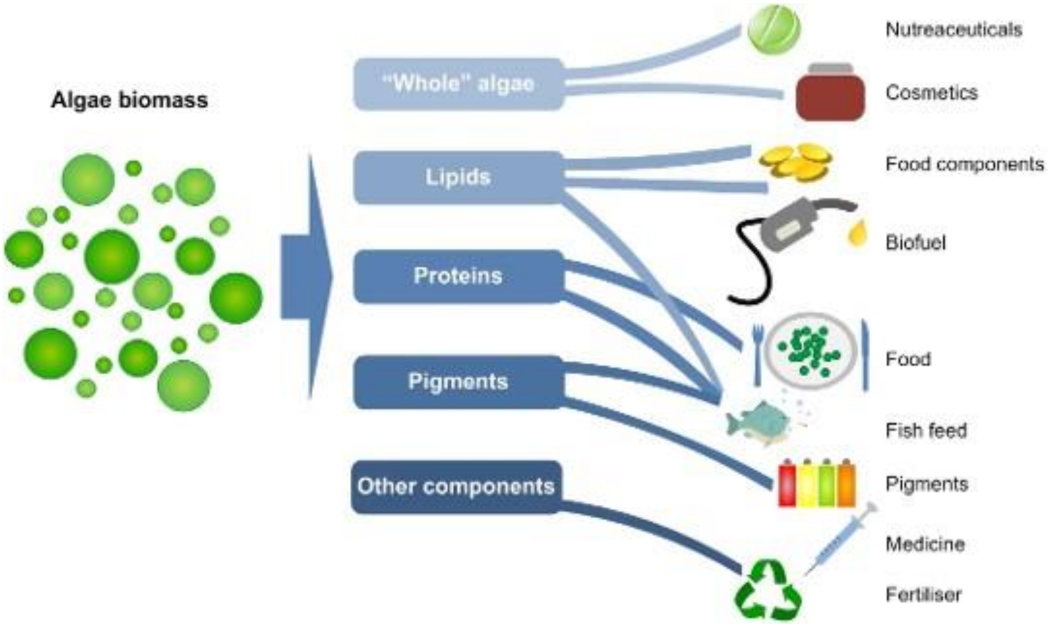


Fig. 1.6. Microalgae and the biorefinery concept

Microalgal Global Companies

The world has been confronted with a food and energy crisis due to accelerated global population growth and the depletion of finite fossil fuel resources (Jacob-Lopes, Queiroz, Ramírez-Mérida, & Zepka, 2015). The increase in nutritional problems along with rising fuel demands and environmental problems have increased the search for nutritional supplements and sustainable sources of energy.

Microalgae have great potential in generating energy from biotechnological processes using renewable sources and do not compromise food production. Microalgae have been of major interest in biofuel production as well as in the feed, chemical and pharmaceutical sectors (Jacob-Lopes, et al., 2015). Depending on the species and growth conditions, microalgae can be selected to produce a wide variety and abundance of lipids, proteins, carbohydrates, and feedstocks important for biofuel and production of nutraceuticals (Jacob-Lopes, et al., 2015). For instance, Table 1.1 below shows different global companies engaged in algae with CO_2 sequestering technology.

Table 1.1. Global algae companies with CO_2 sequestering technology for algae culture

Companies	Countries	Descriptions
AFS BioOil Co.	San Francisco, USA	Founded in 2010. This company uses algae that are fed by nutrients recovered from wastewater treatment plants. Biodiesel, the main product produced in these biorefineries is cost competitive with petroleum products.
AFS Biofarm™	San Francisco, USA	Uses CO_2 sequestered from industrial facilities and power plants for conversion into renewable fuels and other valuable products such as food additives.
Seambiotic Ltd.	Tel Aviv, Israel	Set up 2003. The company aims to develop microalgae biomass for the production of food additives and biofuel using flue gas from coal burning power stations.
Aeon Biogroup	Chile	This company develops biomass production methods with CO_2 capture from winegrowing for production of oil, nutraceuticals, food additives and biochemical compounds.
IGV Biotech	Nuthetal, Germany	Established 1960. IGV Biotech develops microalgae biotechnology processes for the production of several products such as food, pharmaceuticals and chemicals. This company uses advanced technology for the cultivation of photosynthetic microorganisms and CO_2 capture.
Algenol Biofuels	Florida, USA	Started in 2006. The company uses CO_2 and seawater as a culture medium for bioethanol. Nitrogen fixing technology is used to reduce production costs of fertilizers by cyanobacteria.

Adapted from Jacob-Lopes et al. (2015)

1.2. Liquid Fuels from Biomass

The following are common types of processes for the production of liquid fuels from lignocelluloses or other biomasses (Fig.1.7) (Huber & Dumesic, 2006). The first process involves gasification to produce syngas, followed by Fischer-Tropsch synthesis to produce alkanes. The second employs pyrolysis and thermochemical liquefaction for bio-oils production, which are further refined to produce alkanes. The third relies on hydrolysis of lignocellulose for sugar monomer production, and is further converted into ethanol and/or aromatic hydrocarbons through fermentation and/or dehydration. However, gasification was carried out at 1000°C which is energy intensive. Pyrolysis and liquefaction are expensive as refining or upgrading of biooil requires high H_2 pressure (Brennan & Owende, 2010). Further study is required to achieve a better understanding of the economics of biomass thermal conversions for bio-fuel production, as well as resolving issues related to the capabilities of these technologies in industrial application.

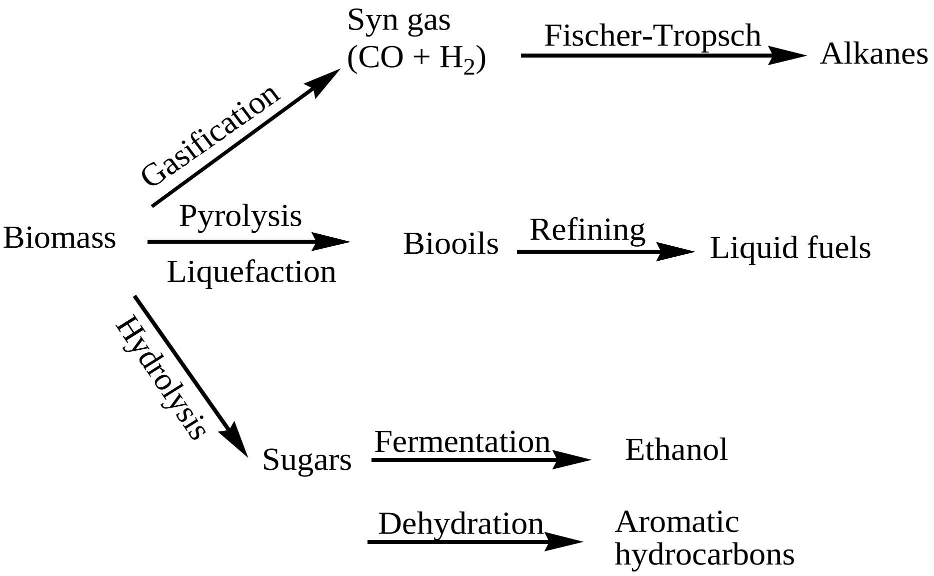


Fig. 1.7. Processes for production of liquid fuels from biomass (Huber & Dumesic, 2006)

1.3. Vegetable Oils to Green Diesel

Despite the fact that most of the current biofuels are produced through a transesterification process, these fatty acid methyl esters (FAMEs) have high oxygen content, poor flow property at low temperatures and poor oxidative stability (Ma & Hanna, 1999). To solve such problems, catalytic cracking (Corma, Huber, Sauvanaud, & O'connor, 2007), hydrotreating (Huber, O’Connor, & Corma, 2007),and deoxygenation (Snåre, Kubickova, Mäki-Arvela, Eränen, & Murzin, 2006) have been attempted to produce green diesel. The claims of green diesel over biodiesel are the following (Zhao, Brück, & Lercher, 2013):

1) Green diesel is sulphur free; 2) Its cetane number and calorific values are higher than biodiesel and even petro-diesel; and 3) Improved cold filter plug point

However, the utilization of cracking method has several drawbacks. It contains large amount of oxygenated and aromatic compounds, whose use is strictly regulated in Europe. Most importantly, zeolite cracking is a non-selective process that produces a wide range of compounds and undesired coke formation (Corma, et al., 2007). The hydrotreating process is much more selective than cracking in producing diesel range hydrocarbons. However, sulfide catalysts contaminate products via sulfur leaching and lead also to catalyst deactivation (Huber, et al., 2007). Compared with the hydrotreating process, catalytic deoxygenation possesses several advantages, including higher selectivity to the target linear hydrocarbons and less additional hydrogen cost because of few hydrogen requirements. Decarboxylation, decarbonylation and hydrodeoxygenation are the main reaction routes. Noble metal supported catalysts (e.g., commercial Pd/C and Pt/C) are usually employed. However, the high price of noble metal also limits the application in large-scale industrial production (Snåre, et al., 2006).

Therefore, until green diesel gets solution to its drawbacks, no doubt that biodiesel will be competitive alternative to petro-diesel. Hence, biodiesel produced from transesterification of vegetable oils is around 80% of the current biofuel market in Europe (Bendz, 2005) and production is increasing exponentially reaching 9,500,000 tons in 2010 (Knothe, 2010).

1.4. Microalgae Biochemistry and Biotechnology

1.4.1. Lipid Classes

Microalgae crude lipids contain two fundamental categories of lipids: neutral lipids (NLs) and polar lipids. The polar lipids comprise mainly phospholipids (PLs) and glycolipids (GLs) (Mayzaud, Boutoute, Perissinotto, & Nichols, 2007; Ryckebosch, Muylaert, & Foubert, 2012; Fangfang Yang et al., 2014). For instance, hydrocarbons (HC), cholesterol, sterols, pigments (i.e., β-carotene, lutein, zeaxanthin, and astaxanthin etc.), tri, di and mono acyl glycerols (TAG, DAG, and MAG), fatty acids (FAs) , GLs and PLs were reported in crude extracts of microalga (Brennan & Owende, 2010; Jones, Manning, Montoya, Keller, & Poenie, 2012).

Neutral Lipids

NLs usually comprise up to 50% microalgae lipid content (Fangfang Yang, et al., 2014). They are water-insoluble, whereas polar lipids have one or more water-soluble functional groups (Mayzaud, et al., 2007; Ryckebosch, et al., 2012; Fangfang Yang, et al., 2014). TAG, DAG, MAG and FA are the main NLs fractions reported of which TAG is dominant in microalgae.

TAG, stored in adipose tissue, is a major form of energy storage in animals and plants (Brasaemle, 2007). NLs can be converted into a biodiesel via transesterification (Bendz, 2005) (Fig.1.8). Transesterification consists of three consecutive reversible reactions of TAG to DAG, followed by DAG to monoacylglycerol, and finally to fatty acid methyl esters (FAMEs) and glycerol (Fig.1.8).

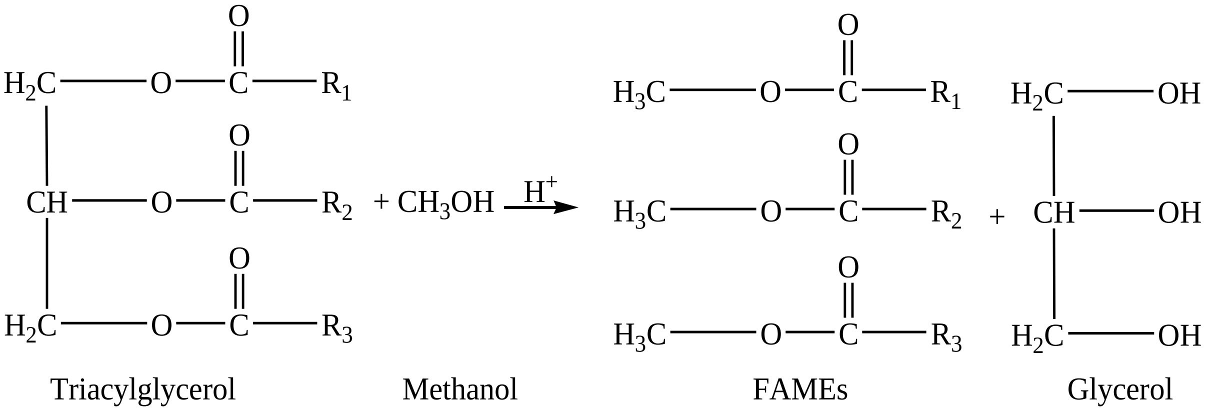


Fig. 1.8. Biodiesel production through transesterification of TAG

The transesterification reaction can be catalysed by alkaline catalysts such as sodium or potassium hydroxide (Knothe, 2005b). Such catalysts lead to complete conversion with 98% ester yields. However, their efficiency decreases when free fatty acids (FFAs) content is higher than 0.5% (w/w%, oil) due to saponification (Lotero et al., 2006; Lotero et al., 2005). On the contrary, acid catalysts offer the advantage that they can catalyse the transesterification of acylglycerols and esterification of FFAs simultaneously.

Glycerol is a by-product of biodiesel, which is a very attractive raw material for fine chemicals due to its relatively low cost and wide availability (Werpy et al., 2004). 26% of glycerol consumption was associated with pharmaceutical, cosmetic and soap industries- wider applications were reviewed and reported at (Fangxia Yang, Hanna, & Sun, 2012).

Phospholipids

PLs are a class of lipids that are a major component of all cell membranes. They can form lipid bilayers because of their amphiphilic nature. The structure of the PLs molecule generally consists of two hydrophobic FA “tails” and a hydrophilic phosphate “head”, joined together by a glycerol molecule (Fig 1.9). They are widely distributed in animals and plants, and the main sources include vegetable oils (e.g. soybean) and animal tissues (e.g. egg yolk) (Wendel, 1995). However, soybean and egg yolk have differences in the contents and types of PLs.

For example: 1) egg yolk lecithin contains high amounts of PC; 2) PLs in egg yolk have long chain polyunsaturated fatty acids (LC-PUFA) of n-6 and n-3 series, primarily arachidonic acid (AA) and docosahexaenoic acid (DHA), which are absent from soybean lecithins; and 3) the saturation level of egg yolk lecithins is higher than that of soybean lecithins (Hager, De Paoli, Ihlo, Farach, & Poole, 1993), so their oxidative stability is better than that of soybean lecithins.

Each individual PLs fraction provides the following functions (Fig 1.9):

- Phosphatidylcholine (PC) has function of nourishing the brain and improving intelligence (Little, Levy, Chuaqui-Kidd, & Hand, 1985);
- Phosphatidylethanolamine (PE) has important role in membrane fusion (Dowhan, Bogdanov, & Mileykovskaya, 2002);
- Phosphatidylserine (PS) improves function of nerve cells and enhancing the memory of brain (Pepeu, Pepeu, & Amaducci, 1996);
- Phosphatidylinositol (PI) plays a very important role in the process of transmission of messages in neural system (Bansal & Majerus, 1990);
- Phosphatidyic acid (PA) is a precursor for the biosynthesis of many other lipids (Delon et al., 2004);
- Phosphatidylglycerol (PG) is a glycerophospholipid found in pulmonary surfactant (King & MacBeth, 1981).

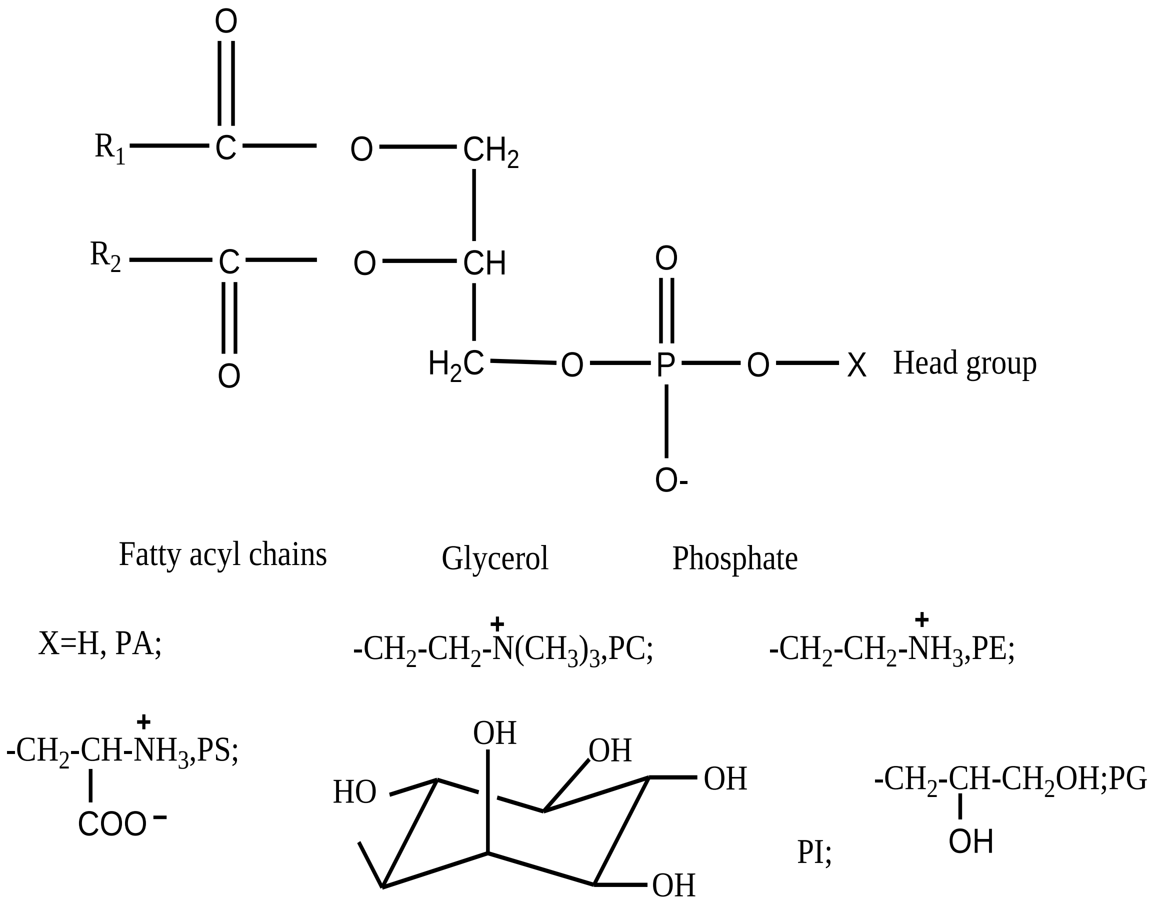


Fig. 1.9. Schematic depiction of the common phospholipid fractions

PLs can also be used as drug delivery system as liposome. Therapeutic agents such as proteins/peptides, nucleic acids, anticarcinogens, and other drugs have the drawbacks of low bioavailability, rapid clearance, and high toxicity. Liposomes have many advantages being able to deliver hydrophilic and lipophilic drugs, targeting, and tissue compatibility, reducing drug toxicity and improving its stability (Fig 1.10). Liposomes can serve as the carriers of antitumor, antifungal, analgesic, in gene therapeutics and vaccines (Delalat et al., 2015; J. Li et al., 2015).

