UiT
THE ARCTIC UNIVERSITY OF NORWAY

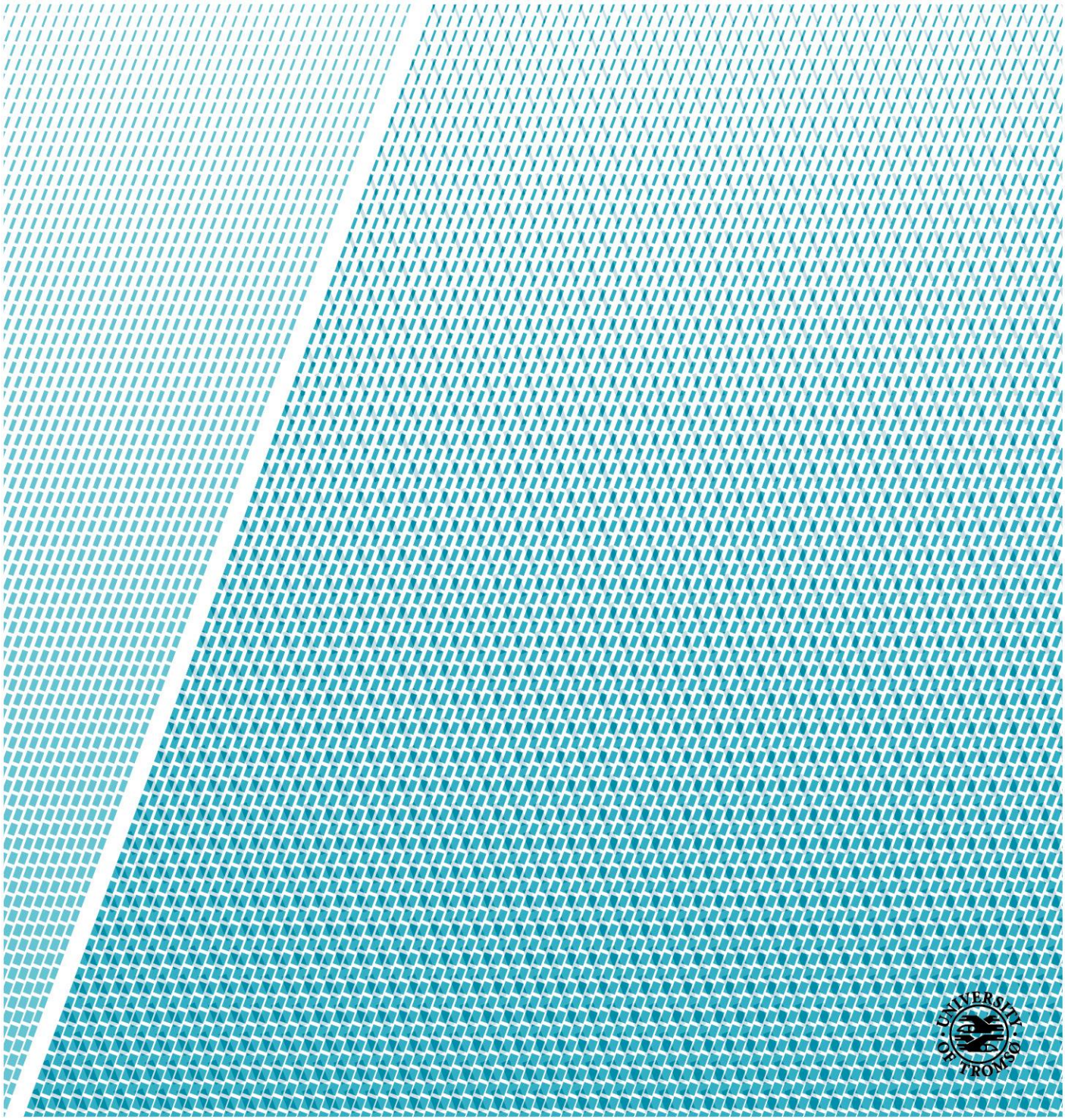
Department of Pharmacy

Large scale cultivation of microalgae at Finnfjord AS: The effect of different storage treatments on lipid and fatty acid stability

—

Simen Aronsen

Master's thesis in Pharmacy May 2018



Acknowledgement

The research presented in this thesis was carried out at the Department of Pharmacy (IFA), UiT The Arctic University of Tromsø in collaboration with The Norwegian College of Fishery Science, and Finnfjord AS.

I would like to thank my supervisor, Associate professor Terje Vasskog for giving me the opportunity to be included in this project. Your patience, guidance, advice, support and help during this project have been invaluable. Then I would like to thank my co-supervisors, Associate professor Terkel Hansen who miraculously fixed the GC-MS in the darkest of times when nothing was working, and PhD candidate Lars Dalheim who replied to all my emails the very moment I sent them. Then I would like to thank my unofficial supervisor, PhD candidate Jon Brage Svenning who served me nothing less than steak with Jerusalem artichoces on our trip to the smeltery in Finnfjordbotn.

I would also like to express my gratitude to everyone involved in the microalgal mass cultivation project, and my fellow student and lab partner Marte who made long days at the laboratory enjoyable.

Finally, I am grateful to my family for their support, encouragement and kindness.

Tromsø, May 2018

Simen Aronsen

Abstract

The Arctic University of Norway and the ferrosilicon smeltery Finnfjord AS has started a microalgal mass cultivation project where sequestration of CO_2 and NOx from factory fumes are used in the production of diatom biomass. Primary application of the produced biomass is fish feed. This requires information regarding lipid content and storage stability. In this study the lipid content, fatty acid composition and the effect of different storage treatments (formic acid, benzoic acid and heat) were investigated for the diatom species *Porosira glacialis*.

The diatoms were cultivated at the factory facilities at Finnfjord AS in Finnfjordbotn. The lipids were extracted using Folch's method with some modifications, and the lipid content was determined gravimetrically. The lipids were transesterified to yield isolated fatty acids methyl esters (FAMEs) which was quantified by GC-MS. To investigate the double bond positions of the fatty acids, the FAMEs were further transesterified to 3-pyridylcarbinol esters and analyzed by GC-MS.

A total of 20 different fatty acids were identified. Double bond position of all fatty acids except 18:3 were determined. The fatty acid composition of *P. glacilis* is highly unsaturated with roughly 80% poly unsaturated fatty acids. These essentially consist of 16:4 (n-1) and EPA (20:5 n-3). Low amounts of omega-6 fatty acids (<1%) were found. The low omega-6 to omega-3 ratio is favorable and makes *P. glacilis* a candidate for fish feed production. Heat treatment seem to be the most promising treatment of the ones tested. 14 days of storage at 4ºC and 20 ºC did not influence the extracted lipid content and fatty acid composition remarkably. Generally, results from the lipid content analysis were conflicting. Hence the analysis should be repeated.

Table of Contents

1 Introduction

1.1 Background

The world is facing several ecological and environmental challenges. With depleting global fish stocks and a growing population, the demand of marine oils rises (1). Consumption of omega-3 fatty acids is a well studied topic because of their beneficial health effects (see paragraph 1.4). However, based on recommended intake, the current supply of omega-3 fatty acids will not be adequate to meet future needs (2). As a consequence, recent research has been directed towards more environmentally friendly and sustainable omega-3 sources.

Finnfjord AS is one of Europe's most energy efficient ferrosilicon smelters (3). They are located in Finnfjordbotn and produce around 100 000 tonnes ferrosilicon annually with ambitions of having zero CO_2 emission. In cooperation with UiT, The Arctic University of Norway, they started a microalgal mass cultivativation project where the CO_2/NOx factory fumes are "fed" to diatoms. The project fills two needs with one deed as the factory emission of greenhouse gasses is reduced, and the main aim of the produced biomass is to be used in fish feed for aquaculture.

Figure 1: Finnfjord AS (4).

The project has received national attention and was donated NOK 18 million by the government in May 2017 for showing promise (5). Although the idea of using microalgae for lipid production is not novel, the use of large cold-water diatoms for this purpose has not been done before, and the results so far seem to offer new solutions for cultivation. Self-shadowing from light is a problem for smaller species with high surface to volume ratio, and require photobioreactors with large surface to volume ratios. With a lower surface to mass ratio, large tanks can be utilized and give lower production costs. Experiments from the pilot project shows that the addition of factory fumes boosts diatom growth while contamination from heavy metals is very low (6). The project is currently being upscaled to a preindustrial scale with a 300 000 L tank being installed.

Half dry biomass from diatoms harvested in its exponential growth phase usually contains 10-25% lipids and 20-40% proteins which is comparable to terrestrial fish feed produced today, but with higher omega-3 content. It is therefore desirable to use the biomass as whole feed, as this can reduce processing costs considerably.

1.2 Diatoms

Diatoms are a major group of microalgae and one of the main primary producers in the ocean. They are photosynthetic and create the basis for marine life; hence they are one of the most significant groups of organisms on earth. There are an estimated 100 000 species of diatoms. They vary in size from 2 μm to about 2 mm, but share the unique feature of being enclosed within a silica wall (frustule) made up of two thecae (7, p. 5-13). Diatoms are single celled organism, but some species can form colonies in the shape of long chains. They reproduce by binary fission, with the daughter cells receiving one theca each. This form of division leads to a size reduction of the offspring. When the cells reach a size about one-third of their maximum size they form an auxospore, usually through sexual reproduction. This give rise to a cell with maximum size and binary fission can continue.

Figure 2: Porosira glacialis diatoms (8).

In order to grow, diatoms require light, dissolved CO_2, nitrogen, silicate, phosphate and trace metals. The major part of the annual growth happens during spring when nutrient and light levels are optimal. Upon depletion of nutrients, usually nitrates and silicates, the diatoms decline fast in number. In some locations an autumn bloom may occur as more nutrition becomes available. Diatom resting spores may be formed as a response to unfavorable environmental conditions. These can sink to the bottom and create seed banks, and germinate when conditions are good (9).

The growth of diatoms is characterized by three phases, the lag phase, the exponential phase, and the stationary phase (see figure 3). In the lag phase, there is little increase in cell density. Physiological adaptions may cause this lag of growth. In the exponential phase the diatoms divide rapidly. When physical or chemical factors such as space or nutrients begins to run out cell division slows down. This is called the stationary phase. In this phase lipid accumulation is triggered.

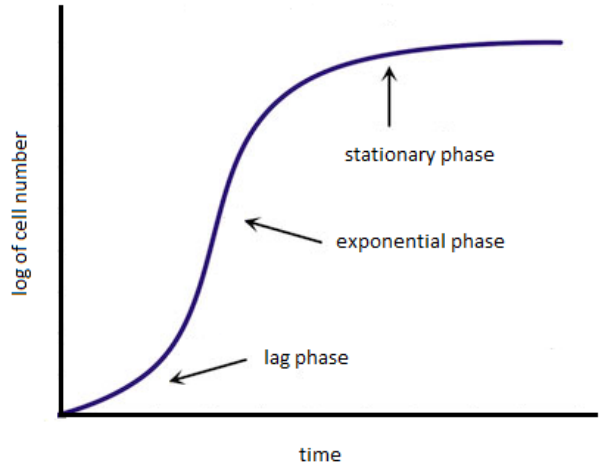


Figure 3: Growth curve of diatoms

1.3 Lipids

Most textbooks and reference works define lipids as a substance of biological origin that is readily dissolved in nonpolar solvents. This include a wide range of compounds, that can have little in common regarding structure and function. Scientists however often restrict the use of the term to fatty acids and their naturally occurring derivatives, as well as compounds closely related biosynthetically or functionally to these derivatives (10, p. 3-16).

Fatty acids are compounds consisting of a carboxylic acid group with a hydrocarbon chain. They are synthesized in nature where Malonyl-CoA in a series of reactions elongates the chain by 2 carbon atoms at a time. Hence most naturally occurring fatty acids contain an even number of carbon atoms. Fatty acids that have no carbon-carbon double bonds are called saturated fatty acids (SFA). The most commonly found saturated fatty acid are compounds with 14, 16, and 18 carbon atoms, although all homologues with carbon atoms ranging from 2 to 36 have been discovered in nature. A fatty acid is monounsaturated (MUFA) if it has one double bond within the hydrocarbon chain, and polyunsaturated (PUFA) if it has more than one double bond within the chain. The double bonds in polyunsaturated fatty acids are usually separated by a single methylene group. The chain length, degree of unsaturation and position of double bonds can easily be described by a shorthand designation; C:D(n-x), where C is the number of carbon atoms, D is the number of double bonds, and x is the position of the first double bond, counting carbons from the terminal methyl carbon (the omega side). By using this nomenclature, it is

implicit that each double bond is separated by one methylene group. E.g. Eicosapentaenoic acid (EPA) is designated 20:5 (n-3)

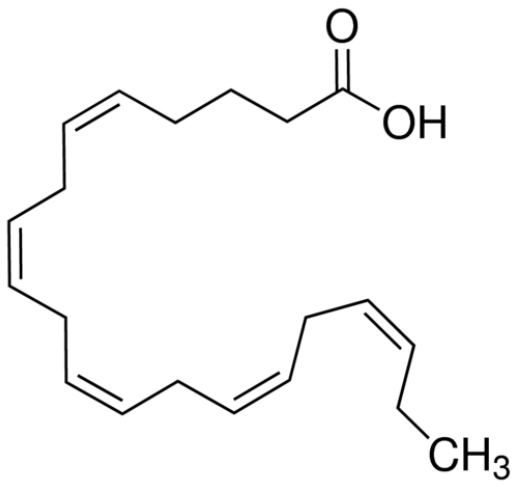


Figure 4: Eicosapentaenoic acid

Generally, fatty acids do not occur as free acids in nature, but as a part of larger lipid molecules. **Acylglycerols** consists of a glycerol group, where the hydroxyl groups are esterified to either one, two, or three fatty acids to form monoacylglycerols, diacylglycerols and triacylglycerols, respectively. The chain length varies but often contains 16, 18, and 20 carbon atoms. Acylglycerols function as energy storage and are usually found as lipid droplets in the cytoplasm. In diatoms, these lipids can amount to more than 60 % of the total lipid content (11).

Glycerophospholipids generally consists of two fatty acids connected via a glycerol phosphate molecule to one of a variety of polar groups. The fatty acids can vary in length but normally contain between 14 and 24 carbon atoms. Glycerophospholipids are the most abundant lipid in biological membranes. The polar headgroup makes them somewhat water soluble in contrast to acylglycerols.

Glyceroglycolipids are somewhat similar to glycerophospholipids, but have a carbohydrate moiety attached to the glycerol backbone instead of a phosphate group. These lipids are found in all animals, but are most abundant in photosynthetic organisms, where they are part of the thylakoid membrane (12).