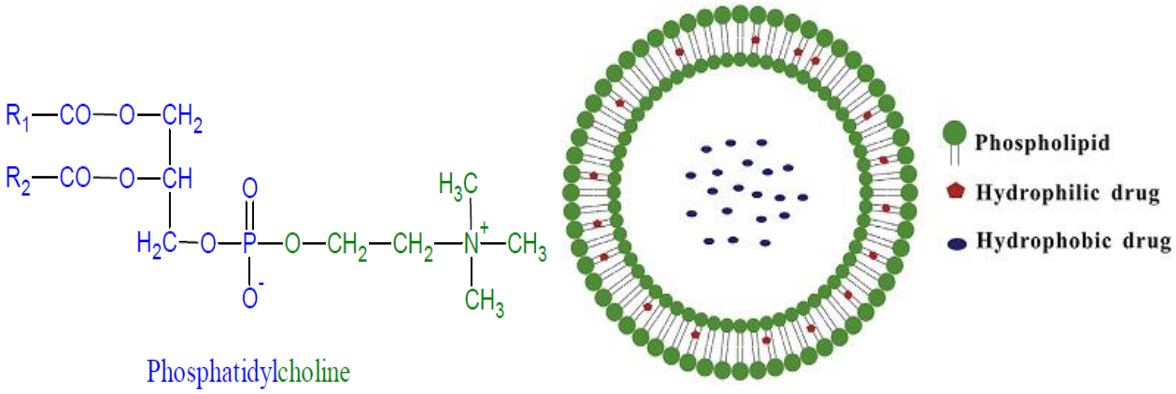


Fig.1.10. Structure of PC (left) and PLs liposome (right) (J. Li, et al., 2015)

Glycolipids

Glycolipids (GLs) are lipids with a carbohydrate attached by a glycosidic bond (Fig 1.11) .Their role is to serve as markers for cellular recognition and also to provide energy. GLs can be converted to biosurfactants (Abdel-Mawgoud, Hausmann, Lépine, Müller, & Déziel, 2011; Kitamoto, Isoda, & Nakahara, 2002). Biosurfactants show unique properties (*e.g.*, mild production conditions, lower toxicity, higher biodegradability and environmental compatibility compared to their chemical counterparts (Kitamoto, et al., 2002). The numerous advantages of biosurfactants have applications not only in the food, cosmetic, and pharmaceutical industries but in environmental protection (soil washing, oil spills cleaning). GLs biosurfactants are the most promising due to high productivity from renewable resources (Kitamoto, et al., 2002).

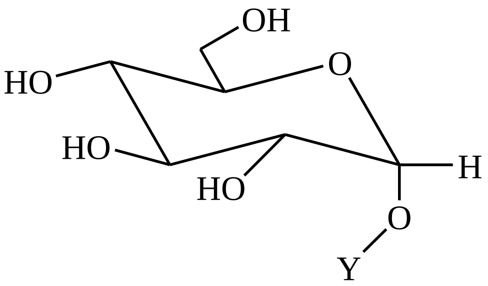


Fig. 1.11. General chemical structure of glycolipid where, ‘Y’ stands for lipids. For instance, if ‘Y’ is glycerol, it is called glycerol glycolipid.

Polar lipids particularly PLs and GLs provide wider applications not only in lipid form but also the fatty acids especially long chain polyunsaturated fatty acids. For instance, Foley et al. (2011) states highly unsaturated lipids occur more frequently in polar lipid fractions. Eicosapentaenoic acid (EPA, C20:5n-3) and DHA (C22:6n-3) are important n-3-PUFA, while AA (C20:4 n-6) is a vital n-6-PUFA and are mainly associated with PLs class of microalgae. EPA and DHA are important in the treatment of atherosclerosis, cancer, and neurodegenerative diseases such as Alzheimer's. AA and DHA are of special importance in the brain and blood vessels and are considered to be essential for pre and post-natal brain and retina development (Crawford, 2000). The eicosanoids, such as prostaglandin, prostacyclin and leukotriene, derived from n-3-PUFAs are important for infant development, modulatory vascular resistance and wound healing (Sijtsma & De Swaaf, 2004; Simopoulos, 1999). Modern nutritional theory is focusing on the numerous health benefits of maintaining sufficient levels of n-3-PUFAs (V. Patil & Gislerod, 2006).

The principal dietary source of DHA and EPA is marine fish. Fish is a declining resource, and there are serious environmental consequences related to the continued exploitation of fish stocks in order to meet the demands of an expanding market.

Moreover, fish accumulate pollutants. Also, the extracted oil often has an unpleasant odour, and the proportion of specific FAs in its TAG is difficult to control. The global aquaculture production was 547 million tonnes in 2003 (V. Patil, Källqvist, Olsen, Vogt, & Gislerød, 2007) and it has been growing by roughly 9% annually (Naylor & Burke, 2005). Feed is the largest production cost for commercial aquaculture plants, so improving feed efficiency in industrial systems has high priority. Considerable evidence has indicated that n-3-PUFAs in fish oils are actually derived via the marine food chain from zooplankton consuming n-3-PUFAs - synthesising microalgae (Yongmanitchai & Ward, 1989).

Therefore, marine microalgae are some of the most important feed sources in aquaculture (live feed for larvae of bivalves, and marine fish; food for rotifers and shrimps), due to their nutritional value and their ability to synthesise and accumulate great amounts of n-3-PUFA. Specific algal species are suitable for preparation of animal feed supplements. Algae species such as *Chlorella, Scenedesmus, and Haematococcus* have beneficial aspects including improved immune response and fertility, and better weight control (Brennan & Owende, 2010).

1.4.2. Fatty Acid Composition

Higher plants oil has suitable FAs for biodiesel production (Table 1.2). The major FAs are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). However, these oil bearing plants compete with food supply.

Table 1.2. Fatty acid composition of typical vegetable oils

Oils	C14:0 [a]	C16:0	C18:0	C18:1	C18:2
Rapeseed	0.1-0.3	5.1-6.1	2.1-3.2	57.9-58.2	24.7-25.5
Corn	-	7.0-13.0	2.5-3.0	30.5-43.0	39.0 -52.0
Soybean	-	2.3-11.0	2.4-6.0	22.0-30.8	49.0 -53.0
Peanut	0.5	6.0 -12.5	2.5-6.0	37.0-61.0	13.0-41.0
Sunflower	-	3.5-6.5	1.3-5.6	14.0-43.0	44.0-68.7

[a] *The nomenclature shows the number of carbon atoms and the number of C=C double bonds: for example, the present sample contained 14 C atoms and no double bonds. Adapted from* Peng (2012).

In algae, the most commonly synthesized FAs have chain lengths that range from C16 to C18, similar to those of higher plants (Hu et al., 2008; Ohlrogge & Browse, 1995). In general, saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) are predominant in most algae (Alonso, Grima, Pérez, Sánchez, & Camacho, 1992). Specifically, the major FAs are C16:0 and C16:1 in the *Bacillariophycea*e, C16:0 and C18:1 in the *Chlorophyceae and Prasinophyceae*, C16:0, C16:1 and C18:1 in the *Chrysophyceae and Prymnesiophyceae*, C16:0 and C20:1 in the *Cryptophyceae*, C14:0, C16:0, C16:1 and C18:1 in *Cyanobacteriaa* (Cobelas & Lechado, 1989).

Polyunsaturated fatty acids (PUFAs) contain two or more double bonds. Based on the number of double bonds, individual FAs are named dienoic, trienoic, tetraenoic, pentaenoic and hexaenoic FAs. Also, depending on the position of the first double bond from the terminal methyl end (n) of the carbon chain, a FA may be either an n-3-PUFAs (i.e., the third carbon from the end of the FA) or an n-6-PUFAs (i.e., the sixth carbon from the end of the fatty acid).

The major PUFAs are C20:5 (n-3) and C22:6 (n-6) in *Bacillarilophyceae*, C18:2 and C18:3 (n-3) in *Euglenophyceae*,C20:3 and C20:4 (n-3) in *Eustigmatophyceae*, C18:5 (n-3) and C22:6 (n-3) in *Dinophyceae*, C18:2, C18:3 (n-3) and C22:6 (n-3) in *Prymnesiophyceae*,C18:2 and C20:5 in *Rhodophyceae* and C16:0, C18:2 and C18:3 (n-3) in *cyanobacteria* (Basova, 2005).

Table 1.3 shows the FAs composition of different microalgae strains. The major FAs are C20:5, palmitoleic acid (C16:1), C16:0, C14:0, and arachidonic acid (C20:4) with lesser amounts of

C18:1 and C18:2. The sum of SFAs and MUFAs ranges from 52-67% and PUFAs from 33-48% in all strains presented.

Table 1.3. Fatty acid composition of transesterified microalgal oils

FAs	*Nitzschia cf. ovalis.*	*Thalassiosira sp*	*Tetraselmis sp.*	*D.pulchellum*	*Stichococcus sp.*
14:0	2.7	4.6	1.3	2.4	2.1
16:0	13.3	19.6	15.3	12.6	17.6
18:0	0.5	0.4	0.4	0.8	0.5
ΣSFAs	16.5	24.6	17.0	15.8	20.2
16:1(n-7)	17.1	31.5	3.2	2.1	-
18:1(n-9)	0.6	0.4	12.3	2.5	2.9
18:1(n-7)	0.3	0.3	2.26	-	1.6
ΣMUFAs	18.0	32.2	17.8	4.6	4.7
16:2(n-4)	2.5	7.5	4.5	3.1	0.2
16:3(n-4)	7.2	2.8	0.4	0.6	-
18:2(n-6)	0.4	2.0	9.7	7.8	14.8
18:3(n-3)	0.4	1.1	16.2	26.5	25.7
18:4(n-3)	2.5	1.8	1.8	-	-
20:4(n-6)	4.4	0.1	0.9	-	-
20:5(n-3)	26.7	16.7	4.7	-	-
22:6(n-3)	4.2	1.3	-	-	-
ΣPUFAs	48.3	33.3	38.2	38.0	45.4

Adapted from Zhao et al. (2013)

Table 1.4 presents a summary of FAs composition of the microalgal strains used in this study. The main FAs in *P. tricornutum* and *N. oculata* are C16:0, C16:1, and C20:5. The sum of SFAs and MUFAs ranges from 52-67% and PUFAs from 33-58% in *N. oculata*. In *P. tricornutum,* the sum of SFAs and MUFAs ranges from 52-86% and PUFAs from 14-48%.

Table 1.4. Fatty acids composition of the microalgae strains used in this study

FAs	*N.oculata*[1]	*N.oculata*[2]	*P.tricornutum*[3]	*P.tricornutum*[4]	*P.tricornutum*[1]
C14:0	4.9	-	7.3	-	-
C16:0	18.4	20.5	26.9	16.6	8.7
C18:0	-	1.8	-	0.6	18.7
ΣSFAs	23.3	22.3	34.2	17.2	27.4
C16:1(n-7)	26.1	25.2	48.2	26.0	26.4
C18:1(n-9)	3.0	3.6	3.4	1.8	2.1
ΣMUFAs	29.1	28.8	51.6	27.8	28.5
16:2(n-4)	-	-	-	-	5.2
16:3(n-4)	-	-	3.2	-	9.3
16:4	-	-	-	-	3.2
18:2(n-6)	6.2	2.2	-	-	5.1
18:3(n-3)	-	0.2	-	0.3	-
18:3(n-6)	-	0.7	-	1.5	-
18:4(n-3)	-	0.1	-	3.3	-
20:4(n-6)	-	-	-	-	-
20:4(n-3)	7.4	-	-	-	-
20:5(n-3)	34.0	29.5	11	28.4	20.9
ΣPUFAs	47.6	32.7	14.2	33.5	44.2

[1]*Gangadhar et al. (2015);* [2]*Bellou et al. (2014);* [3]*Ryckebosch et al. (2012); and* [4]*V. Patil et al. (2007)*

Table 1.4 shows the FAs profiles of the same strains but not identical results. For instance, the sum of PUFAs for *P. tricornutum* ranges from 14 to 48%. FAs profiles of lipid fractions were also reported with widespread results. It is very likely that a different method of oil extraction, lipid sources, strain types, biomass production methods affect FAs distributions depending on the relative proportion of polar and NLs (Ryckebosch, et al., 2012; Wang & Wang, 2012).

FAs profiles of individual lipid classes from ethanolic extracts of *Nannochloropsis* was reported at (Wang & Wang, 2012). The total SFAs in TAG tended to be lower than that in polar lipids, and the TAG total MUFAs tended to be higher than that in polar lipids.

The sum of SFAs and MUFAs are greater than sum of PUFAs in polar lipids. Following the same protocol, the reverse results were reported for *Schizochytrium*. This shows strain types can affect the FAs distribution.

Ryckebosch et al .(2012) also detailed FAs composition (%TFA, w/w) of lipid class of *P. tricornutum* (Table 1.5). The dominant FAs in NLs were C16:0 and C16:1; in PLs were C16:0, C16:1 and C20:5; in GLs were C16:0, C16:1, C16:3 and C20:5. This finding implies that PUFAs especially EPA are associated with polar lipids and SFAs and MUFAs with NLs. However, the cells were grown in batch culture and continuously illuminated with two fluorescent lamps. This results may not be similar with biomass produced under normal growth conditions. In general, growth conditions can affect FAs distribution in both total lipids and lipid fractions. These details were disclosed for both *N.oculata* and *P. tricornutum* at (Shen et al., 2016) grown under nitrogen repletion (N^+) and depletion (N^-).

Table 1.5. FAs composition for lipid classes of *P. tricornutum* (Ryckebosch, et al., 2012)

FAs	NLs	GLs	PLs
C14:0	5.3 ± 0.0	1.2 ± 1.2	9.6 ± 1.1
C15:0	-	-	1.4 ± 0.7
C16:0	30.7 ± 0.1	18.1 ± 2.1	20.4 ± 0.4
C16:1	45.9 ± 0.2	16.1 ± 2.5	25.2 ± 6.4
C16:2	-	3.3 ± 0.3	-
C16:3	-	19.0 ± 2.1	2.2 ± 1.8
C16:4	-	2.2 ± 0.1	-
C18:0	-	6.5 ± 0.4	1.8 ± 0.9
C18:1	7.8 ± 0.1	3.6 ± 0.2	8.1 ± 0.00
C18:2	-	-	1.8 ± 0.3
C18:3(n-3)	-	1.3 ± 0.1	-
C20:5(n-3)	6.1 ± 0.1	28.4 ± 1.1	18.0 ± 6.9
C24:0	-	-	6.5 ± 0.6
C22:6(n-3)	-	-	3.2 ± 2.0

1.5. Problem Statement and Rationale

We are in the century of uncertainty regarding petro-diesel supply as predictions show that they are about to be depleted (Azam, et al., 2005). More than affordability, availability seems pivotal. Consequently, it is time to work on how to reduce the cost of biodiesel production as it appears particularly advantageous for the transportation sector. However, among the factors that affect the cost of biofuels, biomass production seems a challenging venture than other factors and boosted responsibility on professionals specialized in the area (Brennan & Owende, 2010).

Biomass Dewatering and the Way Forward to Reduce the Cost

Energy consumption of drying microalgae biomass accounted for the majority of the total processing energy (Lardon, Helias, Sialve, Steyer, & Bernard, 2009; P. D. Patil, Reddy, et al., 2012).Various researchers have investigated wet lipid extraction methods, including ultrasound-assisted extraction (Adam, Abert-Vian, Peltier, & Chemat, 2012), simultaneous distillation and extraction process (Tanzi, Vian, & Chemat, 2013), microwave-assisted extraction (Cheng, Yu, Li, Zhou, & Cen, 2013), and supercritical fluid extraction (Halim, Gladman, Danquah, & Webley, 2011). Unfortunately, these require high temperature and long times; so they are not ideal for industrial-scale production.

Standard methods for lipid extraction employ chloroform and methanol that are carcinogenic (Bligh & Dyer, 1959; Folch, Lees, & Sloane-Stanley, 1957).

Some alcohols have the potential to extract lipids from microalgae (M. Chen et al., 2012; Fajardo et al., 2007; Fangfang Yang, et al., 2014). FA composition of ethanolic extract was higher when compared to some solvents (Fig.1.12) (Petrick et al., 2013). FAs recovery by ethanol is better than chloroform, propanol, methanol or hexane (Fig.1.12). Ethanol successfully extracts as much as the standard methods for lipid extraction as shown in (Fig.1.12) (Fangfang Yang, et al., 2014). Any physical pre-treatments to induce cell lysis (e.g., sonication or microwaves), which if applied incur additional costs, are not essential and instead extraction twice is sufficient. Extraction can be carried out at reflux or room temperature and is not energy consuming (Fangfang Yang, et al., 2014). Ethanol is a food grade solvent when compared with chloroform and methanol. Unlike higher plants lipids, microalgal lipids are complex for they comprise almost equal proportion of both neutral and polar lipids. Hence, a solvent that has both polar and non-polar nature is more effective at extracting total lipids from wet microalgae biomass than a purely non-polar.

For instance, hexane is not effective at extracting total lipids from wet microalgae biomass but effective at extracting from higher plants. It seems hexane or non-polar may extract mainly the NLs from microalga and the polar are left in spent biomass as waste.

There are some reports where extraction by alcohols from wet biomass containing water is easier than from dried biomass. For example, 2-ethoxyethanol is claimed to be more effective at extracting lipids from wet microalgal biomass than dried as compared to chloroform: methanol or hexane (Jones, et al., 2012).