1.4 The effects of PUFAs

PUFAs have several important functions in the human body. They are constituents of cell membranes where they play a role in regulating the structure, dynamics, permeability and phase transition, as well as modulating the behavior of membrane-bound proteins (13). PUFAs are precursors of other groups of molecules with important biological functions, such as eicosanoids, which are signaling molecules affecting inflammation and several other cellular functions. Mammals lack the ability to introduce double bonds in fatty acid beyond carbon 9 and 10, hence PUFAs are essential in the human diet.

Omega-3 fatty acids are among the most researched substances in modern medicine. Most research focus on the marine fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) as they seem to be the major contributors to potentially beneficial effects. Results of observational studies on healthy adults and randomized trials on patients with coronary heart disease provide moderate evidence that people with modest fish oil consumption (approximately 250mg/day EPA+DHA) are less likely to die of coronary heart disease than people who rarely eat seafood (14). There is also some evidence that EPA and DHA may relieve symptoms in patients with rheumatoid arthritis (15). DHA plays an important role in the development of the brain and eyes (16). Therefore, maternal fish consumption during pregnancy and breastfeeding may have beneficial effects to the child's neurodevelopment and cognitive outcome. Based on the current evidence it is recommended that most adults consume at least one to two meals per week of fish high in omega-3 fatty acids; women who are pregnant or breastfeeding are recommended three (14, 16).

1.5 Stability of lipids in diatoms

A major problem limiting the utilization of lipid rich biomass is the oxidative degradation of lipids. A common reaction is autoxidation where in most instances a hydrogen atom is abstracted to form a lipid radical in the presence of an initiator such as light, transition metals, oxygen reactive species (ROS), and heat (17). The radical reacts with oxygen and proceeds to react with a second lipid yielding a new radical and a lipid hydroperoxide. The chain reaction is terminated when two radicals react and combine their odd electrons to form a new covalent bond. The primary oxidation products decompose into products such as aldehydes, ketones and alcohols. The susceptibility of fatty acids to autoxidation depends on their ability to donate a

hydrogen atom to a peroxy radical. Allylic hydrogens are especially reactive because of the formation of a stable radical which is delocalized in the allylic system. Consequently, PUFAs such as EPA and DHA, are prone to autoxidation.

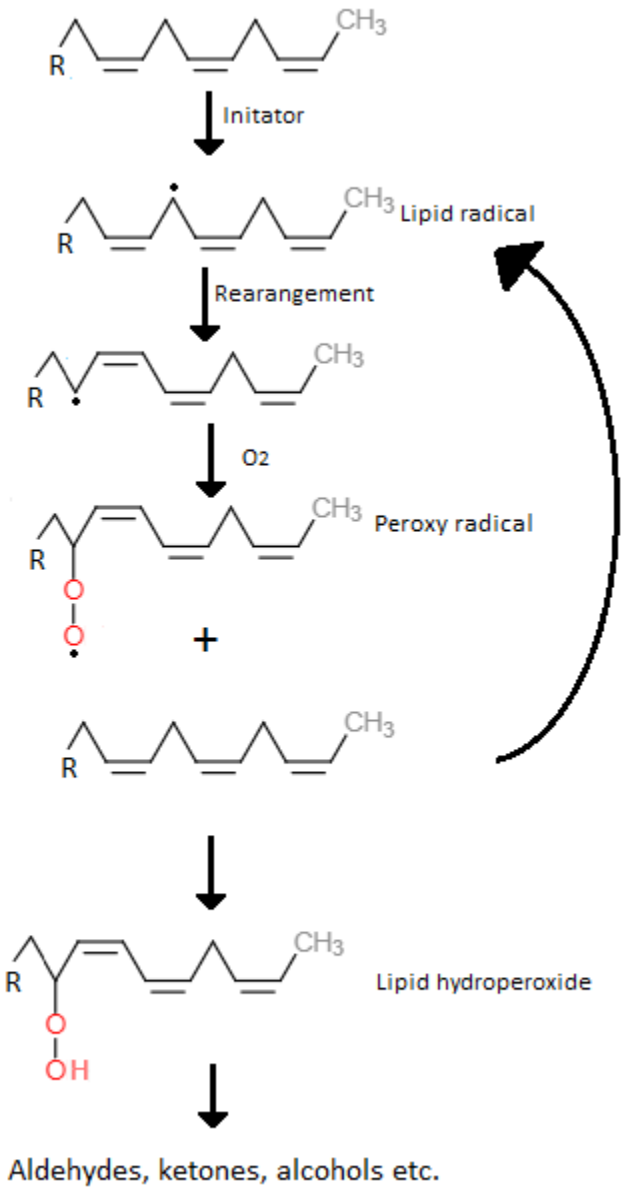


Figure 5: Autoxidation of unsaturated lipids

Lipid stability is a major concern directly related to economic value, nutrition quality and safety. The literature on the stability of omega-3 PUFAs is controversial, partly attributed to

the wide range of factors affecting the stability such as lipid composition, physical state, pro-oxidants and antioxidant content. Several microalgal species have been reported to contain substantial amounts of antioxidants (18) which may delay degradation and yield a high oxidative stability compared to other sources of PUFA-rich biomass.

1.6 Lipid extraction

In order to analyze fatty acids, it is necessary to extract the lipids from the cells and free them from non-lipid contaminants. This is a crucial step regarding quantification and several methods have been described. One of the most popular methods for extracting both neutral and polar lipids is Folch extraction (19). This method uses a system of chloroform, methanol, and water to extract and wash the lipids. The use of dichloromethane instead of chloroform has shown to give equal yields (20), and it is therefore often replaced as there are safety concerns regarding chloroform. The Folch method was developed for analyzing animal tissues. Plant cells normally have a rigid cell wall which needs to be disrupted to allow efficient extraction, otherwise the yield may be lowered. Several disrupting methods such as bead-beating, ultrasonication, and microwaves are often used. However, studies on several different species of microalgae have shown that these techniques do not produce significantly higher yields and the use of them is generally not necessary for lipid extraction on freeze dried microalgae (21, 22)

1.7 Fatty acid derivatives

Gas chromatography is usually the technique of choice when it comes to analyzing fatty acids. However, in their free form fatty acids tend to form hydrogen bond, leading to absorption in the GC column. It is usually necessary to prepare non-reactive derivatives of various kinds. Fatty acid methyl esters (FAME) are the most widely used derivate in general. They are less polar, more volatile and give better resolution in the chromatographic procedure. Although they give limited information regarding the structure of fatty acids (i.e. double bond positions), the molecular mass is often obtainable and can in combination with GC retention time data give valuable information regarding structure. The esterification process can be performed by heating the fatty acids with an excess of anhydrous methanol in the presence of an acidic catalyst (see figure 6). It is not necessary for the fatty acids to be freed from the lipid complex beforehand as most lipids can be directly transesterified (10, p. 205-209).

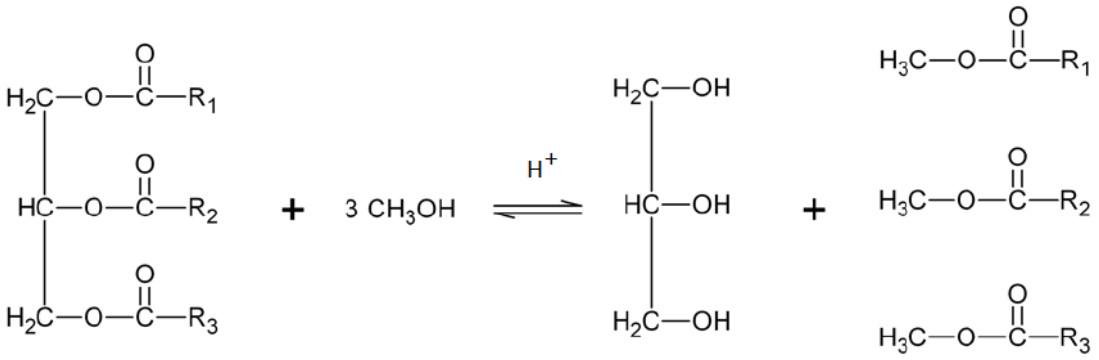


Figure 6: Acid-catalysed transesterification of a triacylglycerol to form fatty acid methyl esters

3-pyridylcarbinol esters, often referred to as picolinyl esters, are important derivatives for structural determination of fatty acids. They do not have optimal properties for GC analysis, but offer distinctive mass spectrometric fragmentation patterns, which is useful for locating double bonds. 3-pyridylcarbinol esters can be prepared from methyl esters or intact lipids by transesterification with 3-hydroxymethylpyridine (23).

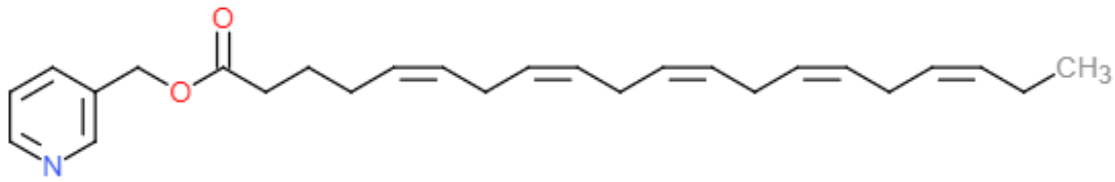


Figure 7: 3-pyridylcarbinol ester of EPA

1.8 GC-MS

GC-MS is an analytical technique that combines gas chromatography for separation and mass spectrometry for detection, identification, and quantification of substances. The principle of a GC/MS system is that volatile analytes are injected onto a heated column containing a stationary phase. A gas under pressure flows through the column and functions as a mobile phase. Separation of analytes occurs as they are carried at different speed through the column because of the difference in distribution ratios between the phases. The analyte molecules are

directly transferred from the column to the ion source, a chamber where a gas or electrons ionize them. The ions are unstable and can split in several different ways to give smaller ionized entities (fragments). The produced ions are accelerated into a magnetic or electronic field and separated according to the mass to charge ratio (m/z), before reaching the detector which measures the signal strength of the different ions. Although these are the basic principles, a GS-MS system can have several configurations.

1.8.1 Injection system

To avoid overloading of capillary columns a split/splitless injection system is often used where a syringe injects the sample to a heated chamber (injector) where the analytes and solvent evaporates. In split mode, only a portion of the sample is brought onto the column by the carrier gas; the rest is vented out. In splitless mode the total sample volume is transferred onto the column, but the analytes get trapped in the condensed solvent as the initial oven temperature is set below the boiling point of the solvent. After the sample is completely transferred onto the column the oven temperature is raised and the solvent evaporates.

1.8.2 Columns

Today most GC separations are preformed using capillary columns due to the fast and efficient separation they can offer. For capillary columns the stationary phase is a thin film bonded or coated on the inner surface of the column. This film must be temperature stable and have very low vapor pressure. Polysiloxanes and polyetylenglycols (PEG) are preferred stationary phases (see figure 8). Polydimethylsiloxane is the most hydrophobic of the polysiloxanes. Substituting the methyl groups with functional groups of varying polarity yields a number of different stationary phases. The PEG stationary phase is polar and can vary in weight. The temperature limit increases for the column as the molecular weight increases.

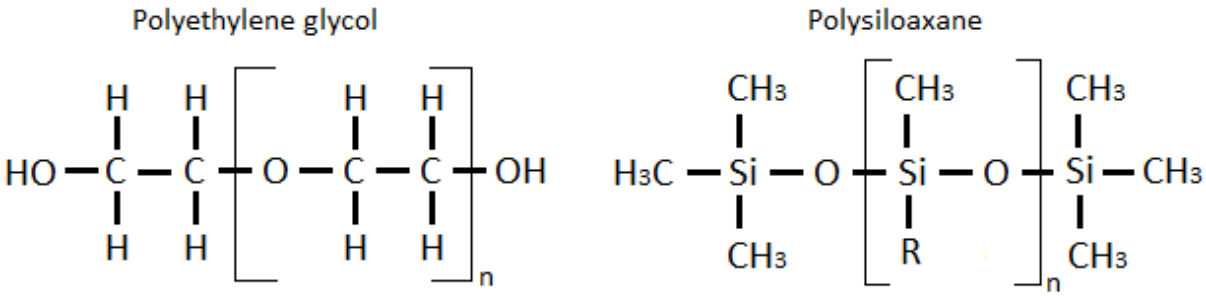


Figure 8: Basic structure of polyethylene glycols and polysiloxanes

The film thickness is typically 0.25 or 0.50 µm but can vary from 0.05 to 10 µm. The advantages of thin films are sharper peaks and reduced column bleed. Both of these benefits increase the signal to noise ratio. In addition, thin films allow analytes to elute with shorter retention and increases the columns maximum operating temperature. Thicker films increase retention time and are used in analysis of very volatile substances and in cases where the sample is highly concentrated, to minimize the risk of compounds overloading. The internal diameter (ID) of the columns is typically 0.25 mm. Sample capacity increases as the column ID increases, but at the cost of the efficiency (N), the ability to yield narrow and well-resolved peaks. Generally, if the sample has several substances with similar retention time the narrowest ID that is practical should be selected. Longer columns provide better resolution but increases retention time and back pressure. Usually a 30-meter column provides optimal balance of resolution, analysis time, and pressure.

1.8.3 Ionization

A frequently used operating principle in the ion source is electron ionization (EI). A wire filament releases electrons which are accelerated in an electric field with a potential of 70 V. As analyte molecules enter the ion source they are bombarded with these electrons; and as a result, some molecules lose an electron, leaving them a molecular ion with a positive charge. For GC-MS, the ions usually carry a single positive charge. Generally, EI gives unstable molecular ions which are likely to decompose into fragment ions. In cases where almost every molecular ion has decomposed, it is difficult to identify the analyte, since the m/z ratio of the molecular ion usually corresponds to the mass of the analyte. One approach to reduce

fragmentation is to lower the electric potential in the ion source. Another approach is to add a reagent gas, usually methane, into the ion source. This is known as chemical ionization (CI). The gas molecules are ionized by the electrons and react with the analyte to form more stable ions. Chemical ionization with methane normally form quasi-molecular ions. They are protonated molecules or ions formed from molecular ions by loss of a hydrogen atom.

1.8.4 Mass analyzer

The most common mass analyzer is the quadrupole. It consists of four parallel rods to which are applied a DC offset voltage and a radio frequency oscillating voltage. The ions travel between the rods and are deflected into a corkscrew trajectory by the oscillating electric field. For a given ratio of voltages only ions of a certain m/z ratio will reach the detector, as other ions have unstable trajectories and collide with the rods. By rapidly varying the applied voltages, the instrument can scan a wide range of m/z values. A triple quadrupole consists of three quadrupoles in which the first and third functions as mass filters and the second being a collision cell where the analyte can fragment further (see figure 9). There are four main scan modes used in tandem mass spectrometry: precursor ion scan, neutral loss scan, product ion scan and selected reaction monitoring. For the precursor ion scan the second quadrupole is set at the mass of a selected ion product, while the first quadrupole is set to scan across a mass range. This results in a spectrum that contain signal of the precursor ions that dissociate to the selected product ion. In neutral loss scan both quadrupoles are set to scan across a specific mass range, but the second quadrupole is offset by a selected neutral loss. Therefore, any molecule that loses the neutral fragment of the selected mass will be transmitted through the second quadrupole and detected. In product ion scan, the first quadrupole is set to the mass of a selected ion. This ion dissociates in the collision cell, and the resultant product ions are scanned. In selected reaction monitoring both quadrupoles are set to scan for selected ions. An ion product from the fragmentation of the selected precursor ion is then selected in the second quadrupole and detected. Utilizing a triple quadrupole can increase both sensitivity and selectivity (24, p. 236-256).

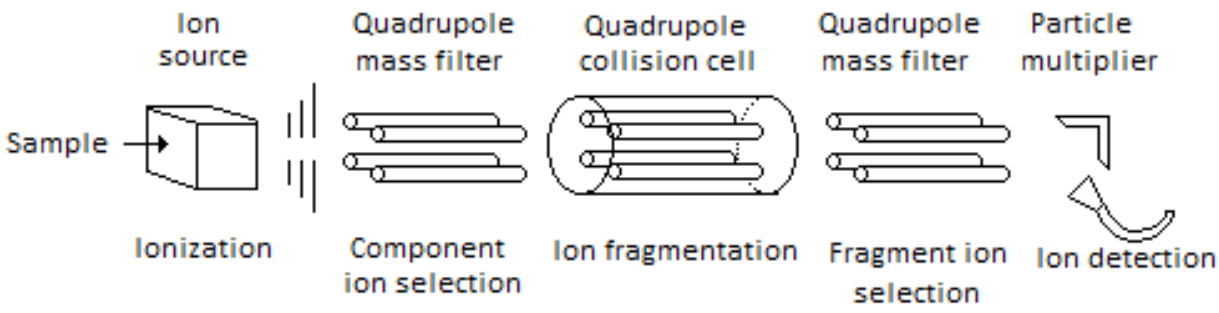


Figure 9: Schematic of a triple quadrupole mass spectrometer

1.9 Quantification

A general method for determining the concentration of an unknown sample is the use of a standard curve. The standard curve is a plot that shows how the detector response changes with the concentration of the analyte. To set up a standard curve a series of calibration samples are made at varying concentrations with a range that include the expected concentration of the analyte. Plotting the detector response of these calibration samples along the y-axis and the concentration along the x-axis usually yields a linear relationship that fit the equation model $y = ax + b$, where **a** is the slope and **b** is the intercept. The unknown analyte concentration (**x**) can be calculated from this equation. With linear regression an R^2 value called the coefficient of determination is given. This value gives information about how far the y-values are from the predicted line, and hence the uncertainty of the concertation calculated form the standard curve. An R^2 value of 1 indicates the model explains all the variability of the y-values around its mean. In cases where the calibration curve exceeds the linear detector response, the R^2 value will be low and the equation cannot be used. The slope indicates how much the signal changes for a change in concentration and hence the sensitivity. A gradual slope indicates less sensitive measurements where small changes in the detector response results in larger changes in the measured analyte concentrations. To improve the precision of quantitative analysis an internal standard can be used.

An internal standard (IS) is a substance that is added to samples and calibration standards in chemical analysis to correct for the uncontrolled loss of analyte, during sample preparation or analysis. Instead of basing the results on the absolute respond of the analyte, they are based on the ratio of responses to the analyte and the IS. In this case the results would be constant regardless of varying sample recovery, under the assumption that any losses would affect the

analyte and the IS proportionally. The use of internal standard can especially be beneficial where there are multiple sample preparation steps in which volumetric recovery may vary to decrease the accuracy of the results (25). However, several criteria must be met when choosing a suitable internal standard. The IS needs to have similar stability, retention time, extraction characteristics and detector response as the analyte, and is therefore often similar in structure. The analytical system needs to be able to measure the size of the chromatographic peaks for both the IS and analyte. For some detectors this means the IS must be chromatographically resolved. It is also important that the IS is chemically stable and do not occur naturally in the sample.

When making the calibrating solutions for a standard curve, each solution can be made separately or by serial dilutions. For serial dilutions a concentrated stock sample is made and diluted in a stepwise manner, where the next sample is made from the previous dilution. In this way only one initial solution is needed. The disadvantage of this is that any potential errors in the making of the solutions such as inaccurate volume transfer get propagated as more solutions are made. This error does not necessarily affect the R^2 value and can be hard to notice. Thus, care must be taken when making the initial solution.

2 The aim of the thesis

The main aim of this master thesis was to identify the fatty acid composition and total lipid content of *Porosira glacialis* and investigate how different storage treatments affect the stability of lipids and fatty acids. To achieve this the following sub goals were set:

- To develop GC methods for separation of FAMEs and 3-pyridylcarbinol esters of fatty acids.
- To develop MS methods for analyzing FAMEs and 3-pyridylcarbinol esters of fatty acids.
    - To test single ion monitoring (SIM) and CI for identification of FAMEs
    - To test product scan of molecular/quasi-molecular ions of FAMEs and 3-pyridylcarbinol esters of fatty acids for identification double bond position
- To assess the reliability of two GC-MS instruments by comparing test results.

3 Materials and methods

3.1 Diatom cultivation and harvesting

A Monoculture of *Porosira glacialis* originally collected in the Barents Sea (N 76° 27.54', E 033° 03.54') in May 2014 was cultivated in a 6000-litre fiberglass tank at the factory facilities at Finnfjord AS in Finnfjordbotn. The tank was supplied with seawater collected at 25 m depth in Finnfjordbotn. A polypropylene filter with 1 μm pore size (Model GX01-9 7/8, GE Power & Water, Minnetonka) was used to filter the water prior to addition. Inorganic nutrients in form of 0.25 g/L kristalon flower (Yara, Oslo, Norway) and 0.035 g/L Sodium metasilicate nonahydrate (Sigma-Aldrich Inc. St Louis, MO, USA) was added. The culture was continuously illuminated by a LED light source with an irradiance of 150-200 $mW\ m^{-2}\ nm^{-1}$ and intermittently aerated with flu gas and pressurized air, maintaining the pH at 7.4-8.1. The diatoms were harvested in the exponential growth phase when cell density reached approximately 15 000 000 cells/L. Harvesting was performed by washing 3 000 L of the culture through a 20 μm pore size plankton net (Sefar Nytal®, Heiden, Switzerland).

During the cultivation process cell density, pH, chlorophyll a and phaeophytin were measured. The density measurements were done by adding a sample of the culture in a 2 mL sedimentation chamber (Nunclon®, Roskilde, Denmark), counting the cells in a transect to calculate the cell density. Chlorophyll a and phaeophytin measurements were done using the method described by Holm-Hansen and Riemann (26), with a small modification where ethanol replaces methanol as the extraction medium.

Cultivation, harvesting and measurements were mainly performed by the technicians at The Norwegian College of Fishery Science (NFH).

3.2 Storage treatments

Application of microalgal biomass as fish feed requires information regarding storage stability. It is of economic interest to minimize the expenditures required to prevent worsening of the nutritional quality. There is limited research available regarding stability of fresh algae biomass prior to the removal of water. In order to investigate the lipid stability and find methods to preserve the nutritional content, different treatments were tested.

Benzoic acid was the first chemical preservation officially allowed in foods in the USA and is still widely used as food preservatives at low concentrations. Benzoic acid acts primarily as a mold and yeast inhibitor but is not effective against bacteria (27). Formic acid has antibacterial properties and is frequently used in animal feed as a preservative (28). Heat treatment is commonly being used during post-harvest storage of many products to inhibit microorganisms and enzymes (29).

Immediately after harvesting, the algae sludge was homogenized and aliquoted in centrifuge tubes (VWR International, USA, PA) for the following separate treatments:

FA: Formic acid (Sigma-Aldrich) was added until pH 3 was reached,

BA: 0.1 % (w/w) benzoic acid (Merck KGaA) was added.

HT: The sample was heated to 70 °C for 30 minutes.

NT: The sample was untreated.

For each treatment, 3 parallel samples were stored for 1, 3, 7 and 14 days at 4°C and 20°C. 3 samples were in addition immediately freeze-dried and prepared for analysis to serve as control samples prior to treatment.

3.3 Lipid extraction

Table 1: Chemicals used for lipid extraction and preparation of FAMEs, 3-pyridylcarbinol esters and calibration standards

Chemical	Purity	CAS No.	Supplier
3-pyridinemethanol	98%	100-55-0	Sigma-Aldrich Inc. St. Louis, MO, USA
Dichlormethane	≥ 99%	75-09-2	Merck KGaA, Darmstadt, Germany
Methanol	≥ 99.9%	67-56-1	Sigma-Aldrich Inc. St. Louis, MO, USA
n-Heptane	≥ 99.9%	142-82-5	Sigma-Aldrich Inc. St. Louis, MO, USA
Potassium tert butoxide solution, 1M inTHF		865-47-4	Sigma-Aldrich Inc. St. Louis, MO, USA
Sodium chloride		7647-14-5	Merck KGaA, Darmstadt, Germany
Sulfuric acid	96%	7664-93-9	Merck KGaA, Darmstadt, Germany

Lipid extraction was performed using Folch's method with some modifications. The biomass sample was freeze dried and crushed into powder using a glass rod. 2 ml dichloromethane-methanol (2:1) and 2 ml 5% NaCl in Milli-Q water (Merck Millipore, Burlington, MA, USA) was added to 100 mg biomass in a centrifuge tube (falcon). The tube was shaken for 30 seconds and centrifuged at 2000G for 4 minutes. The water phase was discarded and the organic liquid phase (bottom phase) containing the lipids was transferred to a pre-weighed dram glass vial. The solid residue was re-extracted using the same procedure. The recovered organic phase was pooled in the same dram glass vial from the first extraction. The solvents were evaporated under a nitrogen stream and the mass of the lipids was determined gravimetrically.

3.4 Internal standard

19-Methylarachidic acid and Isopalmitic acid was chosen as internal standards as they were well resolved and had a reasonable retention time in the GC-MS analysis. The standards were weighed, dissolved in heptane, and stored at -20 °C in airtight containers. Prior to lipid transesterification and esterification of calibration standards, stock solutions containing 100μg/ml of each internal standard were made.

Table 2: Internal standards

IUPAC name	Purity	CAS No.	Supplier
14-methylpentadecanoic acid	≥ 98%	4669-02-7	Sigma-Aldrich Inc. St. Louis, MO, USA
19-methyleicosanoic acid	~98%	59708-73-5	Sigma-Aldrich Inc. St. Louis, MO, USA

3.5 Transesterification of lipids to fatty acid methyl esters

The dry extracted lipids were dissolved in a certain amount of dichlormethane-methanol (2:1 v:v) to give a concentration of 10 mg/ml. 100 μL of the dissolved extract, 100 μL internal standard stock solution, 800 μL dichloromethane and 2 mL 10% H_2SO_4 in methanol was added to a duran glass tube with a screw cap. The glass tube was closed and incubated at 100 °C in an oven for 1 hour. After cooling to room temperature 3 ml 5% NaCl in Milli-Q water and 3 ml

heptane was added, and the sample was shaken for 20-30 seconds. The organic phase (upper phase) containing the FAMEs was transferred to a GC vial and evaporated under a nitrogen stream. The FAMEs were redissolved in 0.5 ml heptane and the GC vial was sealed and stored at -80 °C until analysis on GC-MS.

3.6 Transesterification of fatty acid methyl esters to 3-pyridylcarbinol esters

Already analyzed FAME samples were transferred to duran glass tubes, evaporated under a nitrogen stream, and redissolved in 1ml dichloromethane. Potassium tert-butoxide in THF (1M) was mixed with 3-hydroxymethylpyridine in the proportion 1:2 (v/v) in a dram glass vial to form a derivatization reagent. 0.25 ml derivatization reagent was added to the duran glass tube before it was closed and incubated at 45 °C in a block heater for 45 minutes. After cooling to room temperature 1 ml Milli-Q water and 2 ml heptane was added and the sample was vortexed for some seconds. The organic phase (upper phase) containing the 3-pyridylcarbinol esters was transferred to another duran glass tube and added 1 ml 5% NaCl in Milli-Q water. The sample was vortexed for some seconds and the organic phase was then transferred to a GC vial and evaporated under a nitrogen stream. The 3-pyridylcarbinol esters were redissolved in 0.1 ml heptane and the GC vial was sealed and stored at -80 °C until analysis on GC-MS.

3.7 Preparation of standard curves

18 different dissolved fatty acids/FAMEs (purchased from Larodan AB, Solna, Sweeden) were mixed in a volumetric flask to give each a concentration of 1mg/mL, using heptane:DCM (1:1 v/v) as solvent (stock1). 100µl of this stock was transferred to another 1 mL volumetric flask and filled with DCM, giving each fatty acid/FAME a concentration of 100 µg/mL (stock 2). Due to a high abundance of EPA in the algae, a 3ml solution containing 1 mg/ml EPA in DCM was made as well (stock 3).

Table 3: Stock solution fatty acids and FAMEs

Lipid number	IUPAC name	Purity	CAS No.
14:0	Tetradecanoic acid	>99%	544-63-8
16:0	Hexadecanoic acid	>99%	57-10-3
16:1	(9Z)-Hexadecenoic acid	>99%	373-49-9
16:2	(9Z,12Z)-Hexadecadienoic acid	>98%	5070-03-01
16:3	(7Z,10Z,13Z)-Hexadecatrienoic acid	>98%	7591-64-0
16:4 ME	Methyl (6Z,9Z,12Z,15Z)-Hexadecatetraenoate	>98%	94035-78-6
18:0	Octadecanoic acid	>99%	57-11-4
18:1 ME	Methyl (9Z)-Octadecenoate	>99%	112-62-9
18:2	(9Z,12Z)-Octadecadienoic acid	>99%	60-33-3
18:3	(9Z,12Z,15Z)-Octadecatrienoic acid	>99%	463-40-1
18:4	(6Z,9Z,12Z,15Z)-Octadecatetraenoic acid	>97%	20290-75-9
20:0	Icosanoic acid	>99%	506-30-9
20:4	(5Z,8Z,11Z,14Z)-Icosatetraenoic acid	>99%	506-32-1
20:5	(5Z,8Z,11Z,14Z,17Z)-Icosapentaenoic acid	>99%	10417-94-4
22:0	Docosanoic acid	>99%	112-85-6
22:6	(4Z,7Z,10Z,13Z,16Z,19Z)-Docosahexaenoic acid	>99%	6217-54-5
24:0	Tetracosanoic acid	>99%	557-59-5
24:1	15(Z)-Tetracosenoic acid	>99%	506-37-6

The calibration standards were made up of 7 different concentrations ranging from 10 µg/mL to 1000 µg/mL. For EPA, 2500 µg/mL and 5000 µg/mL calibration standards were made as well. Table 12 (see appendix) shows all the calibration solutions that were made and the volumes that were added prior to esterification. 3 parallels were made for each concentration. Esterification into methyl esters and preparation for GC-MS was done using the same procedure as described for extracted lipids. The calibration standards were stored at -20 °C until they were analyzed on GC-MS.