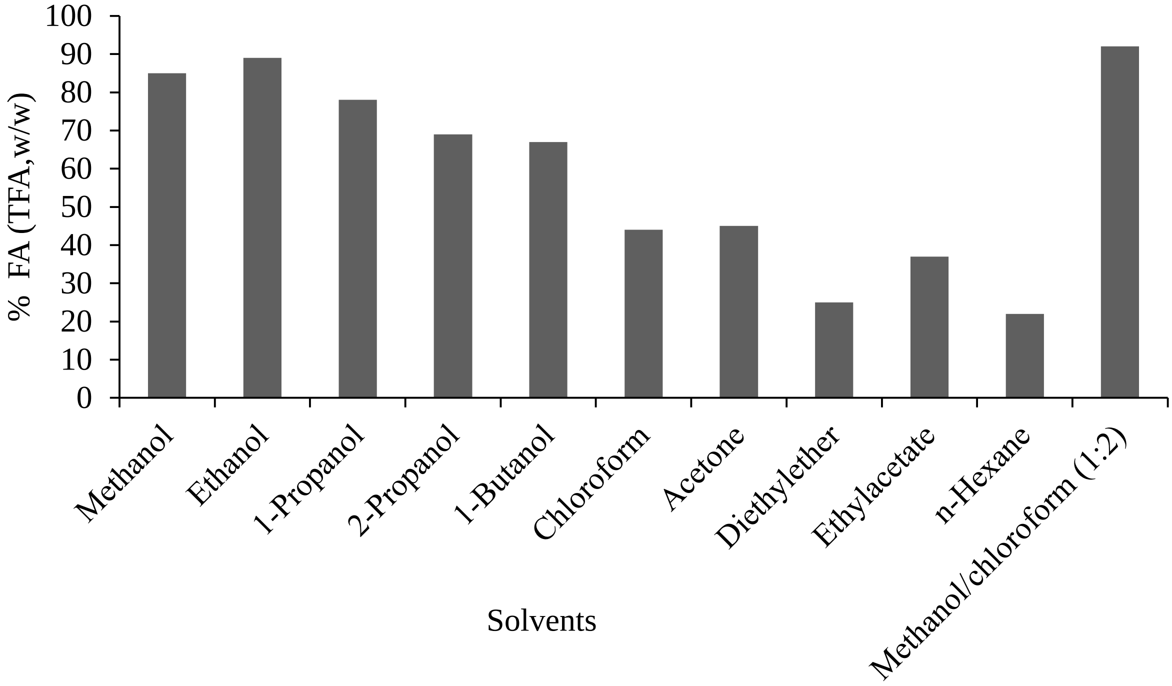


Fig. 1.12. Comparison of lipid extraction using different solvents (Petrick, et al., 2013)

The Merits of Microalgae Lipid Separation

Plant oils, such as palm, rape, sunflower, etc., are TAG containing about 90% FAs (Petkov, Ivanova, Iliev, & Vaseva, 2012). They are relatively easy to be extracted from seeds. Unlike the seeds of many higher plants, microalgal lipids are not only TAG. For example, TAG of the highly oleaginous alga *Parietochloris incisa* represent 42.9% of total lipids, and TAG of *Pavlova lutheri* are 40.3% (Petkov, et al., 2012). The percentages of TAG are lower in the dried lipid extract where chlorophyll, carotenoids, sterols are concentrated, too.

For instance, after hydrolysis of 1 kg microalgal lipids, the usual products are 0.3–0.5 kg FAs, 0.2–0.4 kg unsaponifiable substances (e.g., hydrocarbons, carotenoids, and sterols etc.,), and 0.2–0.3 kg water soluble substances (i.e., glycerol, and phosphoric acid) (Petkov et al., 2012). Algal lipids are predominantly mono- and digalactosyl diacylglycerols, PLs and unsaponifiable substances, and subsequently the FAs content reaches up to 31–32% as a mean value.

Galactose, glycerol, and phosphoric acid, which are esterified in lipid molecules during this treatment, will lose their quality as nutrient components, or will be completely wasted. As a result, microalgal lipids have two to three times lower content of FAs compared to plant oils. It was reported that 15 kg biomass of *Chlorella* and *Scenedesmus* can render 1 kg biodiesel. This shows except 31–32% of FAs, the remaining is waste. Waste has environmental and socio-economic impacts and necessitates the way forward to reduce the impacts.

One approach can be via applying the bio-refinery concept (Cherubini, 2010; Morais & Bogel-Lukasik, 2013).Ethanol or other solvents extract total lipids which contain both neutral and polar lipids (i.e., GLs and PLs). But, separating polar lipids from NLs of microalgae for preparing biodiesel are important for several merits (Iyer, 2016). For example, unsaturated lipids occur more frequently in polar lipid fractions specifically PLs (Foley, Beach, & Zimmerman, 2011). The PUFAs are highly prone to spontaneous oxidation as they contain multiple double bonds (Ostrea, Cepeda, Fleury, & Balun, 1985).

Furthermore, the phosphate moiety in PLs complicates transesterification (Iyer, 2016; Van Gerpen, 2005). Yield of FAMEs was reduced when soybean oil with significant amount of PLs was transesterified with *C. antarctica* lipase (Watanabe, Shimada, Sugihara, & Tominaga, 2002). The presence of 5 and 10% (w/w, DW) PLs in soybean oil decreases the yield of FAMEs by 6.8 and 9.7% (w/w, DW) (Y. Li, Du, & Liu, 2014). The separation of PLs from NLs increased the yield of FAMEs (Freedman, Pryde, & Mounts, 1984). Therefore, separation of the neutral from the polar has dual advantages: it improves biodiesel quality and yield; and polar lipids can be used as value added products. The polar lipids comprise around 50% of the total lipids (Fangfang Yang, et al., 2014).

Although these Omega-3-FAs are obtained from fish oil, they are claimed to be derived from microalgae via food chain (Yongmanitchai & Ward, 1989). EPA and DHA have the potential to change the fluidity and permeability of the membrane (Burri, Hoem, Banni, & Berge, 2012). Liposome from PLs enriched in these FAs are targeting and can deliver both hydrophilic and hydrophobic drugs. Hence, chromatographic separation of these lipids are essential.

Besides, PLs separation from NLs is good to comply with regulations and for the safety of the engine. For instance, ASTM D6751 standards state that phosphorus content should be below 10 ppm. A higher phosphorus content (>10 ppm) in biodiesel would result in phosphorus remaining in exhaust emissions during combustion of FAMEs (biodiesel) poisoning the catalyst in the converter, resulting in serious damage to the catalytic converter (Kaleli, 2001; Tyson & McCormick, 2006).

The presence of polar lipids necessitates using more methanol as they contain acyl groups involving in transesterification. The excess moles of methanol will get solubilized in micelles leading to localized concentration of methanol around the catalyst, resulting in lowering the activity of the catalyst and subsequently reducing the yield of FAMEs (Kaleli, 2001; Tyson & McCormick, 2006).

However, finding industrially feasible lipid separation or oil degumming (first stage in oil refining process) is essential. The most commonly used chemicals (chloroform and methanol) for lipid separation are carcinogenic and requires silica column that cannot be beyond lab scale.

Degumming of crude soybean oil has been carried out by ultrafiltration using polymeric membranes (Pagliero, Ochoa, Marchese, & Mattea, 2001 & 2004). However, the instability of polymeric membranes in organic solvents has been the barriers to oil degumming/refining. Ceramic membrane for oil degumming is energy intensive and so costly (De Souza, Petrus, Gonçalves, & Viotto, 2008).

The drawback of the alkali refining process is the entrainment of significant quantities of neutral oil with the soap stock resulting in a loss of yield. Additional costs are incurred by the soap stock splitting and effluent treatment. Acid degumming process is usually carried out at elevated temperature around 90°C (Mag & Reid, 1980). The organic refining process (Copeland & Belcher, 2000) uses a large excess of citric acid.

In degumming, enzymes are incapable of catalysing the hydrolysis of non-hydratable phosphatides (alkaline earth salts of phosphatidic acid) under industrial conditions (Dijkstra, 2010). Enzymes are expensive and the amount available for industrial scale is not enough, thus its activity can be affected by extreme conditions such as high temperature and organic solvents, for example lower alcohol, methanol or ethanol.

Consequently, in our study we used binary phase system (hexane + water) and separated the polar lipids from NLs employing a process known as Liquid Tri-phase System (LTPS). Hexane is relatively tolerable solvent and water is safer. Less energy consuming mixing process was applied and lipid classes of various FAs and wider applications were separated under the biorefinery scheme. To get optimum amount of distinctly separated lipid classes in short span of time, centrifugation needs to be applied over mixing. The solvents are recovered and reused. In this regard, we hypothesize the cost of biodiesel production can be reduced and our waste foot print and economic loss due to the polar lipids use, too. In our view, this is a contribution to research gaps and first report of oil degumming that can be feasible industrially.

1.6. Objectives

1.6.1. Main Objective

To obtain a neutral lipid rich fraction free of phospholipids and glycolipids to produce biodiesel from microalgae and, at the same time, get a phospholipids and glycolipids rich fraction with biomedical or food and/or feed or other industrial applications under a biorefinery scheme, using an industrially relevant method.

1.6.2. Other Objectives

To extract lipids from wet biomass of *N. oculata* and *P. tricornutum* using ethanol

To separate the colloidal part from the liquid fractions employing the Liquid Tri-phase System.

To analyse the lipid classes and fatty acids composition of the obtained fractions using HPLC-ELSD and GC-MS, respectively.

2. MATERIALS AND METHODS

Experiments were conducted in the laboratory of the Marine Biotechnology group, from the Centre of Marine Sciences (CCMAR) at the University of Algarve, Faro, Portugal.

2.1. Materials

The microalgal biomass from *N. oculata* and *P. tricornutum* was supplied by NECTON S.A., Portugal. Both cultures were grown outdoors in flat panel and tubular photobioreactors. Biomass harvesting was performed by centrifugation, and the resulting microalga paste was frozen at -20°C until use.

All chemicals used in this experiment were of analytical grade and HPLC grade solvents were used only for HPLC-ELSD and GC-MS.

2.2. Methods

2.2.1. Moisture Content

Prior to lipid extraction, 1g of microalgae biomass was incubated in an oven at 60°C for 72 h (n=3) in order to determine its moisture content. The percentage moisture in the biomass was calculated using (Eq .2.1):

Moisture content = $[(W_1 - W_2)/ (W_1 - W_p)] * 100\ \%$ --------------------------------------- (**Eq. 2.1**)

Where, W_1 = Original weight of the sample (with plate) before drying

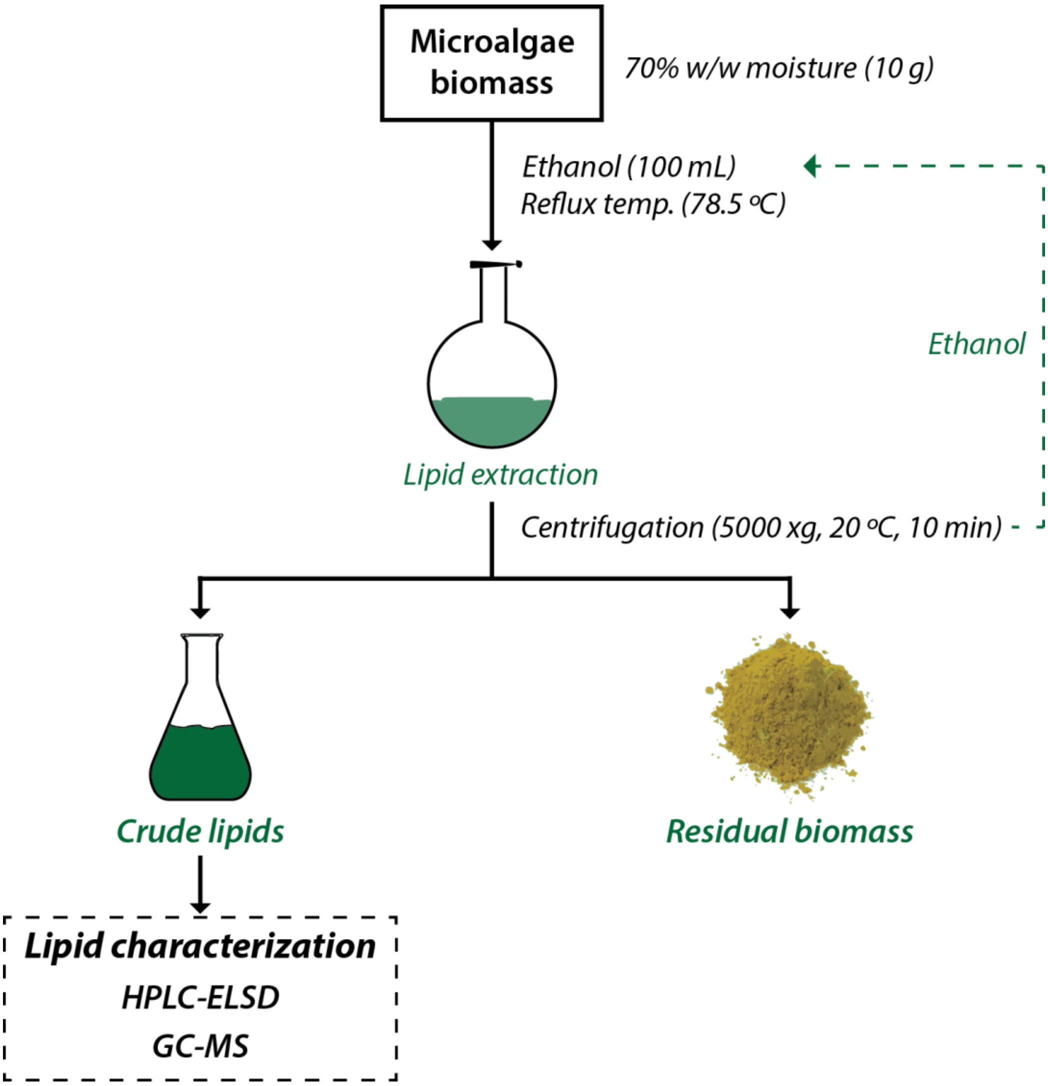
W_2 = Weight of the sample (with plate) after drying and Wp = Plate weight

2.2.2. Total Lipid Extraction

Lipids were extracted according to the protocol described in (Yao, Gerde, & Wang, 2012) with a few modifications. Briefly, 10.0 g of microalgae paste containing 70.0% water was dispersed in 100 mL of ethanol and stirred at reflux temperature of ethanol (78.5°C) for 90 min (Scheme 2.1). After refluxing, the mixture was centrifuged at 5,000 rpm and 20°C for 10 min. The supernatant (Ethanol (ET)-1) was separated from the pellet by decanting. The insoluble matter was further extracted with ethanol under reflux conditions for 30 min (ET-2, 50 mL) and 15 min (ET-3, 25 mL), followed by centrifugation under the same conditions and filtered through Whatman no. 4 filter paper. Ethanol and water were recovered from the mixture using a rotatory evaporator under reduced pressure. The lipid content was estimated using (Eq. 2.2).

Biomass lipid content = $[W_o/W_s] * 100\ \%$ --- (**Eq. 2.2**)

Where, W_o = weight of lipid extracted; W_s = weight of sample



Scheme 2.1. Lipids extraction from wet microalgae using ethanol

2.2.3. Lipid Characterization

Lipid Profiles

Lipids were separated into NLs, GLs and PLs using column chromatography applying the method described at (Wang & Wang, 2012) with little modification (Scheme 2.2). Briefly, 2.0 g of lipid, 100 mL of chloroform: methanol (1:1, v/v) and 2.0-5.0 g of silica were mixed in 500 mL rounded bottom conical flask. Then, the mixture was rotary-evaporated until dryness. Next, it was loaded on top of a 1.4 cm I.D. x 18 cm length glass column filled with 30 g silica gel (60µm). The NLs were first eluted using 300 mL chloroform, then 300 mL of acetone: methanol (9:1, v/v) for GLs; and lastly 300 mL methanol for PLs. The solvents were recovered from each lipid classes and the weight of the lipids was measured and calculated (% w/w, total lipids).

Microalgae crude extracts and lipid fractions were characterized by HPLC (KNAUER) coupled with an Evaporative Light Scattering Detector (ELSD; Agilent Technologies, Varian 380-LC) to analyse the major lipid classes.

Before use, HPLC grade solvents were filtered with (Nylon membrane, 0.45µm, and 47mm) and degassed. After optimization of the parameters for the ELSD, evaporator temperature was set at 50^{o}C and nebulizer at 45^{o}C. The flow rate of the carrier gas (compressed pure nitrogen) was maintained at 1.60 SLM. Gain and smoothing factor was fixed at 2 and 30, respectively.

Lipid standards and microalgae extracts were resolved using Phenomenex sphereClone column (250 mm x 4.6 mm ID, 5µm particle size). Data acquisition was performed using Clarity software (3.1.7) (VWR, Darmstadt, Germany).

The separation of lipid fractions was achieved with a gradient programme shown below (Table 2.1). A combination of four different solvents was used as follows, hexane: ethylacetate:water: isopropanol. Briefly, samples were injected at time 0.0 and were eluted with hexane-ethylacetate-isopropanol (98:1:1, v/v/v) for 10.0 min at a linear flow rate of 1.0 mL/min. Solvents were changed from 98:1:1: to isopropanol (100%) at flow rate of 0.8mL/min over the next 15min. After 25min, the elution solvent was changed to isopropanol : water (75:25; v/v using the same flow rate 0.8mL/min for 5.0 min. In the next 10.0 min, 100.0% isopropanol was used to remove the polar contaminants (if any). After 40min the first solvent mixtures were run again to equilibrate the column for 10 to 15min and the next samples were injected.