3.8 GC-MS analysis

The GC-MS analyses were performed on a Quattro micro GC (Waters co. Milford, MA, USA) (integrated GC [Agilent 6890] and autosampler [Agilent 7683]), equipped with a TG-FAMEwax column (30m, 0.25mm ID, 0.25µm film thickness, Thermo Fisher Scientific, Waltham, MA, USA). Injection port temperature was kept at 350 °C and the injection (1µL) was set to splitless mode. Helium 5.0 (AGA, Oslo, Norway) was used as carrier gas with a

constant flow of 1.4 mL/min. An electron ionization source set to 70 eV was used, with a source temperature of 200 °C. Heptane:methanol:DCM(1:1:1 v:v:v) and heptane were used as washing solvents for the autosampler syringe. The autosampler was set to 5 pre-washes and 5 washes for both solvents and 3 sample washes.

3.8.1 FAMEs

For FAMEs the initial oven temperature was set to 70 °C for 2 minutes, thereafter a ramp at 2 °C/min to 226 °C and a final ramp at 6 °C/min to 250°C which was held for 2 minutes. The MS was operating in full scan mode, recording m/z 45.5-400. The calibration standards were injected 3 times each and in ascending order (by concentration). Blank samples were injected between the runs of the parallel series, to ensure carry over was not taking place. Each parallel of the algae samples was injected 2 times. Blank samples were injected between every algae sample.

Quantification was performed using the analyte area ratio relative to that of the internal standard closest in retention time. (Isopalmitic acid was applied as IS to 18:0, 18:1 and the fatty acid with shorter chain length, while 19-Methylarachidic acid applied to 18:2. 18:3, 18:4 and the fatty acids with longer chain length). Fatty acids that were identified in the algae but missing from the standard solutions were quantified using the standard curve for the fatty acid isomer of the same carbon chain length.

Due to difficulties locating the molecular ion for some of the peaks in the FAME spectra, different MS operation principles were tested. The electron energy potential in the ion source was lowered to 60, 50, 40, and 30eV. One standard FAME sample was run for each of the lowered voltages to investigate changes in molecular ion intensity.

In addition, a selected ion monitoring (SIM) method was used to identify selected fatty acid. Chemical ionization was applied using methane (AGA, Oslo, Norway) as reagent gas at 70% flowrate. The mass of the quasi-molecular ions of the FAMEs were determined by running a standard FAME sample in full scan mode. The quasi-molecular ions of each FAME were recorded around the retention time of the corresponding peaks in algae samples. For the unidentified peaks, the anticipated quasi-molecular ions were recorded.

3.8.2 3-pyridylcarbinol esters

For 3-pyridylcarbinol esters the initial oven temperature was set to 60 °C for 2 minutes, thereafter a ramp at 10 °C/min to 150 °C and a final ramp at 5 °C/min to 250 °C which was held for 50 minutes. The MS was operating in full scan mode, recording m/z 80-500. One parallel of each algae sample was injected. Pre-concentrating of the samples were done prior to analysis, due to difficulties locating the peaks and interpreting the spectra of the scarce fatty acid derivatives. For the same reason a ZB-FAME column (30m, 0.25mm ID, 0.25µm film thickness, Phenomenex, Torrance, CA, USA) was installed and tested as well. For this column initial oven temperature was set to 70 °C for 2 minutes, followed by a ramp at 5 °C/min to 190 °C and a final ramp at 2 °C/min to 280 °C.

3.8.3 Product ion scan

The daughter ions of the molecular- /quasi-molecular ions of a FAME and 3-pyridylcarbinol ester standard were recorded using collision-induced dissociation with argon 5.0 (AGA, Oslo, Norway) in combination with EI and CI. The samples were run on several collision energies (15V, 30V, 40V, 50V). Otherwise the settings were as described above.

3.9 Interlaboratory comparison

The prepared algae FAME samples and calibration standards were also analyzed by NFH at Norut Northern Research institute. This analysis was performed on Quattro Premier GC (Waters) equipped with FAMEWAX column (30m, 0.25mm ID, 0.25µm film thickness, Restek, Bellefonte, PA, USA). To assess the reliability of the instruments, the results obtained by NFH at Norut were compared to the results obtained by the instrument at the Department of Pharmacy (IFA).

3.10 Interpretation of 3-pyridylcarbinol esters mass spectra

The approach used to determine the positions of the double bonds was to identify the molecular ion and progress downwards the fatty acid chain. Loss of a terminal methyl group would result in a fragment ion 15 amu lighter than the molecular ion. Fragment ions 14 amu apart corresponds to cleavage of methylene groups. Upon reaching a double bond there is a 26 amu

gap (see figure 10). This gap can sometimes be difficult to identify, but a 40 amu gap, corresponding to a double bond and the adjacent methylene group can readily be seen. Whether this methylene group is distal or proximal to the double bond must however be verified by other diagnostic features. A useful feature is the abundant doublet of fragment ions 14 amu apart on the distal side of the double bound (closer to the terminal methyl end). A series of ions 40 amu apart is indicative of methylene interrupted double bonds. This feature is especially useful in cases where at least one of the double bonds clearly can be identified.

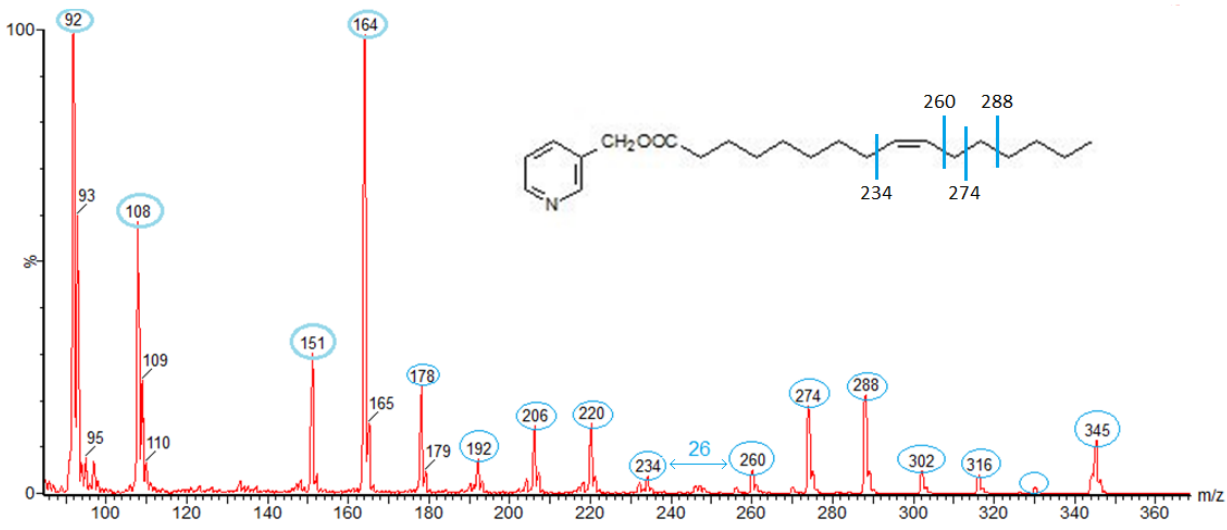


Figure 10: 3-Pyridylcarbinol 9-hexadecenoate. The double bond is located by the gap of 26 amu between the ions at 234 and 260 m/z, with the abundant doublet of ions at m/z 274 and 288 providing supporting evidence. The abundant ions at 92, 108, 151, and 164 m/z are characteristic for 3-Pyridylcarbinol esters and are all fragments close to the pyridine ring. Ions below 92 m/z can usually be ignored.

Double bonds close to either ends of the carbon chain are not easily identifiable, as rearrangement is likely to arise, and the distinctive features are not always present. Reference spectra available from The LipidWeb (30) was used to identify the fatty acids in such cases. Although interpreting mass spectra of 3-pyridylcarbinyl esters of polyenoic fatty acids with several double bonds is more difficult, the spectra are rather distinctive and serve as fingerprints for the fatty acids. Therefore, in cases where not every double bond was seen, the reference spectra were used to determine whether the double bonds were conjugated, methylene-, bis-methylene-, or poly-methylene-interrupted.

4 Results and discussion

4.1 Total lipid content

The results from the total lipid analysis is shown in figure 11 and 12 (see appendix for SD). Lipid content for the untreated control (day 0) was 8.4%.

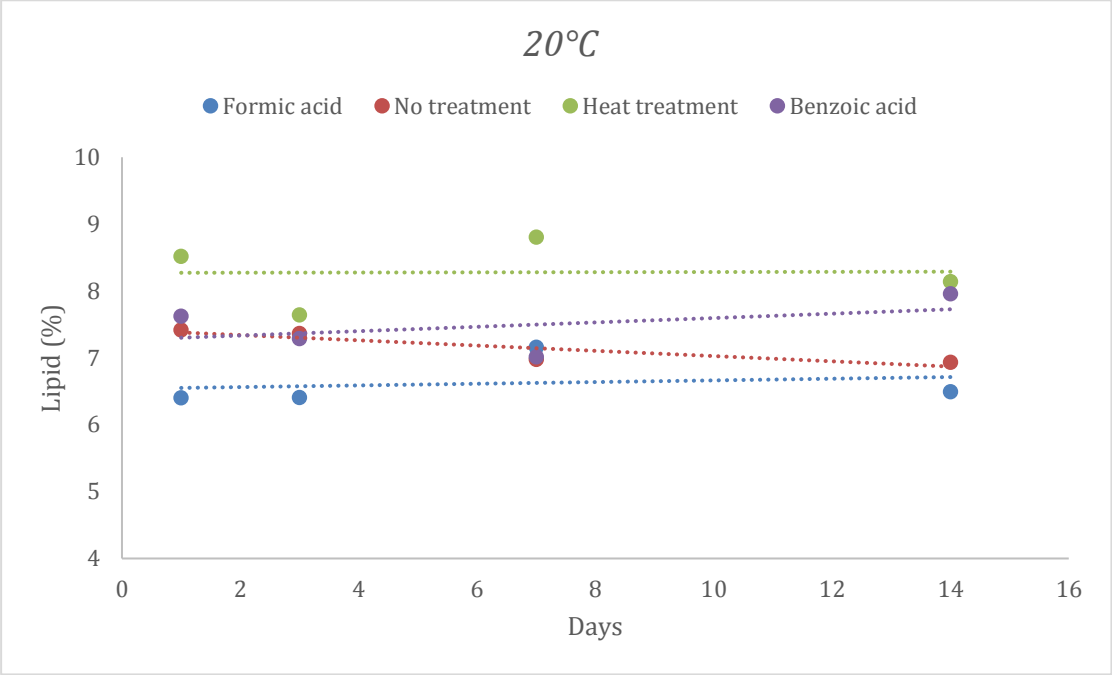


Figure 11: Total lipid content for the different algal samples stored at 20 °C given in percentage of the algal sample dry weight.

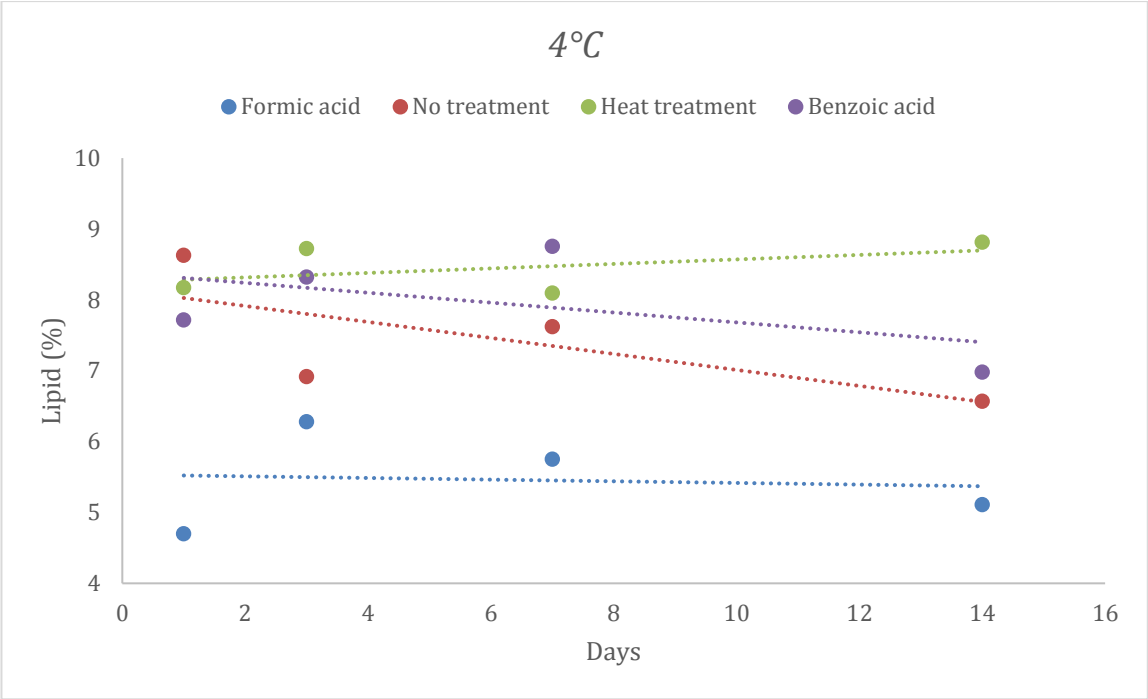


Figure 12: Total lipid content for the different algal samples stored at 4 °C given in percentage of the algal sample dry weight.

The results are hard to interpret as the trend in lipid content varied from day to day. This indicate a significant variance in the extraction yields and questions the validity and of the results. It did not seem that the different storage treatments remarkably affected the extracted amounts of lipids. In general, storage temperature did not seem to be an important parameter for the extracted amounts as no clear trend were seen. E.g. the lipid content in the untreated sample stored for 1 day at 4 °C was higher than at the one stored 20 °C, while the opposite was true for the formic acid treated samples stored for 1 day.

However, heat treatment seems to be the most promising treatments of the ones tested, as the extracted yields were consistently higher than for the other treatments. The results indicate that during 14 days, both time and temperature did not seem to influence the extracted amount of lipids remarkably for heat treated *P. Glacialis*. The lipid content was high throughout the storage time; close to that of the control sample (8,4% ± 0.23). This can possibly be explained by inactivation or reduction of lipases, enzymes that catalyzes the hydrolysis of lipids which results in the formation of free fatty acids. The water content of the stored biomass is high which makes the lipids more prone to enzymatic hydrolysis (31) The remaining glycerol backbones with or without carbohydrate and phosphate moieties, have high polarity and are not

extracted by the modified Folch method that was used; hence active lipases would likely result in a decline in lipid content over time which was the case for the untreated samples but not for the heat-treated samples.

Formation of free fatty acids is troublesome because they are more prone to oxidation and downgrade the nutritional quality. High concentrations of free fatty acids in blood is associated with the development of type 2 diabetes, hypertension, atherosclerosis and obesity (32).

Plunging small samples of plants into boiling water as pre-treatment has previously been suggested for de-activating enzymes and prolonging shelf life. In the diatom species *Skeletonema costatum* boiling water reduced production of free fatty acids thus confirming the presence of active lipases (33). Another study showed that spray drying of the diatom *Phaeodactylum tricornutum* inactivated the lipases but on the expense of decomposition of naturally occurring antioxidants, possibly due to thermal composition (34). Although heat treatment may yield a high short-term stability, it does not necessarily influence long-term stability in the same manner. Further investigations on long time storage of heat treated biomass is required to validate the effectiveness of this treatment.

Although oxidation of lipids yields volatile compounds such as aldehydes and ketones, the extracted lipids may include oxidation products, and the amount of free fatty acids is unknown. Furthermore, the different storage treatments may influence the extractability. Thus, measurements of free fatty acids, naturally occurring antioxidants and peroxide values would be required to evaluate the lipid stability in the extracted samples. This is especially true due to the varying lipid content measured.

A variety of environmental factors can affect the lipid stability and thus the validity of the results. An example of this would be if the water content varies between the sample centrifuge tubes, influencing the enzymatic activity and microbial growth. Diatoms can under stress conditions activate enzymes and change their lipid content as well as composition. Depletion of EPA and other PUFAs in some diatoms have been observed within minutes after cell disruption (35). Hence, proper handling of the diatoms after harvesting is extremely important to obtain valid results.

There are numerous methods for lipid content analysis. The traditional gravimetrical methods are time consuming and require volumetric and gravimetric precision to obtain good results. Only small changes in the proportions of the solvents used as extraction medium may lead to

selective losses of lipids. The more recent method, time-domain nuclear magnetic resonance (TD-NMR), has shown to be applicable for determining lipid content in microalgae with high accuracy and reproducibility (36). TD-NMR is fast and non-invasive, and may in combination with other methods support evidence in determining the lipid content of diatoms.

4.2 Fatty acid identification

20 different fatty acids were found in each of the different treated *P. Glacialis* samples (see table 4). For the two 18:3 isomers found, the double bond positions could not be identified.

Table 4: Fatty acids found in the P. Glacialis samples

Fatty acids:
14:0
16:0
16:1 (n-7)
16:1 (n-5)
16:2 (n-7)
16:2 (n-4)
16:3 (n-4)
16:4 (n-1)
18:0
18:1 (n-7)
18:2 (n-6)
18:3
18:3
18:4 (n-3)
20:0
20:5 (n-3)
22:0
22:6 (n-3)
24:0
24:1 (n-11)

4.2.1 Analysis of 3-pyridylcarbinol esters

The spectra obtained from the analysis of 3-pyridylcarbinol esters using EI were of great value, despite the fact that several peaks were partly overlapping and barely visible in the chromatograms (see figure 13). Some peaks for the late eluting derivatives were more than a

minute broad. This causes loss of MS sensitivity and selectivity, and hence influence the mass spectra obtained.

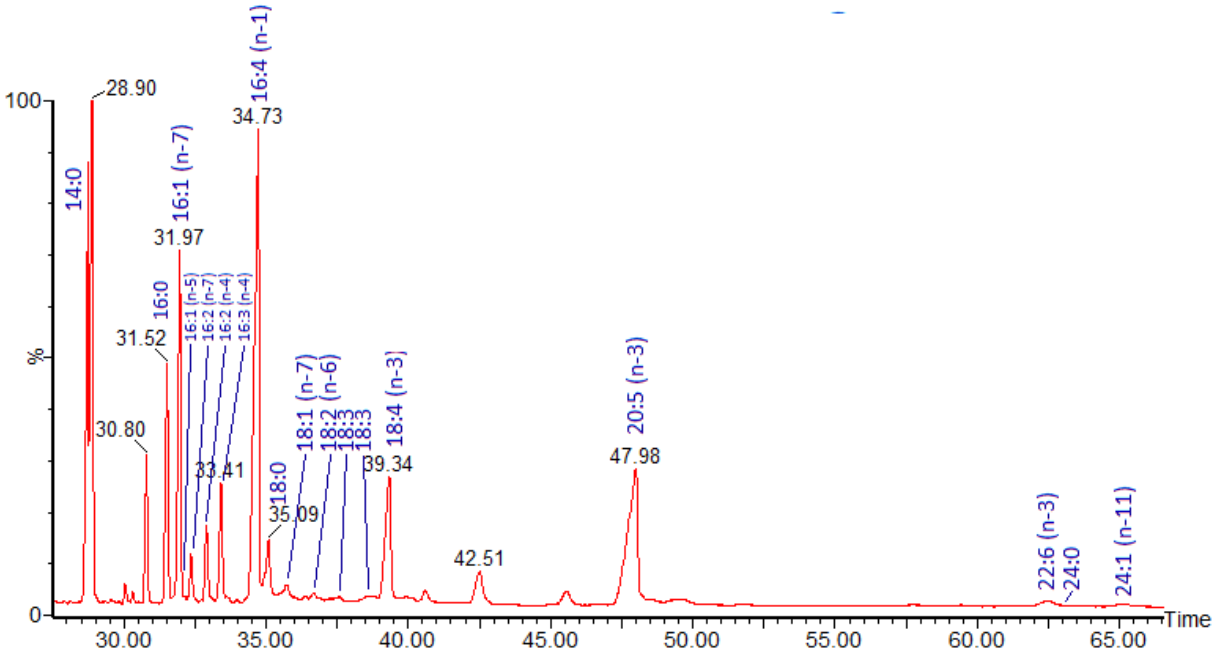


Figure 13: 3-pyridylcarbinol ester chromatogram of algae sample (TG-FAMEwax column).

However, identification seemed to be possible as long as the concentration was adequate, and the analytes did not co-elute with relative high abundance of contaminants. This seemed to be the problem for both 18:3 isomers where the signal was low and disturbed by contaminants. The molecular ions and some characteristic fragment ions were visible, but no clear diagnostic ions for determining double bond positions could be distinguished. Long oven temperature programs and pre-concentrating of the analytes failed to improve selectivity and resolution for the 18:3 fatty acids to be identified. Pre-concentrating did however help in pinpointing the scarce 3-pyridylcarbinol esters in the chromatograms. The ZB-FAME column yielded narrower peaks but did not seem to improve the selectivity adequately for both 18:3 isomers as the mass spectra obtained still contained fragments of unknown components. The stationary phase of the ZB-FAME column consists of highly polar cyanopropyl which yielded great separation within a chain-length group, but consequently affected the elution order relative to fatty acids derivatives of other chain lengths. For the medium polar TG-FAMEwax column a more predictable elution order was obtained where all fatty acids derivatives of a given chain length eluted before those of longer chain length. The results do however suggest that the fatty acids

that were found can be adequately resolved for identification purposes in the form of 3-pyridylcarbinol esters on both columns. Figure 14-17 shows the spectra obtained by the TG-FAMEwax column of the most abundant 3-pyridylcarbinol esters when EI at 70eV was applied. Characteristic fragments are marked with a circle. The other spectra obtained are interpreted in the same way and added to the appendix (see figure 29-42).

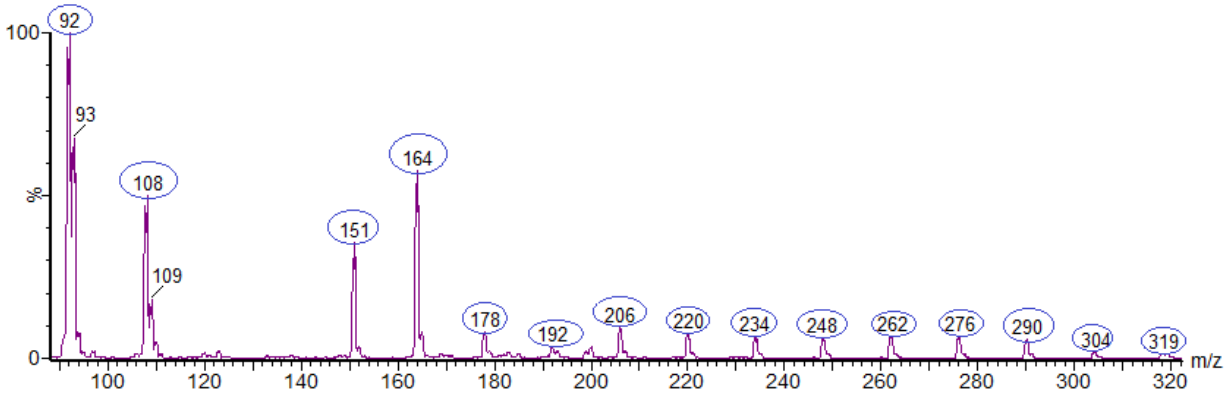


Figure 14: 14:0. The molecular ion is visible at m/z 319. The fragment ion at m/z 304 corresponds to cleavage of the terminal methyl group. The 14 amu gaps indicate cleavage of methylene groups and that no double bonds are present.

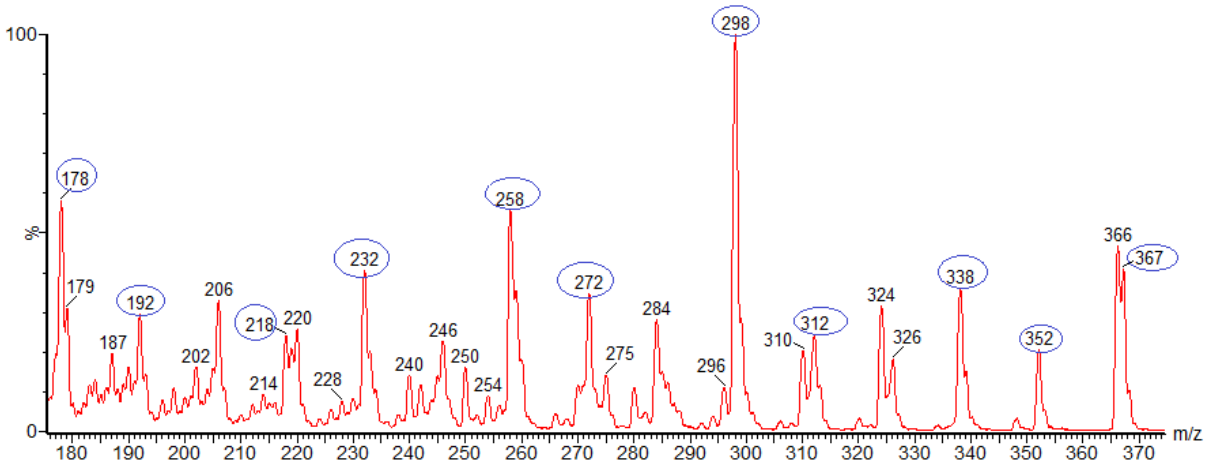


Figure 15: 18:4 (n-3): From the molecular ion at m/z 367 there is a loss of a methyl group to m/z 352 followed by the loss of a methylene group to m/z 338. The double bonds can be located by the 26 amu gaps between m/z 338 and 212; m/z 298 and 272; m/z 258 and 232; and m/z 218 and 192. The two double bonds closest to the methyl end were not easily located as other 26 amu gaps are seen (e.g. between m/z 324 and 298). Reference spectra from the literature was used to be sure of the exact position of the double bonds.

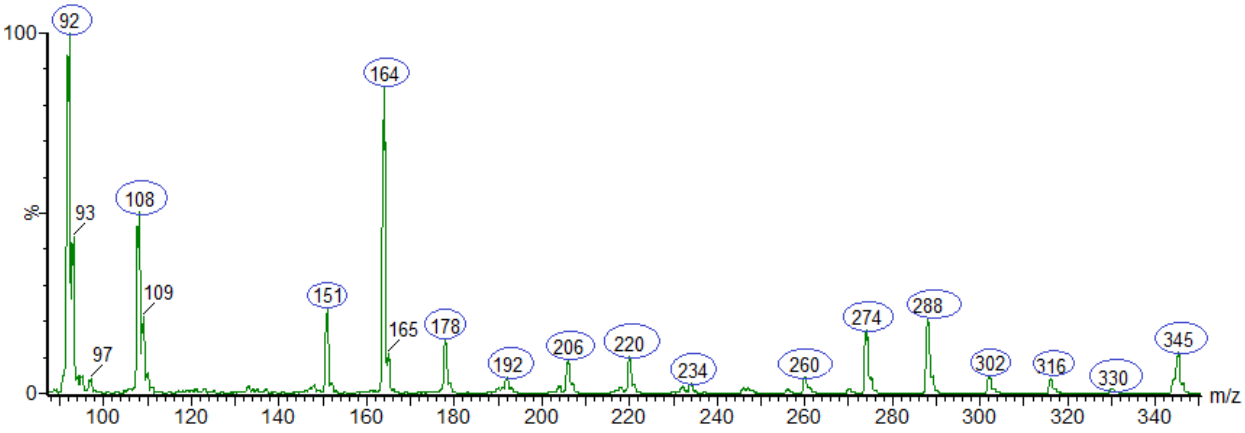


Figure 16: 16:1 (n-7). From the molecular ion at m/z 345 there is loss of a methyl group to m/z 345 followed by a series of ions 14 amu apart corresponding to successive loss of methylene groups. The double bond can be located by the 26 amu gap between m/z 234 and 360. The abundant doublet at m/z 274 and 288 provide evidence of this position.