Table 2.1. HPLC gradient procedure to separate microalgae lipids

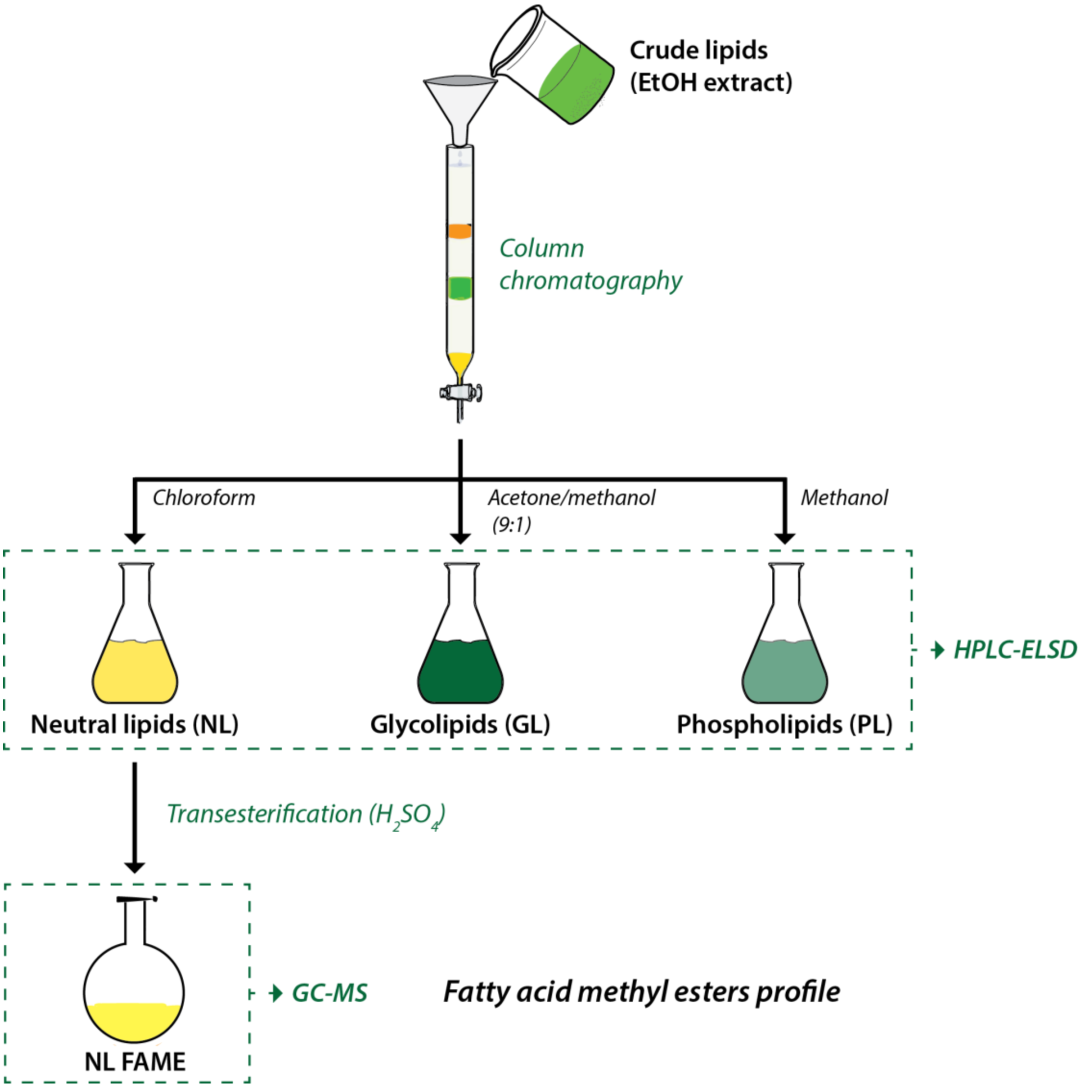
Time (min)	Hexane (%)	Ethylacetate (%)	Water (%)	Isopropanol (%)	Flow rate (mL/min)
0.0	98.0	1.0	0.0	1.0	1.0
10.0	98.0	1.0	0.0	1.0	1.0
15.0	0.0	0.0	0.0	100.0	0.8
25.0	0.0	0.0	25.0	75.0	0.7
30.0	0.0	0.0	0.0	100.0	0.8
35.0	98.0	1.0	1.0	1.0	1.0
40.0	98.0	1.0	1.0	1.0	1.0

2.2.4. Fatty Acid Composition

Lipids (i.e., crude extracts and NLs fractions) were converted into their corresponding FAMEs using methanol by an acid catalysed method. The FAMEs profile was then characterized employing GC-MS.

Transesterification was done according to the method reported by Lam & Lee (2013) with little modifications. Briefly microalgae lipids were mixed with a solution of methanol and 2% H_2SO_4 in a 500 mL rounded bottom flask and stirred at reflux temperature (64–69°C) for 4h. The reaction was monitored by thin-layer chromatography (TLC) using hexane and ethylacetate (95:5, v/ v). After reaction completion, solvent was removed up to 1/4 of the volume to reduce emulsions and sequentially extracted three times with hexane. Hexane fractions were transferred to a separating funnel and washed with distilled water until the acid was neutralized, followed by a saturated NaCl solution treatment. It was dried over anhydrous Na_2SO_4, filtered, and recovered using a rotary evaporator under reduced pressure to obtain FAMEs.

The FAMEs profile was determined in an Agilent GC-MS (Agilent 6890 Network GC System, 5973 Inert Mass Selective Detector) equipped with a DB5-MS capillary column (25 m×0.25 mm internal diameter, 0.25 µm film thickness, Agilent). Helium was used as a carrier gas, while the injector and detector were maintained at 300°C. The oven temperature was programmed for 60°C (1 min), 30°C min^{-1} to 120°C, 5°C min^{-1} to 250°C, and 20°C min^{-1} to 300°C (2 min). Identification and quantification of FAMEs (total ion mode) were performed by comparing the retention times of FAMEs samples with an external standard (Supelco 37 Component FAMEs Mix, Sigma-Aldrich) and further confirmed by comparison of the MS spectra. For quantification purposes, a separate calibration curve was generated for each of the FAMEs in the standard.

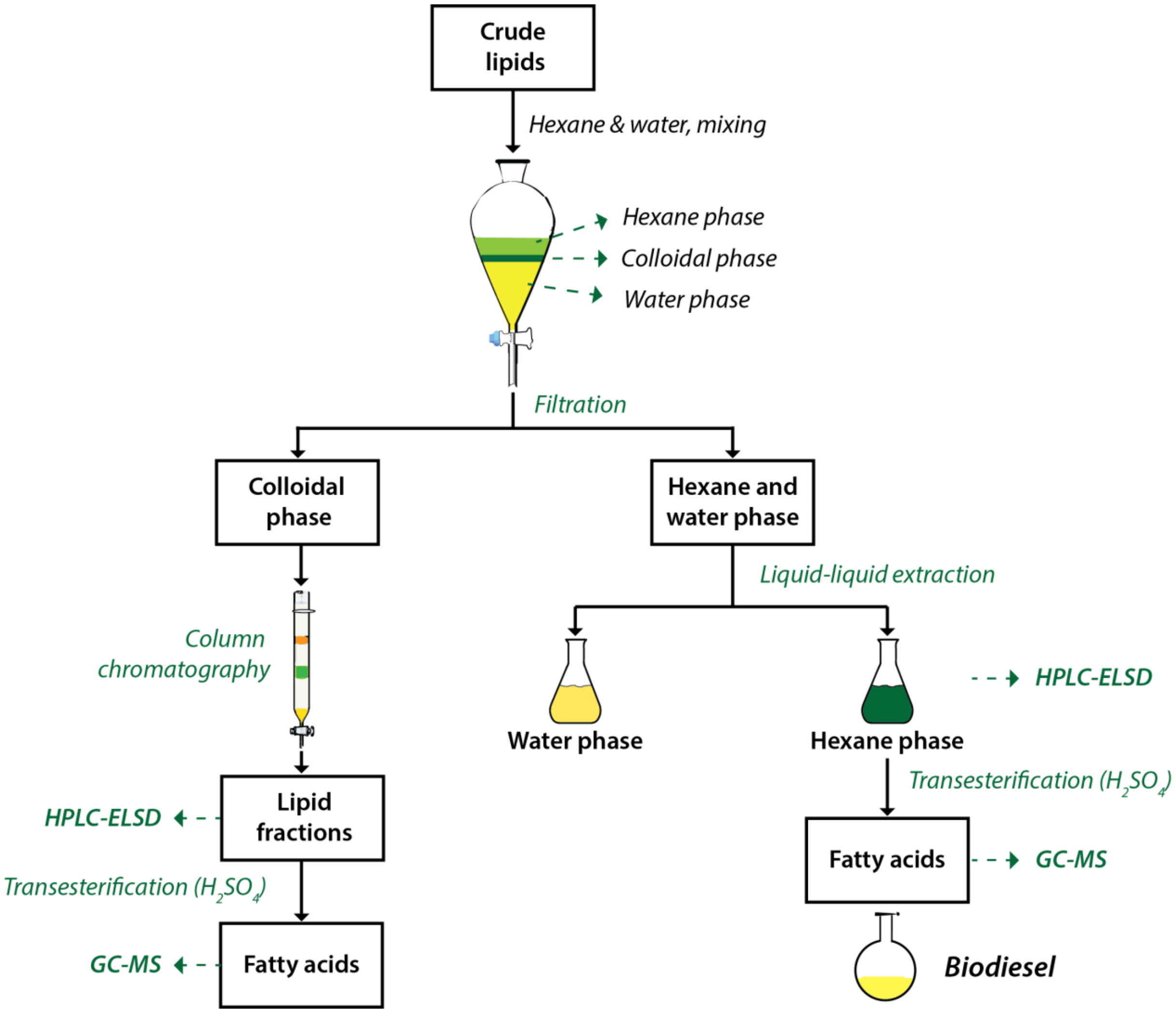


Scheme 2.2. Lipid separation using column chromatography

2.3. Liquid Tri-phase System (LTPS)

In LTPS, we used binary phase system (hexane + water) and separate the total lipids of microalgae into three major classes. Hexane is relatively tolerable solvent and water is almost green. Crude ethanolic extract of microalgae (1.83 g) was re-suspended in a mixture of hexane and water (160 mL, 1:1, v/v) and shaken for 1-3 min. Three phases were observed after mixing and, especially after centrifugation (5,000 rpm, 20°C, and 10 min) (Scheme 2.3): the upper hexane phase, the middle colloidal phase and the lower water phase. The mixture was then filtrated. The colloidal fraction was left on filter paper and the filtrate containing hexane and water phases were collected and repeated for 3-4 times under similar conditions as mentioned above; further transferred to a separating funnel and separated into hexane and water phases as shown in Scheme 2.3.

The lipid profiles of the hexane, colloidal and water phases were further determined with HPLC-ELSD as described in section 2.2.3. The FA compositions of the former two LTPS lipid fractions were analysed following the same procedures detailed under section 2.2.4 and the water phase was left for future study.



Scheme 2.3. Lipid partitioning using LTPS followed by HPLC-ELSD and GC-MS analysis

2.4. Data Analysis

The data were analysed statistically using SPSS vs. 20 (SPSS Inc., Chicago, IL, USA) or Excel whenever appropriate.

3. RESULTS AND DISCUSSION

3.1. Moisture Content and Lipid Extraction

Lipids were extracted with ethanol from the microalgal paste containing 70.0% (w/w) water. *N. oculata* showed higher ethanol extract yield of 38.2 % (w/w, dry weight (DW)) followed by that of *P. tricornutum* (30.3%), which is in line with the yields reported by Rajvanshi and Sharma (2012). This finding for *N. oculata* is similar to that reported previously for isopropanol extracts of the same strain (42.0%) (Gangadhar, et al., 2015) and a modified Bligh-Dyer standard extract (39.1%) (Bondioli, et al., 2012); however, other author's obtained lower yields for ethanolic extract (22.0%) (Wang & Wang, 2012). Similarly, the result reported for *P. tricornutum* (20-30%) is in line with (Singh, Carlson, Fell, & Poolman, 2015); but not to Gangadhar et al.(2015) whose result was (37.0%). In general, ethanolic extraction provided better or nearly the same yield as standard method (Bligh-Dyer) for lipid extraction for different microalga strains such as *Picochlorum sp* , *Selenastrum rinoi* and others (Bligh & Dyer, 1959; Petrick, et al., 2013; Fangfang Yang, et al., 2014).

The use of ethanol has several advantages. It is a greener alternative to carcinogenic solvents (e.g., chloroform-methanol or hexane commonly used for lipid extraction); being a food-grade solvent, it enables the use of product or by-product in food and/or feed applications in the interest of spent biomass; it minimizes the high energy intensive dewatering costs (Brennan & Owende, 2010; Lardon et al., 2009; P. D. Patil, Gude, et al., 2012) as extraction can be done directly from wet biomass; high lipid yields can be obtained at ambient temperature (ethanol reflux temperature, 78.5^{o}C) or room temperature (Fangfang Yang et al., 2014) unlike biomass gasification (1000^{o}C) or hydrothermal liquefaction (300^{o}C) or flash pyrolysis (Brennan & Owende, 2010). Physical pre-treatments for example sonication or microwaves can be avoided to induce cell lysis, which if employed acquire additional costs, are not essential and instead extracting for duplicate is sufficient. It was also reported that physical pre-treatments are not mandatory and extraction twice is sufficient for maximum lipid recovery using methanol: chloroform (1:1, v/v) (Ryckebosch et al., 2012).

3.2. Lipid Characterization

3.2.1. Lipid Profiles

The total lipids extracted from microalgae were separated into lipid classes (i.e. NLs, GLs and PLs) using silica gel column chromatography**.** Lipid class distribution (% w/w, total lipids (TL)) of *N. oculata* was 45.1, 22.1 and 32.8% for NLs, GLs and PLs, respectively (Fig 3.1). These results are in agreement with the findings of Wang and Wang (2012) for the same strain and of Fangfang Yang et al.(2014) for *Picochlorum sp*. And, *P. tricornutum* lipid distribution was 23.2, 49.1 and 27.7% for NLs, GLs and PLs, respectively (Fig 3.1). This result is in line with the report of Miranda et al. (2015) for the same microalgae strain. It contains more GLs but less NLs than *N. oculata*. The PLs contents of both strains are nearly the same.

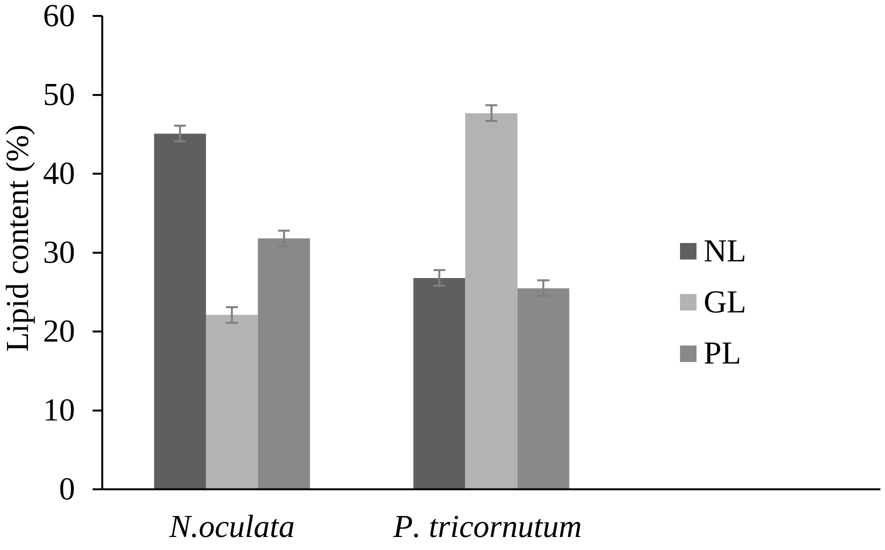


Fig. 3.1. *N. oculata* and *P. tricornutum* lipid classes separated by silica gel column chromatography. NL-neutral lipid, GL-glycolipid, PL-phospholipid

HPLC-ELSD was used to determine the lipid fractions present in the microalgae crude extracts and in the fractions separated by silica gel column chromatography (i.e., NLs, GLs and PLs). In this regard, we achieved the separation of standards TAG, DAG, FA, MG and PLs in just 40 minutes (Fig. 3.2). The more non-polar, TAG, was eluted first, followed by DAG, FA and MAG. PLs were the last to elute in this gradient program.

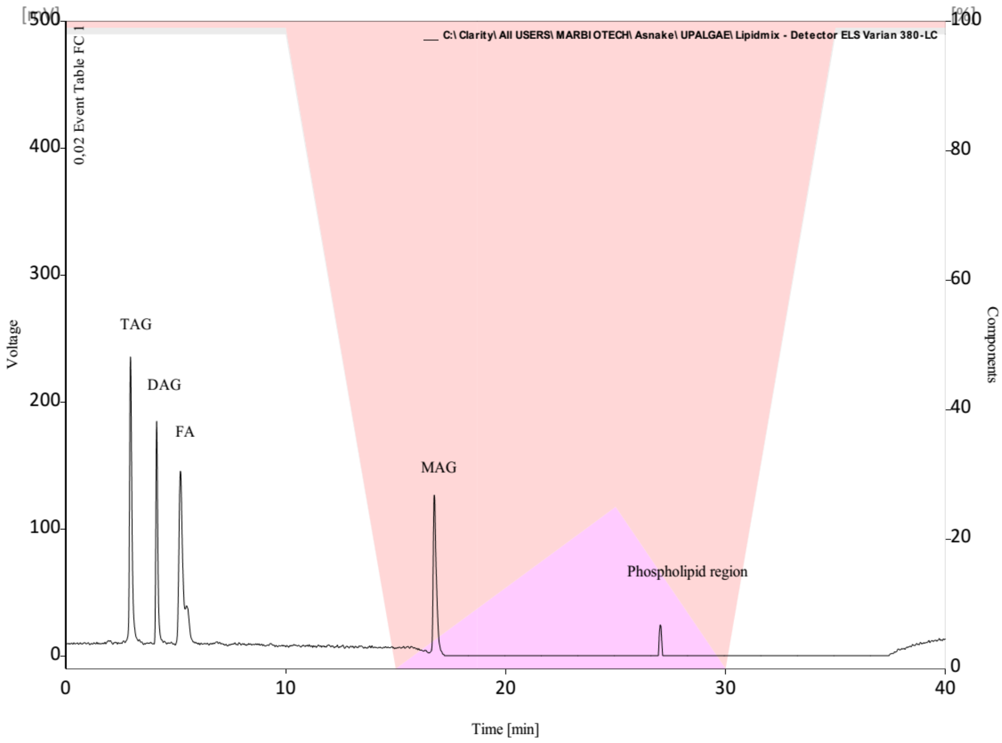


Fig. 3.2. Separation of standard lipids by gradient elution on HPLC-ELSD

TAG-triacylglycerol, DAG -diacylglycerol, FA- Fatty acid, MAG- monoacylglycerol

Crude Extract

The newly developed program fairly resolved *N. oculata* NLs such as TAG, DAG, FA and MAG (Fig.3.3). Closely related finding was reported for *Chlorella* and *C. kessleri* (Jones, et al., 2012; Kobayashi et al., 2013) and marine zooplankton (Graeve & Janssen, 2009). However, there are unidentified fractions which need more standards and further analysis. GLs was separated well; but necessitates standard to identify the fraction types.

On the other hand, the PLs fractions have very close retention times and a full separation would require further method optimization. Despite the peaks need further resolution, it is possible to ascertain that we achieved to obtain a NLs fraction free of polar lipids for biodiesel production; and at the same time, a polar lipids rich fraction for value added chemicals (Fig. 3.4 to 3.6).