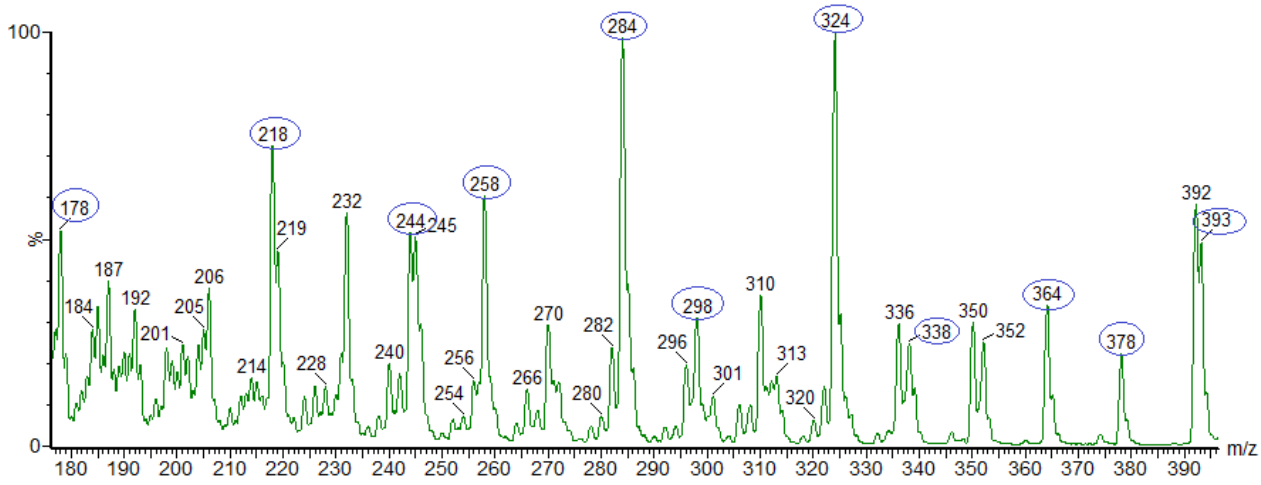


Figure 17: 20:5 (n-3). From the molecular ion at m/z 393 there is a loss of a methyl group to m/z 378 followed by the loss of a methylene group to m/z 364. The double bonds can be located by the 26 amu gaps between m/z 364 and 338; m/z 324 and 298; m/z 284 and 258; m/z 244 and 218; and the 40 amu gap between m/z 218 and 178. Since the 40 amu gap is prominent it is indicative that the double bond is methylene interrupted. If this double bond was bis-methylene interrupted other fragments would have been prominent. Reference spectra from the literature was used for confirmation.

Collision induced dissociation of quasi-molecular ions of 3-pyridylcarbinol esters did not give characteristic fragmentation patterns (see figure 18). Increased collision energy resulted in more fragmentation, but most daughter ions had a low m/z ratio and no diagnostic value. Collision induced dissociation of molecular ions at 15 V did give characteristic fragmentation with the 26 amu gaps corresponding to cleavage at the double bonds for some of the fatty acid derivatives. However, it did not seem to be consistent as the spectrum of 3-pyridylcarbinyl (9Z,12Z,15Z)-Octadecatrienoate (18:3 (n-3)) from the standard sample did not match with the EI reference spectrum from the literature (see figure 19).

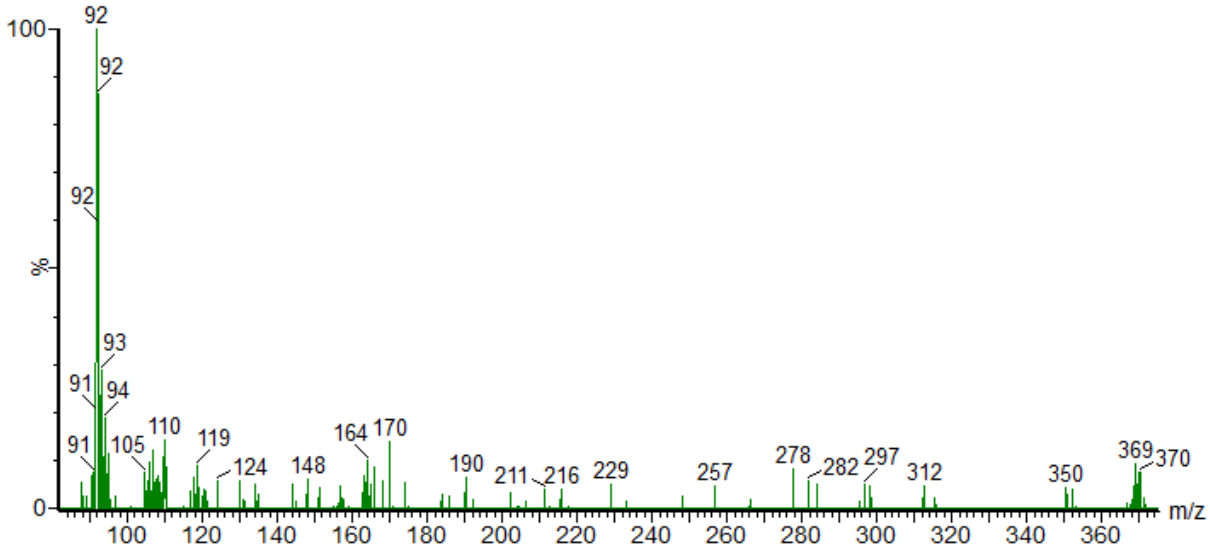


Figure 18: Spectrum of 18:3 (n-3) from a calibration standard (1000 µg/ml). Obtained by CI and CID, 30V.

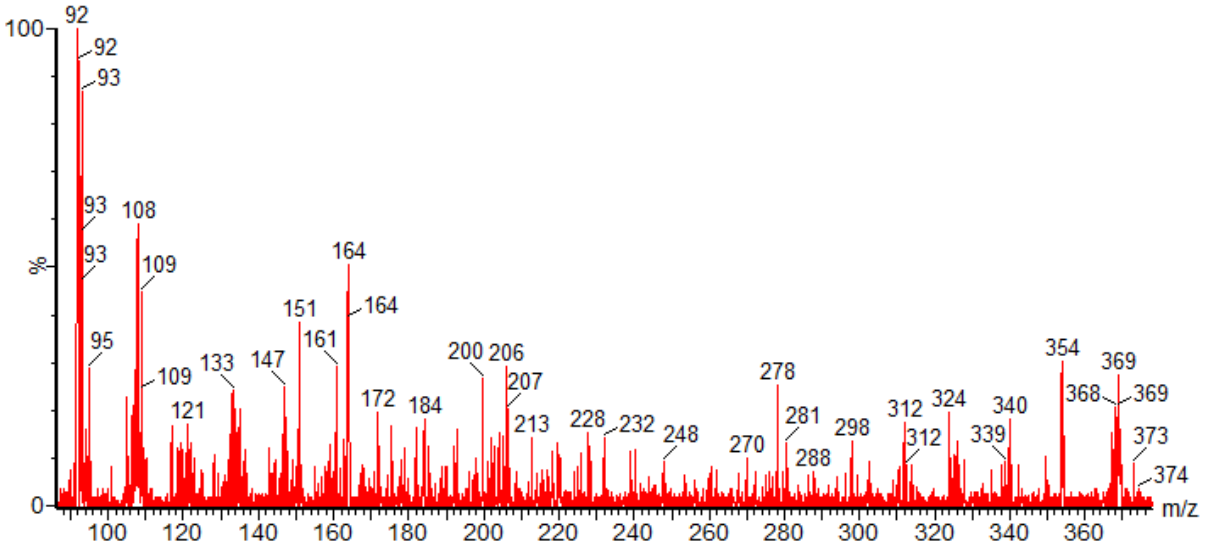


Figure 19: Spectrum of 18:3 (n-3) from a calibration standard (1000 µg/ml). Obtained by EI and CID, 15V. The ions corresponding to cleavage of the terminal methyl group and the adjacent methylene group is seen at m/z 354 and 340. However, the characteristic gaps of 40 amu between m/z 340, 300, 260 and 200 is not seen.

4.2.2 Analysis of FAMEs

The molecular ion was not obtainable from all the FAME spectra, which made it challenging to be certain of the corresponding 3-pyridylcarbinol ester. Reducing the electron energy potential in the ion source did not increase the abundance of molecular ion for any of the voltages tested. However the use of CI did result in a sufficient amount of quasi-molecular ions and was, in combination with SIM, helpful in distinguishing the FAME peaks from other components. The SIM chromatograms indicated that 20:0 and 22:0 were present in the algae sample at low concentrations (see figure 20). The corresponding peaks were not found in the 3-pyridylcarbinol esters spectra, and barely visible in the FAME full scan chromatogram (figure 21), but matching retention times and FAME spectra with the standards gave supporting evidence of their presence. However, 20:0 and 22:0 was not quantified and accounted for in the fatty acid composition determination (see paragraph 3.3) due to the their barely visible peaks.

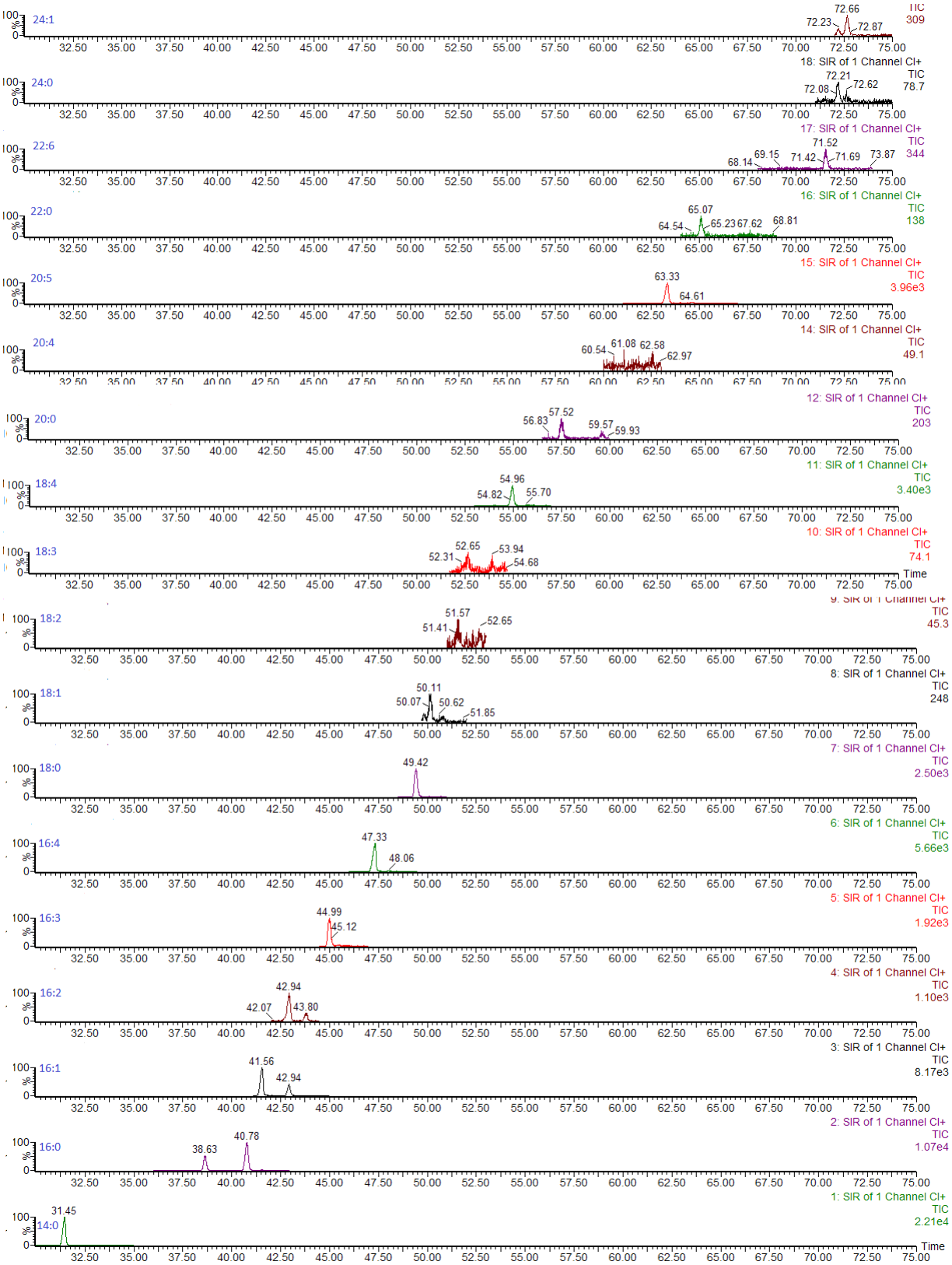


Figure 20: FAME chromatograms obtained by SIM in combination with CI for a heat-treated algae sample (stored 1 day at 4 °C). Note that the first peak in the 16:0 scan is IS 1.

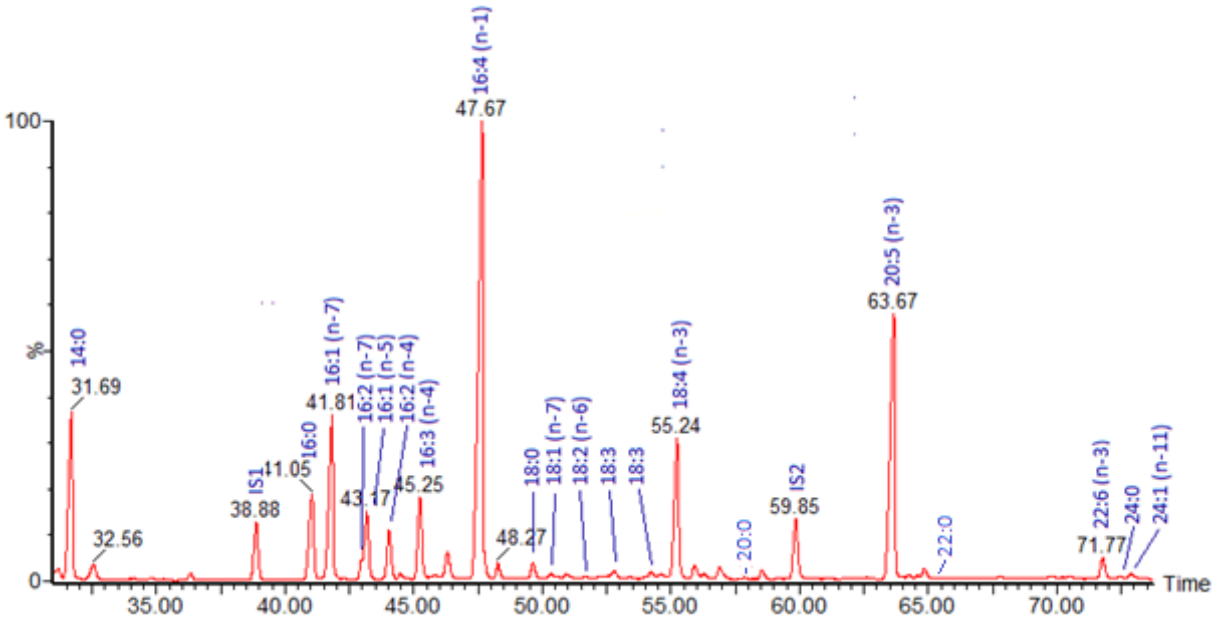


Figure 21: FAME chromatogram from full scan of a heat-treated algae sample (stored 1 day at 4 °C).