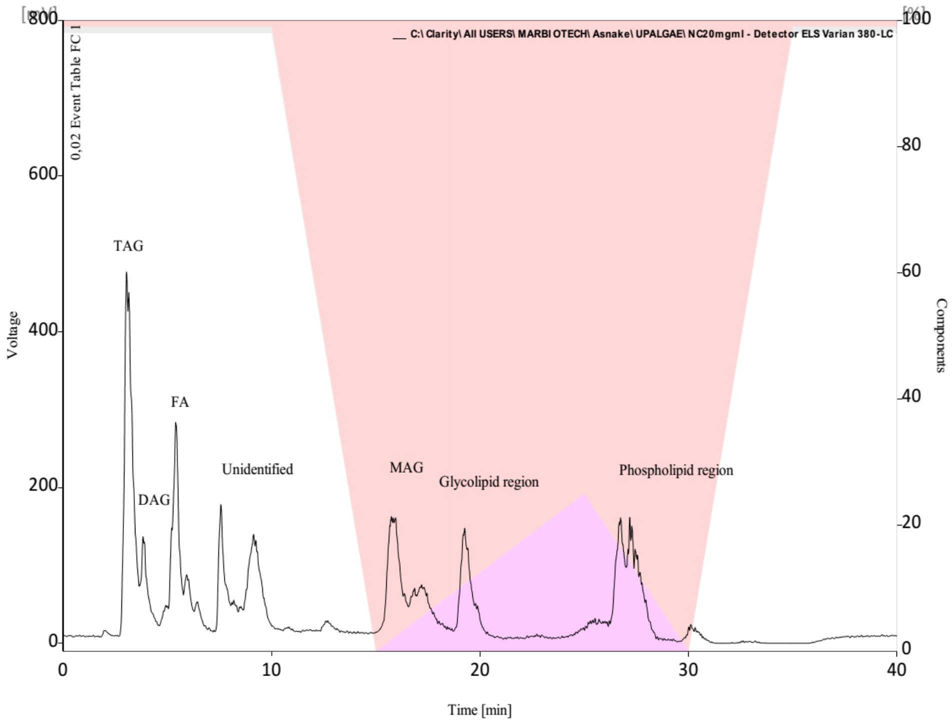


Fig. 3.3. HPLC-ELSD chromatogram of *N. oculata* crude extract showing the separation of lipid classes

Lipid Fractions

Figure 3.4 confirms the separation of NLs from both PLs and GLs in the fractions obtained from silica gel column chromatography.

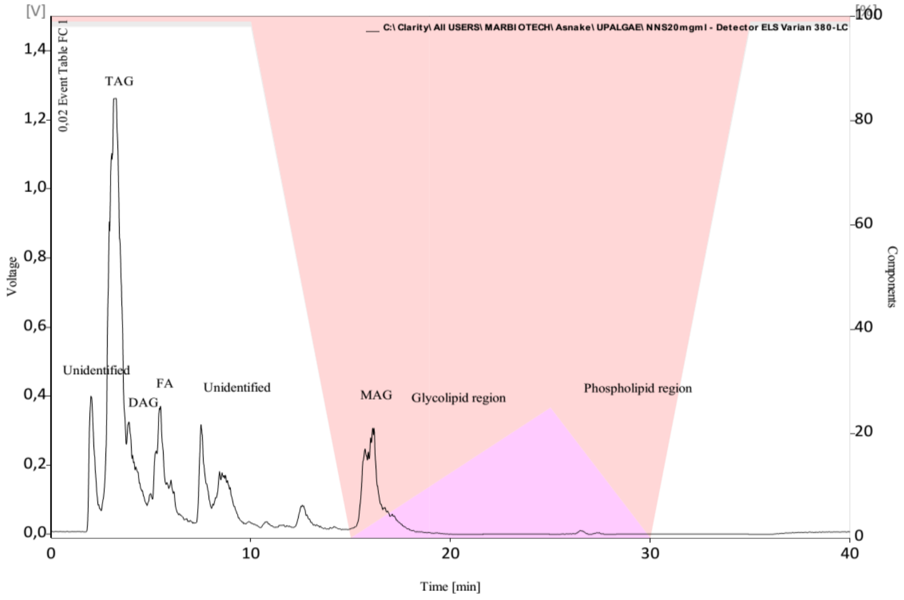


Fig. 3.4. HPLC-ELSD chromatogram of the NLs fraction of *N. oculata*

The area percentage of NLs was 42.2%, which is close to the one estimated from column chromatography (45.1%). The amounts of TAG, DAG, FA and MAG in NLs were 48.2, 8.51, 12.1 and 13.9%, respectively. TAG is the dominant fraction in NL. The remaining 17.3% were unidentified lipids.

Figure 3.5 confirms separation of the GLs from both the NLs and PLs by silica gel column chromatography. GLs accounted for 22.1% of *N. oculata* total lipids. GLs can be converted to non-ionic biosurfactants (Abdel-Mawgoud, et al., 2011; Kitamoto, et al., 2002). Biosurfactants are less toxic and biodegradable compared to their chemical counterparts (Kitamoto, et al., 2002). They have prompted applications in the food, cosmetic, pharmaceutical industries and environmental protection (soil washing, oil spills cleaning) (Kitamoto, et al., 2002).

On the contrary, nonylphenol ethoxylate and other alkylphenol ethoxylates are widely used non-ionic surfactants. They are converted in the environment to nonylphenol. Nonylphenol is an endocrine disruptor with estrogenic activity via mimicking female sex hormone (Ying, Williams, & Kookana, 2002). They are bioaccumulative, therefore, finding alternative, safer, renewable and after use degradable surfactants is vital.

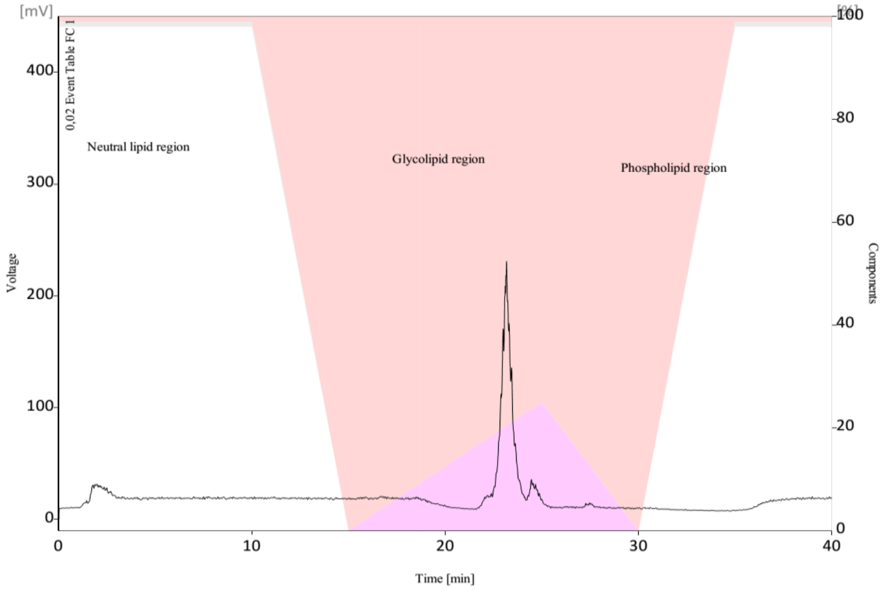


Fig. 3.5. HPLC-ELSD chromatogram of the GLs fraction of *N. oculata*

PLs were also well separated from the remaining lipids by silica gel column chromatography and accounted for 33.0% of the whole lipids (Fig.3.6). The main advantages of separating NLs from polar lipids in general and PLs in particular were detailed under section 1.5. Their wider applications were further elaborated in section 3.3.

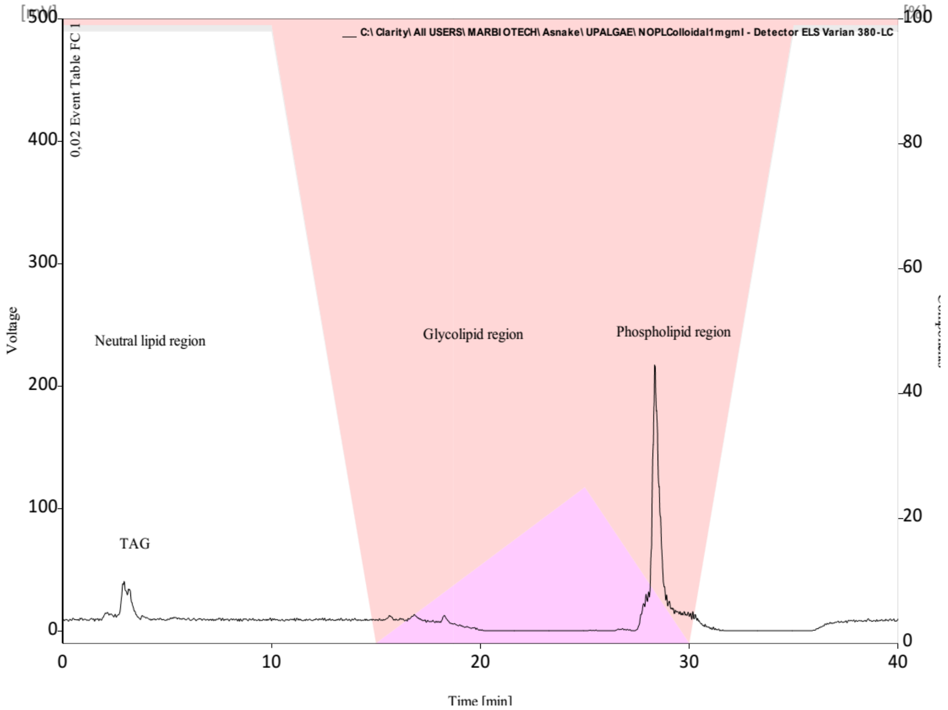


Fig. 3.6. HPLC-ELSD chromatogram of the PLs fraction of *N. oculata*

3.2.2. Fatty Acid Composition

FAs composition of the crude lipid extract and the NLs fraction were calculated in terms of total fatty acids (TFA). The main FAs detected in crude extract of *N. oculata* were myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), and EPA (C20:5) (Table 3.1). This finding is in line with those reported previously (Bellou, et al., 2014; Gangadhar, et al., 2015). SFAs and MUFAs account for more than 58.0%. These FAs are suitable for biodiesel as they are less susceptible to oxidation. The remaining PUFAs like EPA (30.6%) are needed to be separated and optimized for industrial production for biomedical feed and/or food as nutritional supplements (Drevon, Baksaas, & Krokan, 1993; Simopoulos, 1999).

The major FAs detected in the NLs fraction of *N. oculata* were C16:0, C16:1 and C18:1 (Table 3.1). In this fraction, the sum of SFAs and MUFAs are greater than 86.0%. This implies that NLs contain less PUFAs; therefore, it is more suitable for biodiesel production than crude extract which is more prone to oxidation due to 42.0% PUFAs it contains.

Table 3.1. FAs composition of crude extract and NLs fraction of *N. oculata*. Results are presented as mean ± standard deviation (*n* = 2)

Fatty acids	Crude extract	NLs fraction
C14:0	8.1 ± 0.9	8.0 ± 0.7
C16:0	20.3 ± 2.1	30.6 ± 1.4
ΣSFA	28.4	38.6
C16:1(n-6)	25.5 ± 1.6	22.2 ± 0.4
C18:1(n-9)	4.1 ± 0.3	25.7 ± 0.6
ΣMUFA	29.6	47.9
C18:2(n-6)	4.1 ± 1.2	5.7 ± 0.7
C20:4(n-6)	7.3 ± 2.1	1.8 ± 1.2
C20:5(n-3)	30.6 ± 0.3	6.0 ± 1.8
ΣPUFA	42.0	13.5

Similarly, the main FAs found in the crude extracts of *P. tricornutum* were C14:0, C16:0, C16:1 and C20:5 (Table 3.2). These results are closely related to Ryckebosch et al.(2012) and Gangadhar et al.(2015). The sum of SFAs and MUFAs are greater than 71.0%. These FAs are suitable for biodiesel as they are less susceptible to oxidative instability. Therefore, the remaining PUFAs like EPA (22.6%) has to be separated and optimized for industrial production for feed and/or food or biomedical applications (Drevon, et al., 1993; Simopoulos, 1999). *N. oculata* crude extract has more EPA than *P. tricronutum* crude extract. But, NLs fraction of *P. tricronutum* has twice more EPA than *N. oculata* NLs fraction.

The dominant FAs in NLs were C16:0, C16:1 and C18:1 (Table 3.2). The sum of SFAs and MUFAs are greater than 82.5%. The implication is NLs comprise less PUFAs and; therefore, it is suitable candidate for biodiesel production than directly from crude extracts.

Table 3.2. FAs composition of crude extract and NLs fraction of *P. tricornutum.* Results are shown as mean ± standard deviation (*n* = 2)

Fatty acids	Crude extract	Neutral lipids
C14:0	9.3 ± 1.1	6.4 ± 1.7
C16:0	25.9 ± 1.5	21.9 ± 4.1
C18:0	2.1 ± 1.4	2.1 ± 0.9
ΣSFA	37.3	30.4
C16:1(n-6)	29.1 ± 1.4	21.7 ± 2.1
C18:1(n-9)	4.7 ± 0.8	30.9 ± 2.1
ΣMUFA	33.8	52.6
C18:2(n-6)	3.7 ± 0.8	1.6 ± 0.1
C20:4(n-6)	2.7 ± 0.9	1.8 ± 0.3
C20:5(n-3)	22.6 ± 3.4	13.6 ± 3.6
ΣPUFA	28.9	17.0

3.3. Liquid Tri-phase System (LTPS)

3.3.1. Lipid Classes

Figure 3.7 shows the partition of the studied microalgae lipids partitioned between the used binary solvent system (hexane and water). Three phases (upper hexane, middle colloidal and bottom water) were observed after mixing; but centrifugation enhanced the formation of the three distinct phases. The yields of the different fractions were, for *N. oculata,* 27.4, 53.4 and 19.2% for the hexane, colloidal and water phases, whereas for *P. tricornutum,* 30.6, 55.4 and 14.0%, respectively (Fig. 3.7).

The colloidal phase was further separated using silica gel column chromatography. It was composed of 5.1, 23.4 and 71.5% of NLs, GLs and PLs, respectively for *N. oculata*. Similarly, *P. tricornutum* colloidal consisted of 3.0, 28.7, and 68.3% of NLs, GLs and PLs, respectively. These results reveal that the colloidal phase is a rich source of PLs.

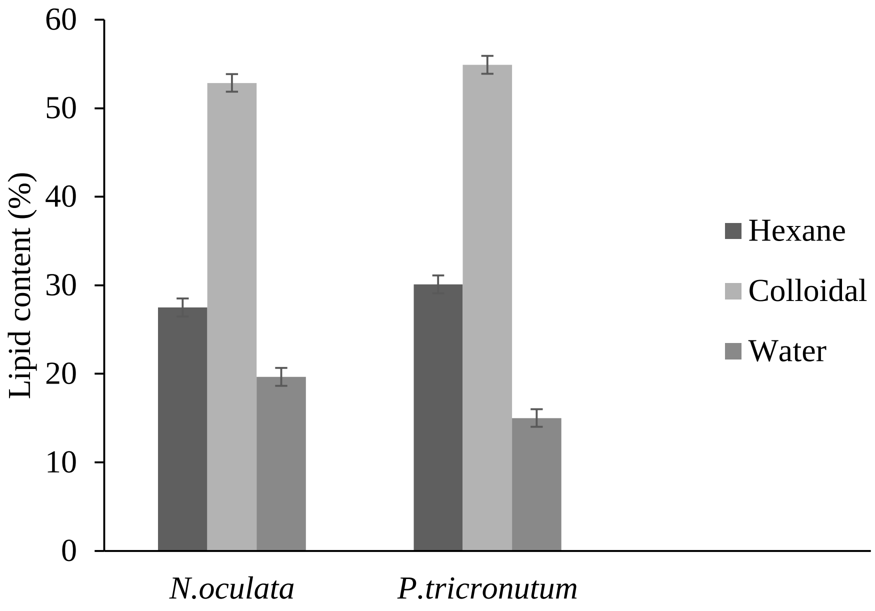


Fig. 3.7. Lipid class separation using Liquid tri-phase system (LTPS)

HPLC-ELSD was used also to analyse the lipid classes of LTPS fractions of *N. oculata* (i.e., hexane, colloidal and water phases) (Fig 3.8 to 3.10). In the hexane fraction (Fig. 3.8), more than 93.7% was composed of NLs and the remaining with PLs (6.3%). GLs were not detected and the PLs fractions are not significant. As a result, the hexane phase is suitable aspirant to be used for biodiesel production as it is almost free from polar lipids.

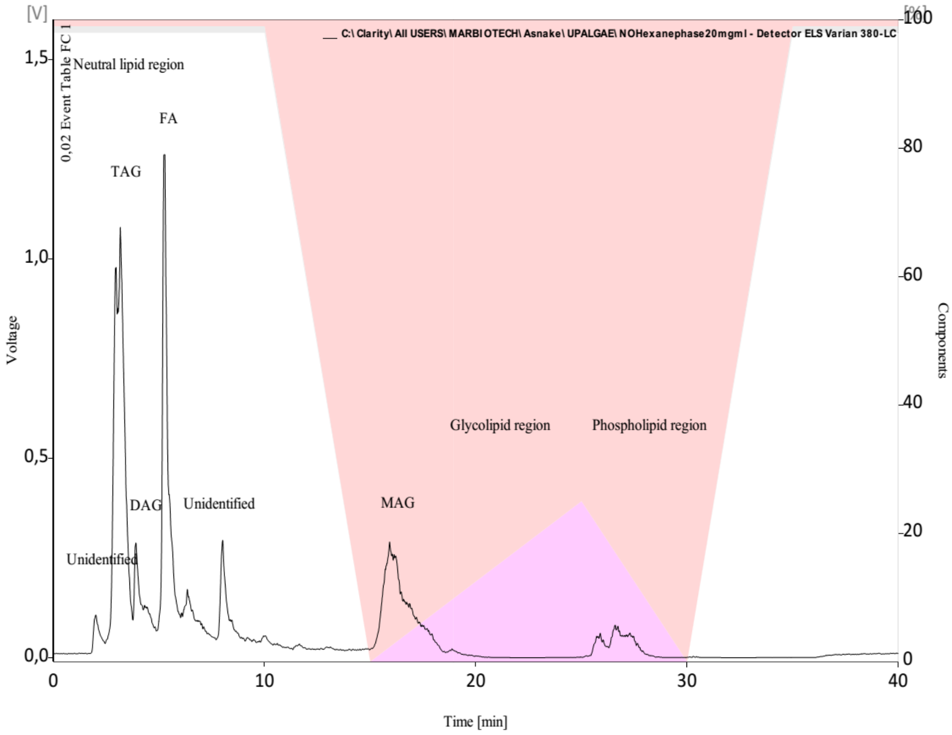


Fig. 3.8. Lipid classes composition of the hexane fraction (LTPS) of *N. oculata*

The lipid classes profile of the colloidal fraction is presented in Fig. 3.9. The approximate area percentages were 8.6, 27.8 and 63.5 for NLs, GLs and PLs, respectively. This was a similar to those obtained from column chromatography (which were 5.1, 23.4, and 71.5 for NLs, GLs and PLs, respectively). This indicates that the colloidal phase was composed of more polar lipids particularly PLs. Hence, this phase can be used for other value added products such as liposome, aquaculture feed or biomedical applications (Drevon, et al., 1993; Simopoulos, 1999). In this regard, the contributions of the present study are to introduce the new amphiphilic or amphipathic phase, the colloidal phase of wider industrial applications, partitioned between the non-polar hexane upper phase and the polar water lower phase.

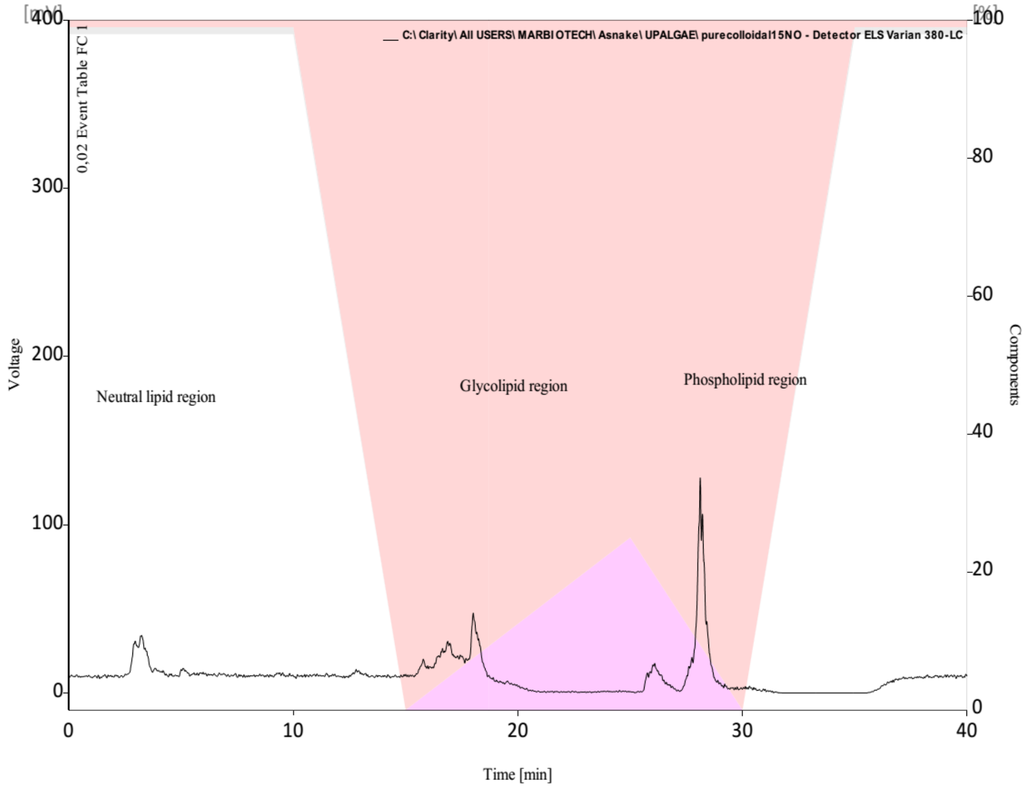


Fig. 3.9. Lipid classes composition of the colloidal fraction (LTPS) *N. oculata*

The lipid profile of the water phase is presented in Fig 3.10. These fractions appeared only in PLs of the chromatograms. These lipids might be highly hydrolysed PLs fractions (e.g., LPC). It might be sugar and/or peptides. Despite logical conclusion that they are polar (by principle of like dissolves like), scientific conclusion demands concrete evidence. Such polar lipids can be used to produce bioalchols (Chen et al., 2013). However, this has been left for further investigation as standards are essential and attention was given to the two phases (i.e., hexane and colloidal) to study their FAs profiles using GC-MS. But, this is an indication of applying biorefinery scheme by using the whole lipids for several kinds of applications.

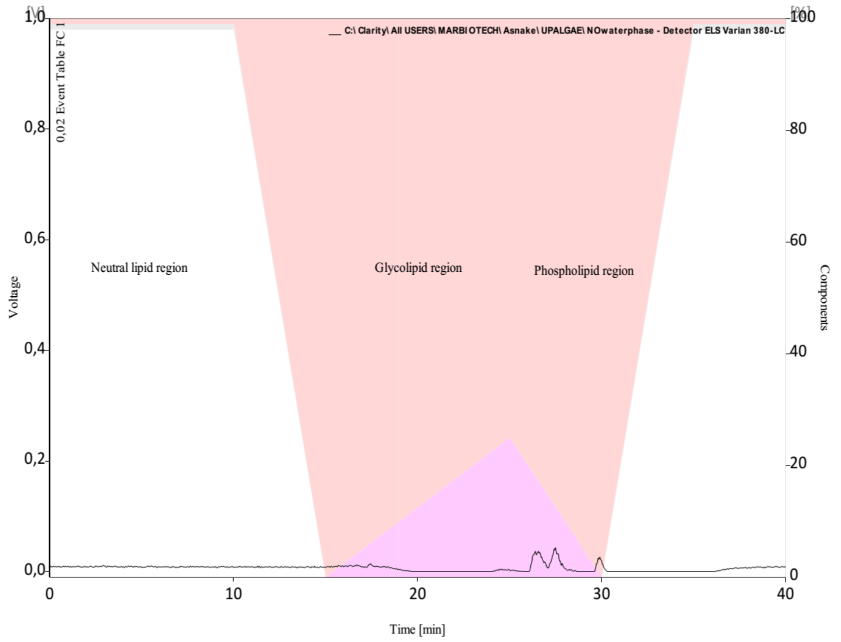


Fig. 3.10. Lipid classes composition of the water fraction (LTPS) *N. oculata*

3.3.2. Fatty Acid Composition

GC-MS analysis for fatty acid profiles was done only for the hexane and colloidal fractions as the water phase contained insignificant amount of lipids in the PLs region. Because the principal objective is biodiesel production from NLs. Besides, a great focus was given to polar lipid rich fractions (i.e., colloidal enriched in PLs and GLs) due to their economic value and high demand in world market for liposome, nutritional supplement to infant formula and biosurfactants as well. Despite this fact, bioethanol produced from the water phase can further reduce the cost of biodiesel from microalgae under the biorefinery scheme. As a result, it is utterly recommended for future study. Furthermore, the spent biomass can be utilized as feed for food in aquaculture or for biogas or bio-fertilizer to complete the biorefinery concept.

Table 3.3 shows the main FAs detected in *N. oculata*. Hexane phase FAs were C14:0, C16:0, C16:1 and C18:1. The sum of SFAs and MUFAs in this phase was more than 88.0%. This has a lot of advantages for biodiesel production from NLs than crude extract. Foley et al. (2011) states highly unsaturated lipids occur more frequently in polar lipid fractions specifically PLs. So, separating the polar lipids from neutral lipids can also enhance the biodiesel quality (i.e, reduce the oxidation) and thus increase the yield of FAMEs (Freedman, et al., 1984; Y. Li, et al., 2014; Watanabe, et al., 2002). Furthermore, it helps to comply with specifications such as EN 14214 and ASTM D6751 standards for phosphorus content should be less than 4ppm according to EN 14214 or 10ppm as per ASTM D6751 specifications (Kaleli, 2001; Tyson & McCormick, 2006).

The colloidal fraction contains some FAs as in hexane phase (Table 3.3**)**. However, it consists of 43.2% of PUFAs such as C20:5, C20:4 and C18:2. As a result, while the hexane phase can be used for biodiesel, the colloidal is able to be used in the treatment of atherosclerosis, cancer, and neurodegenerative diseases such as Alzheimer's because it contains omega-3-FAs (Drevon, et al., 1993; Simopoulos, 1999). It can also be used for infant formula nutritional supplement because of arachidonic acid (Sijtsma & De Swaaf, 2004; Simopoulos, 1999) and aquaculture feed (Brennan & Owende, 2010; Pulz & Gross, 2004; Spolaore, et al., 2006). In addition, it contains conditionally essential FAs like linoleic acid (C18:2). This is an attempt to apply the bio-refinery scheme so that microalgae can be competitive feedstock for biodiesel.

This finding shows LC-PUFAs occur more frequently in polar lipid fractions specifically colloidal phase containing both PLs and GLs. This is in line with Foley et al.(2011) who reported unsaturated FAs are related to polar lipids specially PLs . For example, EPA in *P. tricornutum* is mainly associated with PLs and GLs (Ryckebosch, et al., 2012). Such FAs are available in very few organisms such as fish oil. However, considerable evidences have indicated that n-3-PUFAs in fish oils are actually derived via the marine food chain from zooplankton consuming n-3-PUFAs synthesising microalgae; fish accumulates pollutants up food chain and fish oil is with unpleasant smell (Yongmanitchai & Ward, 1989).Therefore, microalgae are some of the most promising feedstock for biofuels and/or biomaterials. Other applications of PLs fractions include improving intelligence and function of nerve cells (J. Li, et al., 2015; Pepeu, et al., 1996). Therefore, it is very important to separate let alone the general lipid classes even each lipid fractions with in the classes to get wider applications from each chemical or compound entity.

In addition, EPA and DHA have the ability to influence cell membrane fluidity and permeability (Burri, et al., 2012). These omega-3-FAs are rarely available in higher plants lipids but microalgae PLs are enriched in these FAs (Ryckebosch, et al., 2012). This implies that PLs from microalgae can deliver both hydrophilic and hydrophobic drugs to the target site as microalgae contain both non-polar and polar lipids. Such PLs liposomes have many advantages: targeting, tissue compatibility, reducing drug toxicity and improving its stability. Consequently, such liposomes can serve as the carriers of antitumor, antifungal, analgesic, in gene therapeutics and vaccines (Delalat, et al., 2015; J. Li, et al., 2015; Pattni, et al., 2015).

Table 3.3. FAs composition of *N. oculata* fractions obtained by LTPS. Results are expressed as mean ± standard deviation (n = 2)

Fatty acids	Hexane	Colloidal
C14:0	11.3 ± 0.9	11.0
C16:0	36.4 ± 1.1	16.9
ΣSFA	47.7	27.9
C16:1(n-6)	29.8 ± 2.3	16.0
C18:1(n-9)	11.1 ± 0.3	12.9
ΣMUFA	40.9	28.9
C18:2(n-6)	6.0 ± 2.1	8.8
C20:4(n-6)	1.0 ± 1.2	8.1
C20:5(n-3)	4.4 ± 0.2	26.3
ΣPUFA	11.4	43.2

Similarly, Table 3.4 shows the main FAs detected in *P. tricronutum*. Hexane phase FAs were mainly C16:0, C16:1 and C18:1 (Table 3.4). The sum of SFAs and MUFAs in this phase was more than 82.5%. These FAs are good for biodiesel as they are less prone to oxidation when compared with PUFAs which contains more unsaturated bonds.

The colloidal phase contains C14:0, C16:0, C16:1, C18:1 and C20:5 (Table 3.4). These support that the EPA in both strains (*N. oculata* = 26.3%; *P. tricronutum* = 23.0%) can be separated and optimized for industrial scale production using LTPS.

Table 3.4. FAs composition of *P. tricronutum* fractions obtained by LTPS. Results are indicated as mean ± standard deviation (n = 2)

Fatty acids	Hexane	Colloidal
C14:0	8.3 ± 1.7	11.7
C16:0	31.3 ± 0.4	27.0
C18:0	4.8 ± 1.4	2.4
ΣSFA	44.3	38.7
C16:1(n-9)	25.4 ± 1.6	20.9
C18:1(n-9)	12.9 ± 0.6	14.4
ΣMUFA	38.3	35.3
C18:2(n-6)	3.6 ± 1.2	1.0
C20:4(n-6)	1.4 ± 0.7	2.0
C20:5(n-3)	12.3 ± 2.7	23.0
ΣPUFA	17.4	26.0

4. CONCLUSIONS

- Ethanol successfully extracts lipids from wet microalgae biomass. This can reduce the high dewatering cost
- Neutral lipids can be obtained free of polar lipids (i.e. colloidal = phospholipids + glycolipids) to produce biodiesel from microalgae and, at the same time, polar rich fractions can be separated for value added products.
- Neutral lipids comprise fatty acids suitable for biodiesel production but the colloidal phase contains fatty acids suitable for biomedical or feed or food applications.

RECOMMENDED FUTURE STUDIES

- Comparative study of physicochemical properties of biodiesel from microalgae total lipids and its NLs fraction.
- Conversion of GLs fraction to non-ionic bio-surfactant and characterization of its suitability for food or pharmaceutical industries.
- Characterization of colloidal phase for liposome as drug delivery system or infant formula additives.
- Carry out life cycle assessment (LCA) or cost benefit analysis (CBA) of microalgae bio-refinery in comparison to petro-refinery.

BIBLIOGRAPHY

Abdel-Mawgoud, A. M., Hausmann, R., Lépine, F., Müller, M. M., & Déziel, E. (2011). Rhamnolipids: detection, analysis, biosynthesis, genetic regulation, and bioengineering of production *Biosurfactants* (pp. 13-55): Springer.

Adam, F., Abert-Vian, M., Peltier, G., & Chemat, F. (2012). “Solvent-free” ultrasound-assisted extraction of lipids from fresh microalgae cells: a green, clean and scalable process. *Bioresource Technology, 114*, 457-465.

Agarwal, A. K., & Das, L. (2001). Biodiesel development and characterization for use as a fuel in compression ignition engines. *Journal of Engineering for Gas Turbines and Power, 123*(2), 440-447.

Akansu, S. O., Dulger, Z., Kahraman, N., & Veziroğlu, T. N. (2004). Internal combustion engines fueled by natural gas—hydrogen mixtures. *International Journal of Hydrogen Energy, 29*(14), 1527-1539.

Alonso, D. L., Grima, E. M., Pérez, J. S., Sánchez, J. G., & Camacho, F. G. (1992). Isolation of clones of Isochrysis galbana rich in eicosapentaenoic acid. *Aquaculture, 102*(4), 363-371.

Azam, M. M., Waris, A., & Nahar, N. (2005). Prospects and potential of fatty acid methyl esters of some non-traditional seed oils for use as biodiesel in India. *Biomass and Bioenergy, 29*(4), 293-302.

Bansal, V. S., & Majerus, P. W. (1990). Phosphatidylinositol-derived precursors and signals. *Annual Review of Cell Biology, 6*(1), 41-67.

Basova, M. (2005). Fatty acid composition of lipids in microalgae. *International Journal on Algae, 7*(1).

Bellou, S., Baeshen, M. N., Elazzazy, A. M., Aggeli, D., Sayegh, F., & Aggelis, G. (2014). Microalgal lipids biochemistry and biotechnological perspectives. *Biotechnology Advances, 32*(8), 1476-1493.

Bendz, K. (2005). EU-25 Oilseeds and Products Biofuels Situation in the European Union 2005. *GAIN report E, 35058*.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology, 37*(8), 911-917.

Bol, M. B., Bhattacharya, T., & Mishra, T. (2004). Studies on fuel properties of refined soybean oil, soybean ethyl ester and their blends with diesel to assess usefulness as CI engine fuel. *IE (I) Journal-ID, 85*, 17-20.

Bondioli, P., Della Bella, L., Rivolta, G., Zittelli, G. C., Bassi, N., Rodolfi, L., . . . Tredici, M. R. (2012). Oil production by the marine microalgae Nannochloropsis sp. F&M-M24 and Tetraselmis suecica F&M-M33. *Bioresource Technology, 114*, 567-572.

Brasaemle, D. L. (2007). Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *Journal of Lipid Research, 48*(12), 2547-2559.

Brennan, L., & Owende, P. (2010). Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews, 14*(2), 557-577.

Burri, L., Hoem, N., Banni, S., & Berge, K. (2012). Marine omega-3 phospholipids: metabolism and biological activities. *International Journal of Molecular Sciences, 13*(11), 15401-15419.

Cantrell, K. B., Ducey, T., Ro, K. S., & Hunt, P. G. (2008). Livestock waste-to-bioenergy generation opportunities. *Bioresource Technology, 99*(17), 7941-7953.

Chen, C.-Y., Zhao, X.-Q., Yen, H.-W., Ho, S.-H., Cheng, C.-L., Lee, D.-J., . . . Chang, J.-S. (2013). Microalgae-based carbohydrates for biofuel production. *Biochemical Engineering Journal, 78*, 1-10.

Chen, M., Liu, T., Chen, X., Chen, L., Zhang, W., Wang, J., . . . Peng, X. (2012). Subcritical co-solvents extraction of lipid from wet microalgae pastes of *Nannochloropsis* sp. *European Journal of Lipid Science and Technology, 114*(2), 205-212.

Cheng, J., Yu, T., Li, T., Zhou, J., & Cen, K. (2013). Using wet microalgae for direct biodiesel production via microwave irradiation. *Bioresource Technology, 131*, 531-535.

Cherubini, F. (2010). The biorefinery concept: using biomass instead of oil for producing energy and chemicals. *Energy Conversion and Management, 51*(7), 1412-1421.

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances, 25*(3), 294-306.

Chiu, S.-Y., Kao, C.-Y., Tsai, M.-T., Ong, S.-C., Chen, C.-H., & Lin, C.-S. (2009). Lipid accumulation and CO 2 utilization of *Nannochloropsis oculata* in response to CO_2 aeration. *Bioresource Technology, 100*(2), 833-838.

Cobelas, M. A., & Lechado, J. Z. (1989). Lipids in microalgae-a review. 1. Biochemistry. *Grasasy Aceites, 40*(2), 118-145.

Converti, A., Casazza, A. A., Ortiz, E. Y., Perego, P., & Del Borghi, M. (2009). Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chemical Engineering and Processing: Process Intensification, 48*(6), 1146-1151.

Copeland, R., & Belcher, W. (2000). Improved method for refining vegetable oil. *PCT Patent Application WO 00/31219 assigned to AG Processing Inc*.

Corma, A., Huber, G. W., Sauvanaud, L., & O'connor, P. (2007). Processing biomass-derived oxygenates in the oil refinery: catalytic cracking (FCC) reaction pathways and role of catalyst. *Journal of Catalysis, 247*(2), 307-327.

Crawford, M. (2000). Placental delivery of arachidonic and docosahexaenoic acids: implications for the lipid nutrition of preterm infants. *The American Journal of Clinical Nutrition, 71*(1), 275S-284S.

Daroch, M., Geng, S., & Wang, G. (2013). Recent advances in liquid biofuel production from algal feedstocks. *Applied Energy, 102*, 1371-1381.

De Castro, J. F. (2007). Biofuels–an overview. *Final Report. Prepared for DGIS/DMW/IB. Environmental Infrastructure and Impact Division, Environment and Water Department, Directorate-General for International Cooperation (DGIS), the Hague, Netherlands*.

De Souza, M. P., Petrus, J. C. C., Gonçalves, L. A. G., & Viotto, L. A. (2008). Degumming of corn oil/hexane miscella using a ceramic membrane. *Journal of Food Engineering, 86*(4), 557-564.