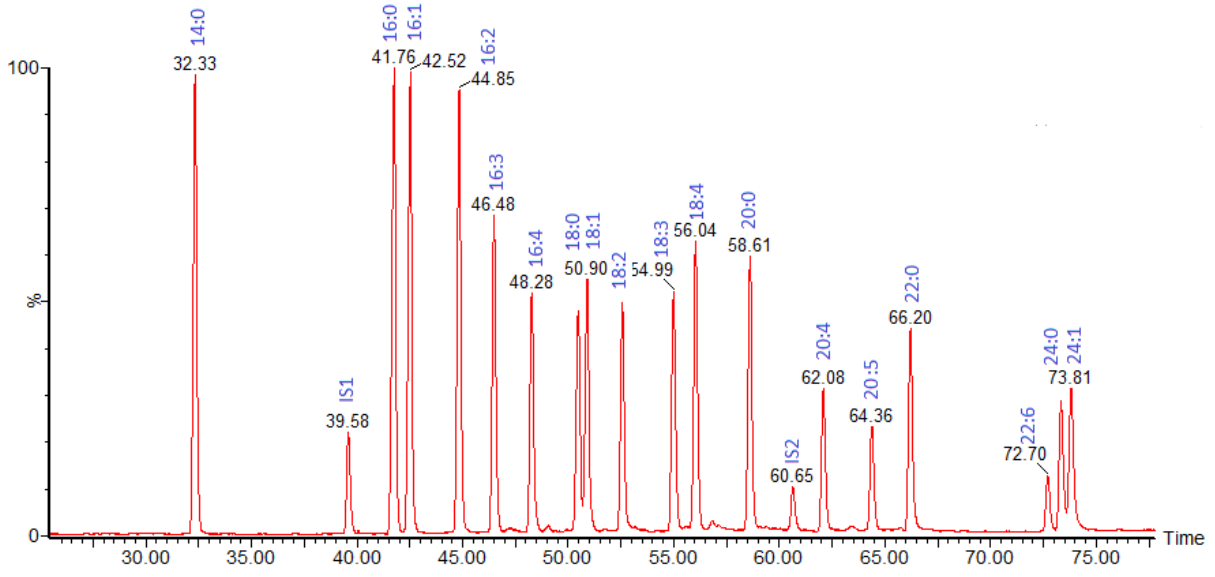


Figure 22: FAME chromatogram from full scan of a calibration standard (500 µg/ml)

As mentioned, FAME spectra offer little help in terms of locating double bonds when EI is applied. The FAME spectra of the 16:1 isomers were virtually identical. Even though small differences related to structural aspects usually can be seen in spectra of di- or polyunsaturated fatty acid, the 16:2 isomers spectra were too similar to use in comparison with reference spectra from the literature (see figure 23)

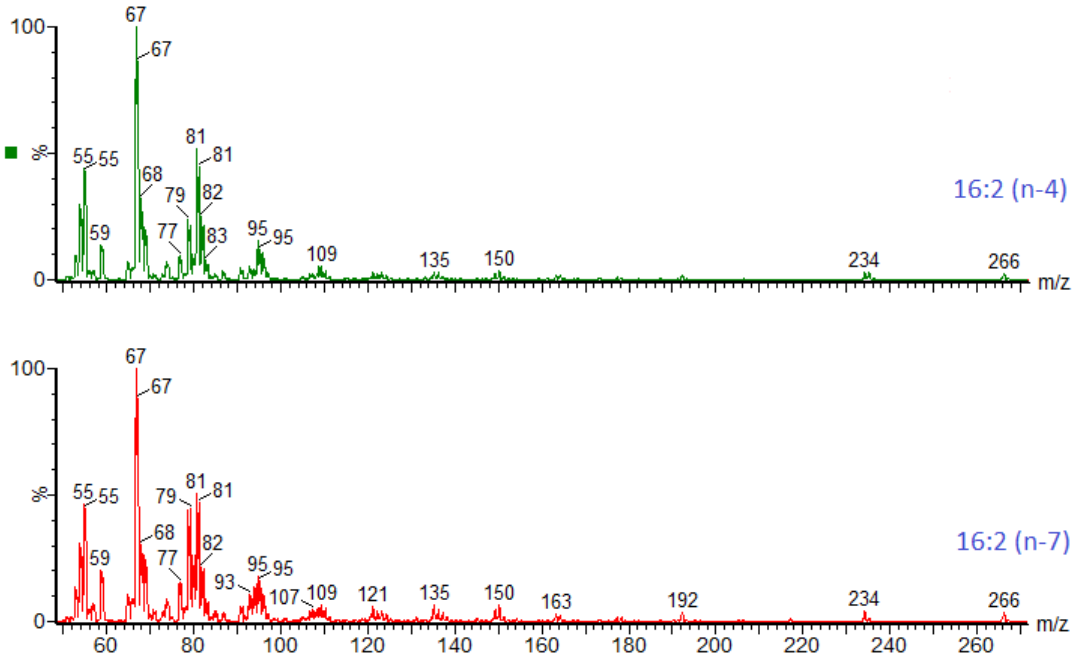


Figure 23: Spectrum of 16:2 (n-4) and 16:2 (n-7) from a heat-treated algae sample (stored 1 day at 4 °C). Obtained by EI, 70 eV.

The spectra obtained from collision induced dissociation of molecular and quasi-molecular ions of standard FAMEs was hard to interpret and did not seem to yield fragments indicative of double bond positions (se figure 24 and 25).

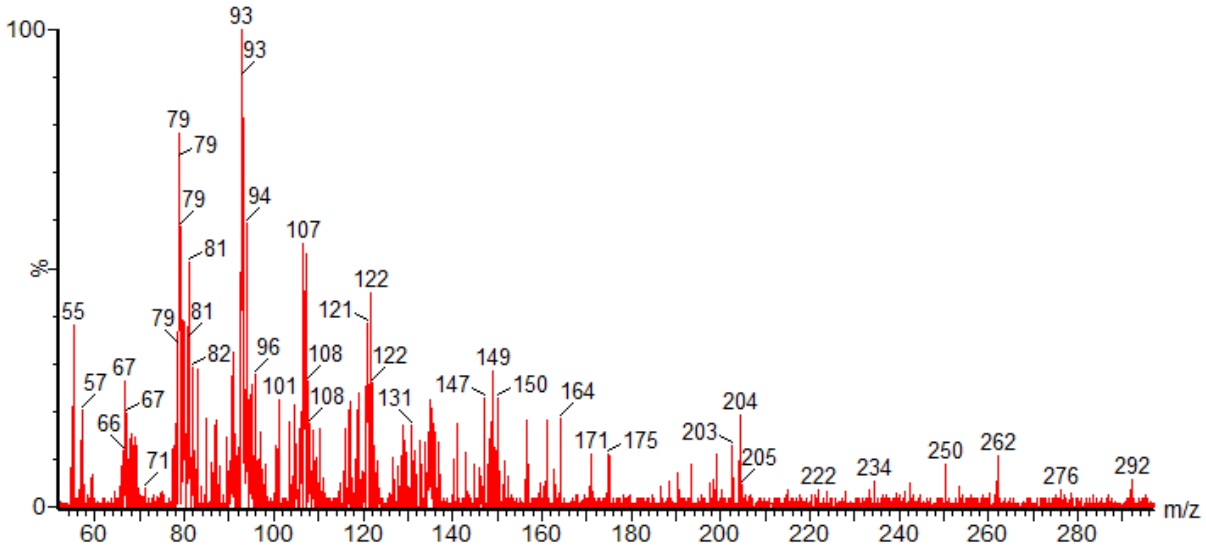


Figure 24: Spectrum of 18:3 (n-3) from a calibration standard (1000 µg/ml). Obtained by EI and CID, 15V.

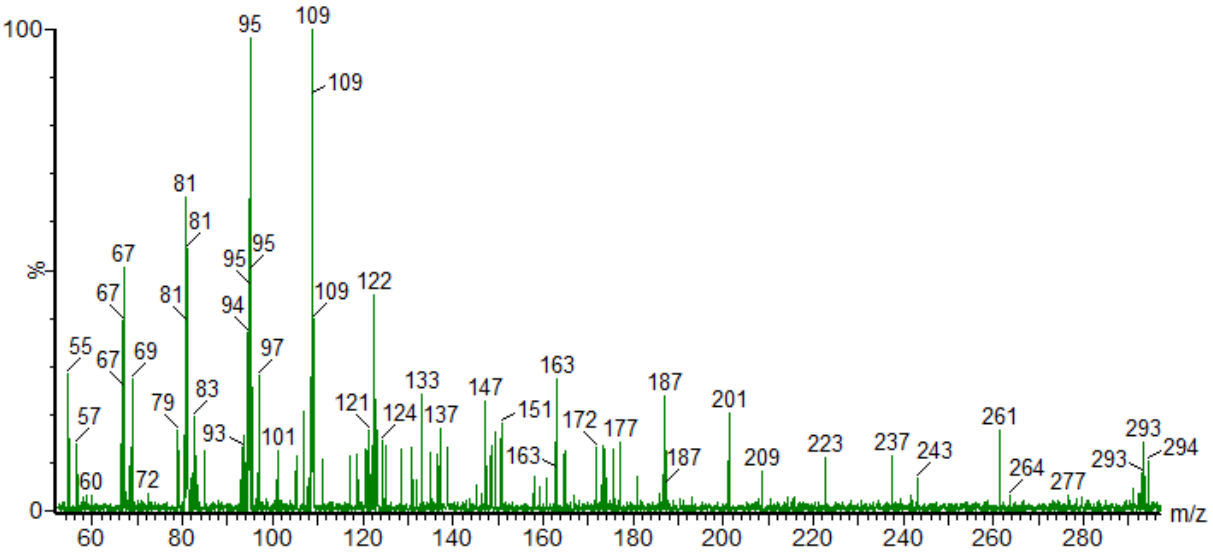


Figure 25: Spectrum of 18:3 (n-3) from a calibration standard (1000 µg/ml). Obtained by CI and CID, 15V.

Designating the FAME isomers with the double bonds positions obtained from the 3-pyridylcarbinol ester spectra was based on the retention time data. The 16:1 and 16:2 isomers eluted in the same order relative to each other as both derivatives. Furthermore, the known standards (16:1 n-7 and 16:2 n-4) which was the same found in the algae, matched in retention time. As some of the fatty acids were different isomers from those found in the algae, a pattern

was found where the isomer with double bonds closer to the carboxyl end (higher omega number) eluted first. Applying this information with the retention time data obtained idicates that the 18:3 isomer with shortest retention time has double bonds located higher up the chain than the omega-3 standard; while the last isomer might be omega-3 as the retention time were similar to the standard.

3-pyridylcarbinol ester are arguably the best derivatives available for identification of fatty acids. Preparing them are however time-consuming and the requirement of different chromatographc conditions compared to FAMEs is inconvenient. An alternative method for unambiguous identification of double bonds is the use of covalent adduct chemical ionization (CACI) tandem MS directly on FAMEs, where acetonitrile is used as reagent gas. The acetonitrile derived reagent ion reacts rapidly with FAME double bonds to form an ion 54 amu above the mass of the parent FAME. Subsequent fragmentation of this ion yields a small number of characteristic ions that are indicative of the double bond position.

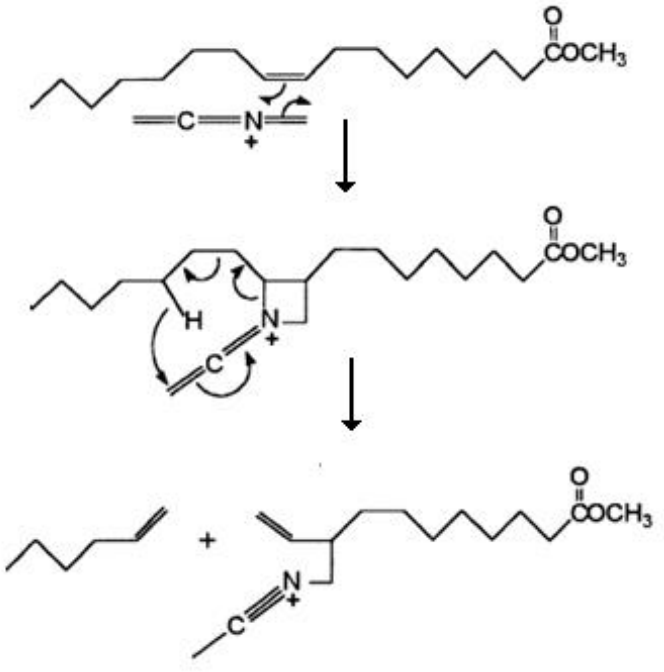


Figure 26: Reaction of a reagent ion with a FAME and subsequent discosiation to form a charachteristic fragment ion.

CACI tandem MS has shown to yield highly characteristic spectra for FAMEs with up to 6 double bonds, including several non-methylene interrupted FAMEs (37, 38).

4.3 Fatty acid composition

Fatty acid composition and how each fatty acid is influenced by different storage treatments after 1 and 14 days is shown in tables 5-9.