Delalat, B., Sheppard, V. C., Ghaemi, S. R., Rao, S., Prestidge, C. A., McPhee, G., . . . Johns, T. G. (2015). Targeted drug delivery using genetically engineered diatom biosilica. *Nature Communications, 6*.

Delon, C., Manifava, M., Wood, E., Thompson, D., Krugmann, S., Pyne, S., & Ktistakis, N. T. (2004). Sphingosine kinase 1 is an intracellular effector of phosphatidic acid. *Journal of Biological Chemistry, 279*(43), 44763-44774.

Dijkstra, A. J. (2010). Enzymatic degumming. *European Journal of Lipid Science and Technology, 112*(11), 1178-1189.

Dismukes, G. C., Carrieri, D., Bennette, N., Ananyev, G. M., & Posewitz, M. C. (2008). Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Current Opinion in Biotechnology, 19*(3), 235-240.

Dowhan, W., Bogdanov, M., & Mileykovskaya, E. (2002). Functional roles of lipids in membranes. *Biochemistry of Lipids, Lipoproteins and Membranes, 5*, 1-37.

Drevon, C. A., Baksaas, I., & Krokan, H. E. (1993). Omega-3 fatty acids: Metabolism and biological effects: Birkhäuser Verlag AG.

Dufey, A., Vermeulen, S., & Vorley, B. (2007). Biofuels: strategic choices for commodity dependent developing countries. *Common Fund for Commodities*.

Fajardo, A. R., Cerdan, L. E., Medina, A. R., Fernández, F. G. A., Moreno, P. A. G., & Grima, E. M. (2007). Lipid extraction from the microalga *Phaeodactylum tricornutum*. *European Journal of Lipid Science and Technology, 109*(2), 120-126.

Folch, J., Lees, M., & Sloane-Stanley, G. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry, 226*(1), 497-509.

Foley, P. M., Beach, E. S., & Zimmerman, J. B. (2011). Algae as a source of renewable chemicals: opportunities and challenges. *Green Chemistry, 13*(6), 1399-1405.

Forján, E., Garbayo, I., Henriques, M., Rocha, J., Vega, J. M., & Vílchez, C. (2011). UV-A mediated modulation of photosynthetic efficiency, xanthophyll cycle and fatty acid production of *Nannochloropsis*. *Marine Biotechnology, 13*(3), 366-375.

Freedman, B., Pryde, E., & Mounts, T. (1984). Variables affecting the yields of fatty esters from transesterified vegetable oils. *Journal of the American Oil Chemists Society, 61*(10), 1638-1643.

Gangadhar, K. N., Pereira, H., Diogo, H. P., dos Santos, R. M. B., Devi, B. P., Prasad, R., . . . Barreira, L. (2015). Assessment and comparison of the properties of biodiesel synthesized from three different types of wet microalgal biomass. *Journal of Applied Phycology*, 1-8.

Graeve, M., & Janssen, D. (2009). Improved separation and quantification of neutral and polar lipid classes by HPLC–ELSD using a monolithic silica phase: application to exceptional marine lipids. *Journal of Chromatography B, 877*(20), 1815-1819.

Group, U. N. E. P. B. W., & Management, U. N. E. P. I. P. f. S. R. (2009). Towards sustainable production and use of resources: assessing biofuels: UNEP/Earthprint.

Gubler, R. (2006). Biodiesel. *The Chemical Economics Handbook*, 205.0000.

Gubler, R. (2007). Subsidies and regulations keep biodiesel markets growing. *Industrial Biotechnology, 3*(1), 22-24.

Hager, A., De Paoli, T., Ihlo, J., Farach, H., & Poole, C. (1993). Stability study of lecithin liposomes during storage using ESR. *Spectrochimica Acta Part A: Molecular Spectroscopy, 49*(13), 1999-2005.

Halim, R., Gladman, B., Danquah, M. K., & Webley, P. A. (2011). Oil extraction from microalgae for biodiesel production. *Bioresource Technology, 102*(1), 178-185.

Houghton, J. T., Ding, Y., Griggs, D. J., Noguer, M., van der Linden, P. J., Dai, X., . . . Johnson, C. (2001). Climate change 2001: The scientific basis.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal, 54*(4), 621-639.

Huber, G. W., & Dumesic, J. A. (2006). An overview of aqueous-phase catalytic processes for production of hydrogen and alkanes in a biorefinery. *Catalysis Today, 111*(1), 119-132.

Huber, G. W., O’Connor, P., & Corma, A. (2007). Processing biomass in conventional oil refineries: production of high quality diesel by hydrotreating vegetable oils in heavy vacuum oil mixtures. *Applied Catalysis A: General, 329*, 120-129.

Iyer, R. (2016). The issue of reducing or removing phospholipids from total lipids of a microalgae and an oleaginous fungus for preparing biodiesel. *Biofuels*, 1-18.

Jacob-Lopes, E., Queiroz, M., Ramírez-Mérida, L., & Zepka, L. Q. (2015). Microalgal biorefineries. *Jacob-Lopes and Queiroz (ed.) Biomass Production and Uses. Rijeka: InTech*, 81-106.

Jiang, H., & Gao, K. (2004). Effects of lowering temperature during culture on the production of polyunsaturated fatty acids in the marine diatom *Phaeodactylum tricornutum* (bacillariophyceae) 1. *Journal of Phycology, 40*(4), 651-654.

Jones, J., Manning, S., Montoya, M., Keller, K., & Poenie, M. (2012). Extraction of algal lipids and their analysis by HPLC and mass spectrometry. *Journal of the American Oil Chemists' Society, 89*(8), 1371-1381.

Kaleli, H. (2001). The impact of crankcase oil containing phosphorus on catalytic converters and engine exhaust emissions. *Industrial Lubrication and Tribology, 53*(6), 237-255.

Kalligeros, S., Zannikos, F., Stournas, S., Lois, E., Anastopoulos, G., Teas, C., & Sakellaropoulos, F. (2003). An investigation of using biodiesel/marine diesel blends on the performance of a stationary diesel engine. *Biomass and Bioenergy, 24*(2), 141-149.

Khan, S. A., Hussain, M. Z., Prasad, S., & Banerjee, U. (2009). Prospects of biodiesel production from microalgae in India. *Renewable and Sustainable Energy Reviews, 13*(9), 2361-2372.

King, R. J., & MacBeth, M. C. (1981). Interaction of the lipid and protein components of pulmonary surfactant Role of phosphatidylglycerol and calcium. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 647*(2), 159-168.

Kitamoto, D., Isoda, H., & Nakahara, T. (2002). Functions and potential applications of glycolipid biosurfactants—from energy-saving materials to gene delivery carriers—. *Journal of Bioscience and Bioengineering, 94*(3), 187-201.

Knothe, G. (2005a). Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Processing Technology, 86*(10), 1059-1070.

Knothe, G. (2005b). The history of vegetable oil-based diesel fuels. *The Biodiesel Handbook*, 4-16.

Knothe, G. (2010). Biodiesel: current trends and properties. *Topics in Catalysis, 53*(11-12), 714-720.

Kobayashi, N., Noel, E. A., Barnes, A., Rosenberg, J., DiRusso, C., Black, P., & Oyler, G. A. (2013). Rapid detection and quantification of triacylglycerol by HPLC–ELSD in *Chlamydomonas reinhardtii* and *Chlorella* strains. *Lipids, 48*(10), 1035-1049.

Koh, L. P., & Wilcove, D. S. (2008). Is oil palm agriculture really destroying tropical biodiversity? *Conservation Letters, 1*(2), 60-64.

Lam, M. K., & Lee, K. T. (2013). Catalytic transesterification of high viscosity crude microalgae lipid to biodiesel: effect of co-solvent. *Fuel Processing Technology, 110*, 242-248.

Lardon, L., Helias, A., Sialve, B., Steyer, J.-P., & Bernard, O. (2009). Life-cycle assessment of biodiesel production from microalgae. *Environmental Science & Technology, 43*(17), 6475-6481.

Li, J., Wang, X., Zhang, T., Wang, C., Huang, Z., Luo, X., & Deng, Y. (2015). A review on phospholipids and their main applications in drug delivery systems. *Asian Journal of Pharmaceutical Sciences, 10*(2), 81-98.

Li, Y., Du, W., & Liu, D. (2014). Exploration on the effect of phospholipids on free lipase-mediated biodiesel production. *Journal of Molecular Catalysis B: Enzymatic, 102*, 88-93.

Little, A., Levy, R., Chuaqui-Kidd, P., & Hand, D. (1985). A double-blind, placebo controlled trial of high-dose lecithin in Alzheimer's disease. *Journal of Neurology, Neurosurgery & Psychiatry, 48*(8), 736-742.

Lotero, E., Goodwin Jr, J. G., Bruce, D. A., Suwannakarn, K., Liu, Y., & Lopez, D. E. (2006). The catalysis of biodiesel synthesis. *Catalysis, 19*(1), 41-83.

Lotero, E., Liu, Y., Lopez, D. E., Suwannakarn, K., Bruce, D. A., & Goodwin, J. G. (2005). Synthesis of biodiesel via acid catalysis. *Industrial & Engineering Chemistry Research, 44*(14), 5353-5363.

Ma, F., & Hanna, M. A. (1999). Biodiesel production: a review. *Bioresource Technology, 70*(1), 1-15.

Mag, T. K., & Reid, M. P. (1980). For removing phosphatides and metals: Google Patents.

Mayzaud, P., Boutoute, M., Perissinotto, R., & Nichols, P. (2007). Polar and neutral lipid composition in the pelagic tunicate *Pyrosoma atlanticum*. *Lipids, 42*(7), 647-657.

Metting Jr, F. (1996). Biodiversity and application of microalgae. *Journal of Industrial Microbiology, 17*(5-6), 477-489.

Miranda, C. T., Pinto, R. F., de Lima, D. V., Viegas, C. V., da Costa, S. M., & Azevedo, S. M. (2015). Microalgae Lipid and Biodiesel Production: A Brazilian Challenge. *American Journal of Plant Sciences, 6*(15), 2522.

Moore, A. (2008). Biofuels are dead: long live biofuels (?)–Part one. *New Biotechnology, 25*(1), 6-12.

Morais, A. R., & Bogel-Lukasik, R. (2013). Green chemistry and the biorefinery concept. *Sustainable Chemical Processes, 1*(1), 18.

Naylor, R., & Burke, M. (2005). Aquaculture and ocean resources: raising tigers of the sea.

Nisbett, R. E., Peng, K., Choi, I., & Norenzayan, A. (2001). Culture and systems of thought: holistic versus analytic cognition. *Psychological Review, 108*(2), 291.

Ohlrogge, J., & Browse, J. (1995). Lipid biosynthesis. *The Plant Cell, 7*(7), 957.

Ostrea, E. M., Cepeda, E. E., Fleury, C. A., & Balun, J. E. (1985). Red cell membrane lipid peroxidation and hemolysis secondary to phototherapy. *Acta Paediatrica, 74*(3), 378-381.

Pacini, H., Silveira, S., & Da Silva Filho, A. C. (2013). The European Biofuels Policy: from where and where to? *European Energy Journal, 3*(1), 1-36.

Pagliero, C., Ochoa, N., Marchese, J., & Mattea, M. (2001). Degumming of crude soybean oil by ultrafiltration using polymeric membranes. *Journal of the American Oil Chemists' Society, 78*(8), 793-796.

Pagliero, C., Ochoa, N., Marchese, J., & Mattea, M. (2004). Vegetable oil degumming with polyimide and polyvinylidenefluoride ultrafiltration membranes. *Journal of Chemical Technology and Biotechnology, 79*(2), 148-152.

Patil, P. D., Gude, V. G., Mannarswamy, A., Cooke, P., Nirmalakhandan, N., Lammers, P., & Deng, S. (2012). Comparison of direct transesterification of algal biomass under supercritical methanol and microwave irradiation conditions. *Fuel, 97*, 822-831.

Patil, P. D., Reddy, H., Muppaneni, T., Mannarswamy, A., Schuab, T., Holguin, F. O., . . . Deng, S. (2012). Power dissipation in microwave-enhanced in situ transesterification of algal biomass to biodiesel. *Green Chemistry, 14*(3), 809-818.

Patil, V., & Gislerod, H. (2006). The importance of omega-3 fatty acids in diet. *Current Science-Bangalore-, 90*(7), 908.

Patil, V., Källqvist, T., Olsen, E., Vogt, G., & Gislerød, H. R. (2007). Fatty acid composition of 12 microalgae for possible use in aquaculture feed. *Aquaculture International, 15*(1), 1-9.

Pattni, B. S., Chupin, V. V., & Torchilin, V. P. (2015). New developments in liposomal drug delivery. *Chemical Reviews, 115*(19), 10938-10966.

Peng, B. (2012). Transformation of triglycerides and fatty acids into biofuels with sulfur-free catalysts. Universität München.

Pepeu, G., Pepeu, I. M., & Amaducci, L. (1996). A review of phosphatidylserine pharmacological and clinical effects. Is phosphatidylserine a drug for the ageing brain? *Pharmacological Research, 33*(2), 73-80.

Petkov, G., Ivanova, A., Iliev, I., & Vaseva, I. (2012). A critical look at the microalgae biodiesel. *European Journal of Lipid Science and Technology, 114*(2), 103-111.

Petrick, I., Dombrowski, L., Kröger, M., Beckert, T., Kuchling, T., & Kureti, S. (2013). Algae Biorefinery–Material and energy use of algae.

Pulz, O., & Gross, W. (2004). Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology, 65*(6), 635-648.

Qin, J. (2005). Bio-hydrocarbons from algae: impacts of temperature, light and salinity on algae growth: Rirdc.

Rajvanshi, S., & Sharma, M. P. (2012). Micro algae: a potential source of biodiesel. *Journal of Sustainable Bioenergy Systems, 2*(03), 49.

Raupach, M. R., Marland, G., Ciais, P., Le Quéré, C., Canadell, J. G., Klepper, G., & Field, C. B. (2007). Global and regional drivers of accelerating CO_2 emissions. *Proceedings of the National Academy of Sciences, 104*(24), 10288-10293.

Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., & Tredici, M. R. (2009). Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering, 102*(1), 100-112.

Ryckebosch, E., Muylaert, K., & Foubert, I. (2012). Optimization of an analytical procedure for extraction of lipids from microalgae. *Journal of the American Oil Chemists' Society, 89*(2), 189-198.

Schenk, P. M., Thomas-Hall, S. R., Stephens, E., Marx, U. C., Mussgnug, J. H., Posten, C., . . . Hankamer, B. (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. *Bioenergy Research, 1*(1), 20-43.

Searchinger, T., Heimlich, R., Houghton, R. A., Dong, F., Elobeid, A., Fabiosa, J., . . . Yu, T.-H. (2008). Use of US croplands for biofuels increases greenhouse gases through emissions from land-use change. *Science, 319*(5867), 1238-1240.

Shell, R. D. (2008). Shell energy scenarios to 2050: An era of revolutionary transitions: Shell.

Shen, P.-L., Wang, H.-T., Pan, Y.-F., Meng, Y.-Y., Wu, P.-C., & Xue, S. (2016). Identification of characteristic fatty acids to quantify triacylglycerols in microalgae. *Frontiers in Plant Science, 7*.

Sijtsma, L., & De Swaaf, M. (2004). Biotechnological production and applications of the ω-3 polyunsaturated fatty acid docosahexaenoic acid. *Applied Microbiology and Biotechnology, 64*(2), 146-153.

Simopoulos, A. P. (1999). Essential fatty acids in health and chronic disease. *The American Journal of Clinical Nutrition, 70*(3), 560s-569s.

Singh, D., Carlson, R., Fell, D., & Poolman, M. (2015). Modelling metabolism of the diatom *Phaeodactylum tricornutum*. *Biochemical Society Transactions, 43*(6), 1182-1186.

Snåre, M., Kubickova, I., Mäki-Arvela, P., Eränen, K., & Murzin, D. Y. (2006). Heterogeneous catalytic deoxygenation of stearic acid for production of biodiesel. *Industrial & Engineering Chemistry Research, 45*(16), 5708-5715.

Spolaore, P., Joannis-Cassan, C., Duran, E., & Isambert, A. (2006). Commercial applications of microalgae. *Journal of Bioscience and Bioengineering, 101*(2), 87-96.

Tanzi, C. D., Vian, M. A., & Chemat, F. (2013). New procedure for extraction of algal lipids from wet biomass: A green clean and scalable process. *Bioresource Technology, 134*, 271-275.

Tyson, K. S., & McCormick, R. L. (2006). Biodiesel handling and use guidelines.

United State Department of Energy **(**US DOE). (2007). With choices like biodiesel and ethanol, what's the best fuel for your vehicle? Retrieved from greenecon.net/...choices-like-biodiesel-and-ethanol-what’s...best-fuel-for-your-vehicle.

US Energy Information Administration (US EIA). (2013). Few transportation fuels surpass the energy densities of gasoline and diesel. Retrieved from www.eia.gov/todayinenergy/detail.cfm?id=9991

Van Gerpen, J. (2005). Biodiesel processing and production. *Fuel Processing Technology, 86*(10), 1097-1107.

Venendaal, R., Jørgensen, U., & Foster, C. (1997). European energy crops: a synthesis. *Biomass and Bioenergy, 13*(3), 147-185.

Wang, G., & Wang, T. (2012). Characterization of lipid components in two microalgae for biofuel application. *Journal of the American Oil Chemists' Society, 89*(1), 135-143.

Watanabe, Y., Shimada, Y., Sugihara, A., & Tominaga, Y. (2002). Conversion of degummed soybean oil to biodiesel fuel with immobilized *Candida antarctica* lipase. *Journal of Molecular Catalysis B: Enzymatic, 17*(3), 151-155.

Wendel, A. (1995). Lecithin. Kirk-Othmer encyclopedia of chemical technology.

Werpy, T., Petersen, G., Aden, A., Bozell, J., Holladay, J., White, J., . . . Jones, S. (2004). Top value added chemicals from biomass. Volume 1-Results of screening for potential candidates from sugars and synthesis gas: DTIC Document.

Yang, F., Hanna, M. A., & Sun, R. (2012). Value-added uses for crude glycerol--a byproduct of biodiesel production. *Biotechnology for Biofuels, 5*(1), 1.

Yang, F., Xiang, W., Sun, X., Wu, H., Li, T., & Long, L. (2014). A novel lipid extraction method from wet microalga *Picochlorum* sp. at room temperature. *Marine Drugs, 12*(3), 1258-1270.

Yao, L., Gerde, J. A., & Wang, T. (2012). Oil extraction from microalga *Nannochloropsis* sp. with isopropyl alcohol. *Journal of the American Oil Chemists' Society, 89*(12), 2279-2287.

Ying, G.-G., Williams, B., & Kookana, R. (2002). Environmental fate of alkylphenols and alkylphenol ethoxylates—a review. *Environment International, 28*(3), 215-226.

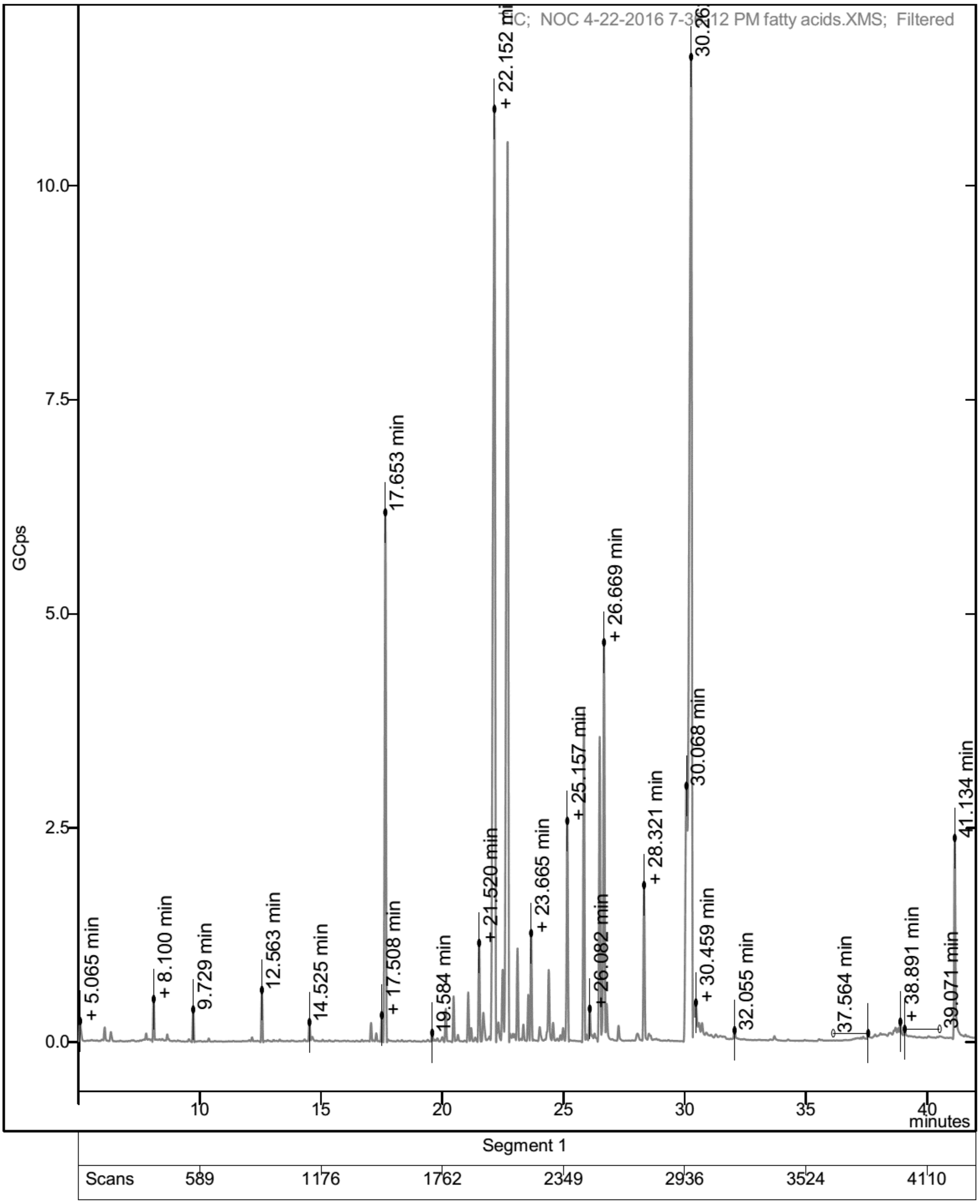
Yongmanitchai, W., & Ward, O. (1989). Omega-3 fatty acids: alternative sources of production. *Process Biochemistry, 24*(4), 117-125.

Zhao, C., Brück, T., & Lercher, J. A. (2013). Catalytic deoxygenation of microalgae oil to green hydrocarbons. *Green Chemistry, 15*(7), 1720-1739.

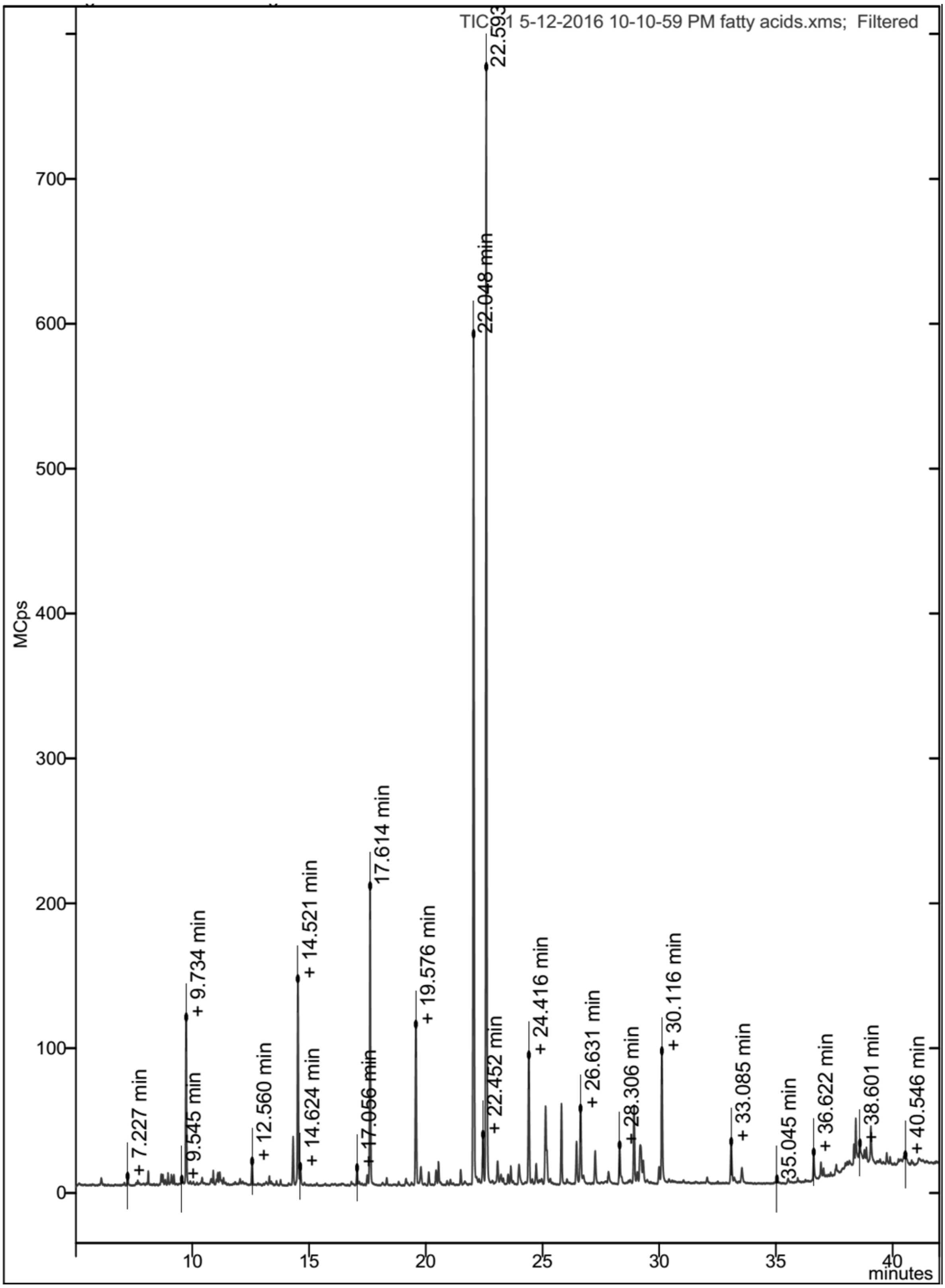
Zilinskas, B., & Zilinskas, B. (1974). Light absorption, emission and photosynthesis. Algal physiology and biochemistry: Oxford: Blackwell Scientiic Publications.

ANNEXES

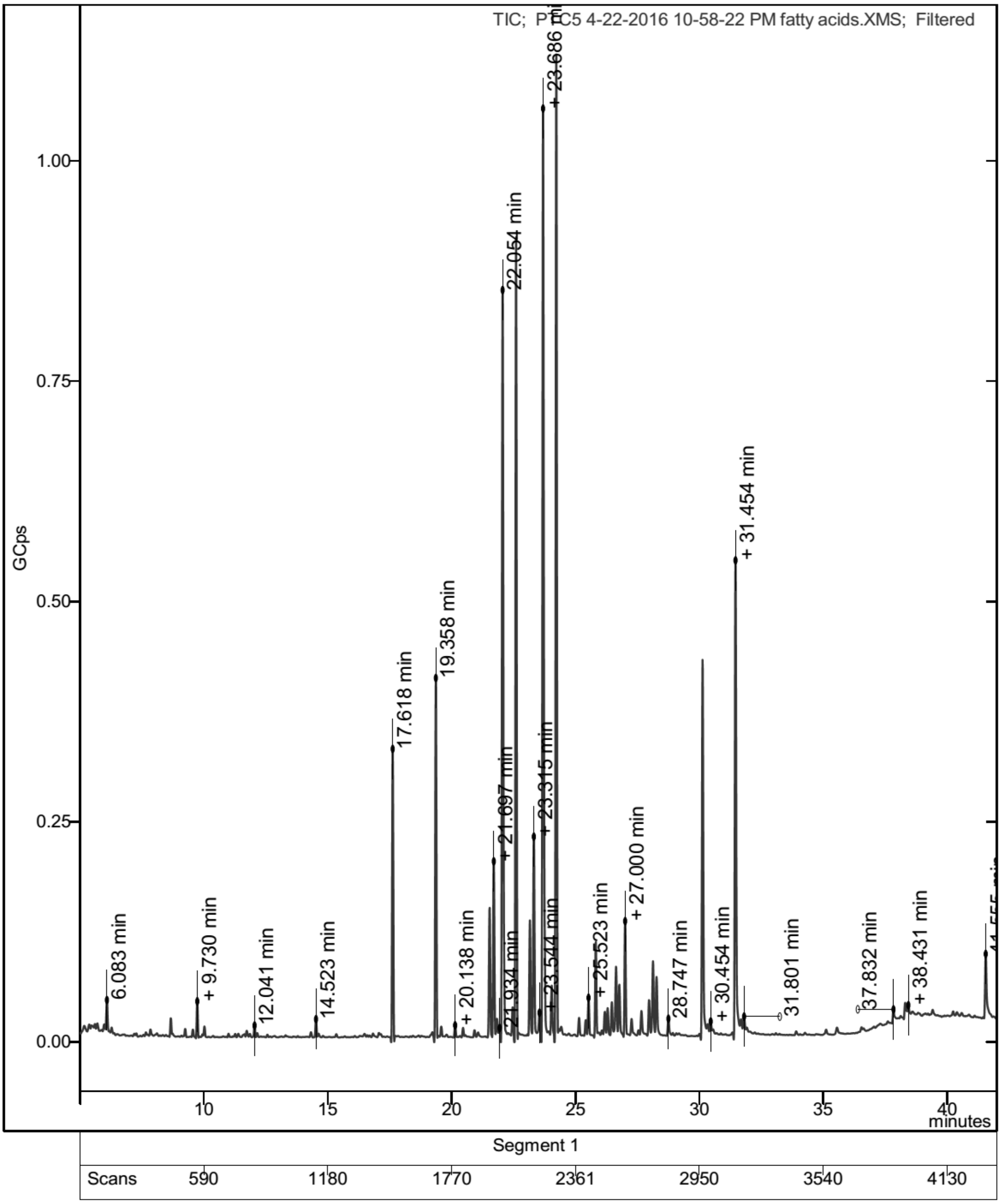
Annex. 1. GC-MS Chromatogram of *N. oculata* crude extract



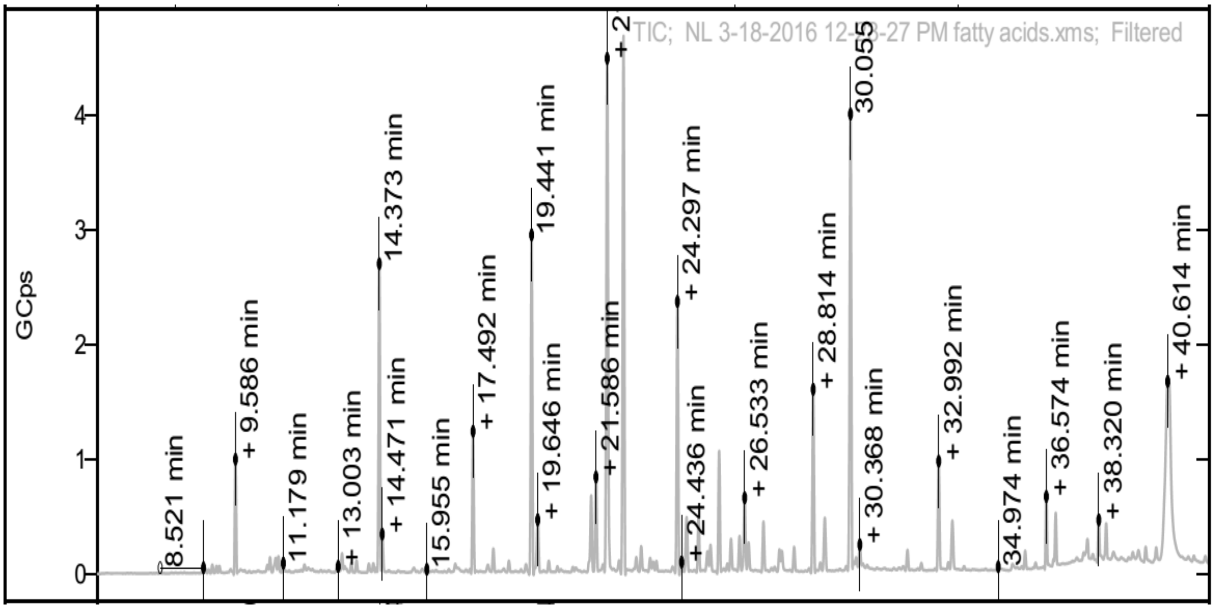
Annex. 2. GC-MS chromatograms *of N. oculata* NLs lipid fraction



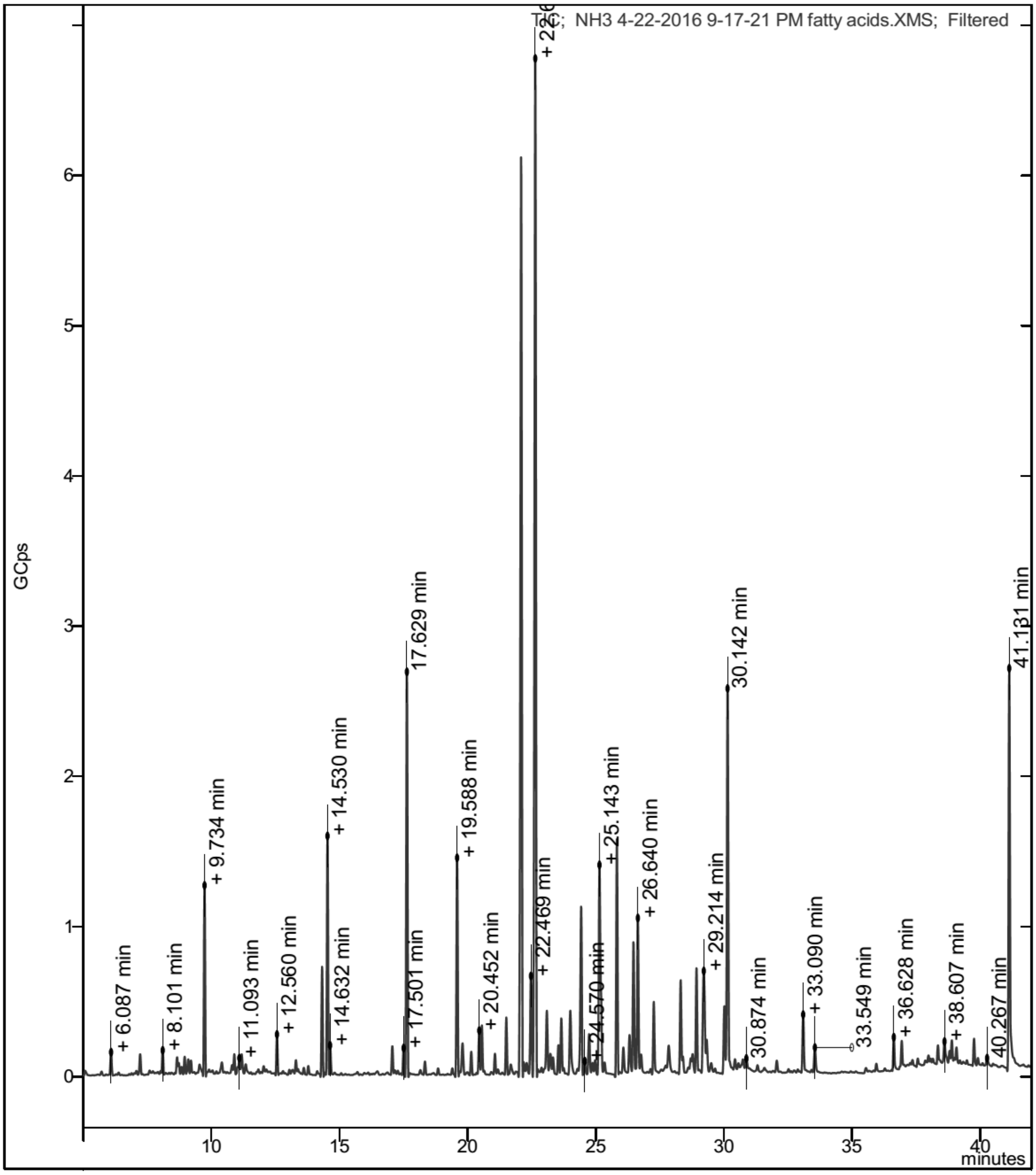
Annex. 3. GC-MS Chromatogram of *P. tricronutum* crude extract



Annex. 4. GC-MS Chromatogram of *P. tricronutum* NLs lipid fraction



Annex. 5. GC-MS chromatogram of *N. oculata* LTPS hexane phase



Annex. 6. GC-MS chromatogram of *P. tricronutum* LTPS hexane phase