*Table 5: Saturated fatty acids given in percentage of total fatty acids, with standard deviation. * denotes significant difference between 1 and 14 days of storage (p-value < 0.05, Independent samples t-test)*

	14:0	16:0	18:0	24:0
Benzoic acid				
20 °C, 1 day	5.5 ± 0.06	3.8 ± 0.08	1.22 ± 0.11	0.34 ± 0.02
20 °C, 14 days	6.34 ± 0.58*	4.45 ± 0.27*	1.33 ± 0.08	0.36 ± 0.02
4 °C, 1 day	4.71 ± 0.31	3.76 ± 0.16	1.29 ± 0.19	0.34 ± 0.02
4 °C, 14 days	5.63 ± 0.36*	4.29 ± 0.16*	1.49 ± 0.12*	0.35 ± 0.02
Formic acid				
20 °C, 1 day	4.1 ± 0.16	3.68 ± 0.31	1.91 ± 0.24	0.34 ± 0.05
20 °C, 14 days	4.36 ± 0.49	3.28 ± 0.17*	1.58 ± 0.07*	0.32 ± 0.01
4 °C, 1 day	3.45 ± 0.17	3.4 ± 0.19	1.7 ± 0.09	0.34 ± 0.01
4 °C, 14 days	8.05 ± 0.90*	7.16 ± 0.54*	3.43 ± 1.08*	0.51 ± 0.06*
Heat Trt.				
20 °C, 1 day	5.73 ± 0.27	3.79 ± 0.28	1.33 ± 0.19	0.35 ± 0.02
20 °C, 14 days	4.49 ± 0.38*	4.21 ± 0.26*	1.32 ± 0.14	0.31 ± 0.04*
4 °C, 1 day	6.34 ± 0.21	4.1 ± 0.31	1.32 ± 0.17	0.32 ± 0.01
4 °C, 14 days	6.08 ± 0.34	4.1 ± 0.33	1.44 ± 0.29	0.37 ± 0.01*
No Trt.				
20 °C, 1 day	5.44 ± 0.18	3.96 ± 0.26	1.46 ± 0.06	0.36 ± 0.01
20 °C, 14 days	7.05 ± 0.86*	5.29 ± 0.94*	1.71 ± 0.33	0.37 ± 0.02
4 °C, 1 day	4.63 ± 0.13	3.56 ± 0.12	1.21 ± 0.11	0.39 ± 0.01
4 °C, 14 days	5.35 ± 0.41*	4.38 ± 0.24*	1.64 ± 0.21*	0.35 ± 0.02*

*Table 6: Monounsaturated fatty acids given in percentage of total fatty acids, with standard deviation. * denotes significant difference between 1 and 14 days of storage (p-value < 0.05, Independent samples t-test)*

	16:1 (n-7)	16:1 (n-5)	18:1 (n-7)	24:1 (n-11)
Benzoic acid				
20 °C, 1 day	6.29 ± 0.11	2.47 ± 0.03	0.67 ± 0.03	0.57 ± 0.05
20 °C, 14 days	7.09 ± 0.51*	2.71 ± 0.21*	0.9 ± 0.09*	0.56 ± 0.05
4 °C, 1 day	6.01 ± 0.24	2.21 ± 0.13	0.63 ± 0.02	0.58 ± 0.05
4 °C, 14 days	6.87 ± 0.17*	2.59 ± 0.06*	0.76 ± 0.05*	0.58 ± 0.03
Formic acid				
20 °C, 1 day	5.62 ± 0.44	1.92 ± 0.20	0.66 ± 0.02	0.44 ± 0.10
20 °C, 14 days	5.95 ± 0.36	1.62 ± 0.16*	0.63 ± 0.02*	0.40 ± 0.03
4 °C, 1 day	5.33 ± 0.34	1.63 ± 0.06	0.63 ± 0.03	0.40 ± 0.03
4 °C, 14 days	10.87 ± 0.67*	2.75 ± 0.18*	0.88 ± 0.08*	0.87 ± 0.08*
Heat Trt.				
20 °C, 1 day	6.17 ± 0.23	2.46 ± 0.11	0.67 ± 0.03	0.56 ± 0.02
20 °C, 14 days	6.55 ± 0.29*	2.71 ± 0.09*	0.68 ± 0.02	0.46 ± 0.03*
4 °C, 1 day	6.61 ± 0.24	2.61 ± 0.06	0.63 ± 0.02	0.55 ± 0.02
4 °C, 14 days	6.45 ± 0.30	2.58 ± 0.15	0.71 ± 0.03*	0.56 ± 0.02
No Trt.				
20 °C, 1 day	6.10 ± 0.22	2.49 ± 0.10	0.75 ± 0.01	0.58 ± 0.02
20 °C, 14 days	7.86 ± 1.12*	3.03 ± 0.19*	0.93 ± 0.18	0.61 ± 0.05
4 °C, 1 day	5.76 ± 0.14	2.26 ± 0.14	0.63 ± 0.02	0.65 ± 0.05
4 °C, 14 days	6.86 ± 0.29*	2.61 ± 0.11*	0.81 ± 0.03*	0.55 ± 0.05*

*Table 7: Polyunsaturated fatty acids given in percentage of total fatty acids, with standard deviation. * denotes significant difference between 1 and 14 days of storage (p-value < 0.05, Independent samples t-test)*

	16:2 (n-7)	16:2 (n-4)	16:3 (n-4)	16:4 (n-1)	18:2 (n-6)
Benzoic acid					
20 °C, 1 day	1.37 ± 0.05	2.20 ± 0.02	4.41 ± 0.08	30.39 ± 0.94	0.42 ± 0.05
20 °C, 14 days	1.50 ± 0.10*	2.37 ± 0.07	4.44 ± 0.13	29.83 ± 0.72	0.47 ± 0.04*
4 °C, 1 day	1.39 ± 0.10	2.21 ± 0.09	3.77 ± 1.41	30.71 ± 0.92	0.45 ± 0.05
4 °C, 14 days	1.46 ± 0.05*	2.42 ± 0.05*	4.62 ± 0.09	31.19 ± 0.95	0.46 ± 0.03
Formic acid					
20 °C, 1 day	1.34 ± 0.20	2.28 ± 0.13	4.84 ± 0.18	32.12 ± 1.74	0.47 ± 0.12
20 °C, 14 days	1.15 ± 0.10*	2.06 ± 0.09*	4.36 ± 0.08*	28.84 ± 0.22*	0.43 ± 0.02
4 °C, 1 day	1.17 ± 0.07	2.00 ± 0.07	4.69 ± 0.15	34.18 ± 0.48	0.43 ± 0.05
4 °C, 14 days	2.01 ± 0.07*	3.06 ± 0.09*	4.96 ± 0.13*	26.02 ± 0.76*	0.92 ± 0.07*
Heat Trt.					
20 °C, 1 day	1.41 ± 0.03	2.31 ± 0.05	4.5 ± 0.14	31.51 ± 0.87	0.45 ± 0.02
20 °C, 14 days	1.46 ± 0.04*	2.34 ± 0.04	4.63 ± 0.06	31.30 ± 0.45	0.44 ± 0.02
4 °C, 1 day	1.6 ± 0.53	2.34 ± 0.02	4.52 ± 0.03	31.51 ± 0.88	0.46 ± 0.01
4 °C, 14 days	1.17 ± 0.31	2.38 ± 0.05	4.59 ± 0.15	31.87 ± 0.89	0.48 ± 0.01*
No Trt.					
20 °C, 1 day	1.39 ± 0.06	2.22 ± 0.05	4.43 ± 0.05	30.01 ± 0.78	0.43 ± 0.03
20 °C, 14 days	1.51 ± 0.04*	2.39 ± 0.07*	4.65 ± 0.23	27.9 ± 2.93	0.48 ± 0.03*
4 °C, 1 day	1.44 ± 0.08	2.23 ± 0.06	4.38 ± 0.07	29.05 ± 1.07	0.50 ± 0.07
4 °C, 14 days	1.39 ± 0.07	2.42 ± 0.16*	4.74 ± 0.21*	31.97 ± 0.67*	0.40 ± 0.03*

*Table 8: Polyunsaturated fatty acids given in percentage of total fatty acids, with standard deviation. * denotes significant difference between 1 and 14 days of storage (p-value < 0.05, Independent samples t-test)*

	18:3	18:3	18:4 (n-3)	20:5 (n-3)	22:6 (n-3)
Benzoic acid					
20 °C, 1 day	0.74 ± 0.05	0.57 ± 0.07	4.43 ± 0.17	31.11 ± 0.64	3.50 ± 0.09
20 °C, 14 days	0.68 ± 0.16	0.71 ± 0.07*	4.28 ± 0.12	28.61 ± 1.66*	3.38 ± 0.13
4 °C, 1 day	0.77 ± 0.07	0.68 ± 0.13	4.49 ± 0.20	32.58 ± 1.11	3.43 ± 0.11
4 °C, 14 days	0.72 ± 0.04	0.84 ± 0.07*	4.14 ± 0.23*	28.47 ± 1.22*	3.13 ± 0.11*
Formic acid					
20 °C, 1 day	0.81 ± 0.14	0.89 ± 0.21	4.46 ± 0.33	31.00 ± 1.38	3.11 ± 0.10
20 °C, 14 days	0.82 ± 0.03	0.71 ± 0.12*	4.36 ± 0.03	36.07 ± 1.55*	3.05 ± 0.05
4 °C, 1 day	0.83 ± 0.07	0.58 ± 0.12	4.58 ± 0.09	31.68 ± 1.38	2.98 ± 0.21
4 °C, 14 days	1.18 ± 0.07*	1.00 ± 0.07*	4.31 ± 0.15*	19.71 ± 0.20*	2.32 ± 0.07*
Heat Trt.					
20 °C, 1 day	0.74 ± 0.02	0.71 ± 0.12	4.3 ± 0.19	29.65 ± 1.71	3.38 ± 0.12
20 °C, 14 days	0.70 ± 0.03*	0.69 ± 0.10	3.96 ± 0.07*	30.32 ± 1.25	3.42 ± 0.14
4 °C, 1 day	0.75 ± 0.03	0.59 ± 0.01	4.26 ± 0.14	28.39 ± 1.02	3.08 ± 0.14
4 °C, 14 days	0.77 ± 0.005	0.69 ± 0.18	4.24 ± 0.17	28.29 ± 1.92	3.21 ± 0.15
No Trt.					
20 °C, 1 day	0.75 ± 0.05	0.64 ± 0.09	4.38 ± 0.14	30.95 ± 0.61	3.67 ± 0.08
20 °C, 14 days	0.71 ± 0.04	0.78 ± 0.04*	4.01 ± 0.14*	27.19 ± 0.42*	3.51 ± 0.14*
4 °C, 1 day	0.82 ± 0.07	0.76 ± 0.06	4.69 ± 0.23	33.27 ± 0.75	3.77 ± 0.07
4 °C, 14 days	0.66 ± 0.02*	0.76 ± 0.03	3.95 ± 0.18*	27.98 ± 1.76*	3.17 ± 0.15*

Table 9: Fatty acid composition by class, given in percentage of total fatty acids.

	SFA	MUFA	PUFA
Benzoic acid			
20 °C, 1 day	10.86	10.00	79.14
20 °C, 14 days	12.46	11.26	76.27
4 °C, 1 day	10.10	9.43	80.47
4 °C, 14 days	11.76	10.79	77.45
Formic acid			
20 °C, 1 day	10.02	8.65	81.32
20 °C, 14 days	9.55	8.60	81.85
4 °C, 1 day	8.89	7.99	83.12
4 °C, 14 days	19.14	15.37	65.49
Heat Trt.			
20 °C, 1 day	11.19	9.86	78.95
20 °C, 14 days	10.33	10.39	79.28
4 °C, 1 day	12.07	10.41	77.52
4 °C, 14 days	11.99	10.30	77.70
No Trt.			
20 °C, 1 day	11.21	9.91	78.88
20 °C, 14 days	14.42	12.44	73.14
4 °C, 1 day	10.49	9.24	80.28
4 °C, 14 days	11.72	10.83	77.45

The results show that the fatty acid profile in *P. glacialis* is highly unsaturated with roughly 80% PUFAs. The PUFAs essentially consist of 16:4 (n-1) and EPA (20:5 n-3), while 16:1 (n-7) is the most abundant MUFA. The content of EPA did not decrease for the heat-treated samples at both storage temperatures. For the samples that did not receive treatment and the benzoic acid treated sample, a moderate decrease in EPA was seen. This decrease was statistical significant (p-value < 0.05) for both storage temperatures. The results from the formic acid treatment are conflicting and difficult to interpret. Storage at 4 °C resulted in considerable drop of EPA from 31.68% to 19.71%, while storage at 20 °C resulted in an increase from 31.00% to 36.07%. These results are probably caused by an error during sample preparation or analysis as storage temperature did not seem to influence the composition of the other fatty acids to a similar extent. DHA is of low abundance in *P. glacialis* and did not change significantly for the heat-treated samples while a slight decrease was seen for the other treatments and for the

untreated samples. The general increase in SFAs content for the benzoic treated and untreated samples is intelligible as the content of the more prone PUFAs decreased.

The results provide evidence to the effectiveness of heat treatments as the relative amount of PUFAs and omega-3 fatty acids did not decrease during storage. The results also suggest that treatment with benzoic acid might be ineffective, as the decrease in PUFAs and EPA was similar to that of no treatment.

The high content of omega-3 fatty acids makes *P. glacialis* an attractive candidate for fish feed production. The aquaculture is struggling with fish oil deficiency. 90% of the ingredients in salmon fish feed were of marine origin in 1990. Today, biomass from terrestrial plants, has replaced most of the marine ingredients. As a consequence, the EPA and DHA content in the salmon has decreased significantly (39). Soybean and rapeseed oil are major ingredients in terrestrial fish feed. Rapeseed oil typically contains around 60 % oleic acid (18:1 n-9), 20% linoleic acid (18:2 n-6) and 10% alpha linoleic acid (ALA, 18:3 n-3), while soybean oil typically contains around 20% oleic acid, 50% linoleic acid and less than 10% ALA (40). The high omega-6 to omega-3 fatty acid ratio in these oils raises concern both for fish health and for the beneficial health effects of fish for the consumer. The replacement of fish oils with vegetable oils in fish feed can affect fish health in terms of suppressing the immune system and alter the stress response (41). A high omega-6 to omega-3 ratio, as found in today's western diets, is associated with the development of many diseases including cardiovascular and autoimmune diseases (42). The low amount of omega-6 fatty acid (<1%) found in *P. glacialis* yields a favorable ratio that would promote the content of healthier fatty acid in farmed fish and overall help in balancing the omega-6 to amoega-3 ratio in human diets.

Lipids amount to about 30% of the total ingredients in salmon fish feed, hence *P. glacialis* harvested in the exponential growth phase may be more applicable as an ingredient in fish feed rather than whole feed. Due to the shortage of fish oil on the market it is undesirable to use more omega-3 fatty acids than necessary to maintain good fish health and quality. A study showed that fully replacing fish oil with *Schizochytrium sp.* microalgae in the feed of juvenile Nile tilapia did not change the fish survival rate but yielded higher weight gain, protein efficiency ratio, feed intake, and DHA fillet content (43). Similar results were found for small salmon where partially or fully replacing fish oil with algae meal resulted in increased feed intake, higher omega-3 content in fillets, and no negative health effects (44).

4.3.1 Interlaboratory comparison

To assess the reliability of the instruments to yield precise measurements, data from the quantification of a selection of the fatty acids were compared. The fatty acids selected had different chain length, retention time, detector response and concentration. This was done to evaluate the instruments ability to obtain precise results for a variety of fatty acids. The results are shown in table 10.

*Table 10: Fatty acid concentration in dissolved lipid (µg/mL) with standard deviation determined by different GC-MS instruments. * denotes significant difference in concentration obtained by the two different instruments (p-value < 0.05, Independent samples t-test)*

	16:1 (n-7)		18:0		20:5 (n-3)	
	IFA	*Norut*	*IFA*	*Norut*	*IFA*	*Norut*
Benzoic acid						
20 °C, 1 day	287 ± 42	330 ± 59	55.6 ± 8.4	58.9 ± 10.5	1419 ± 158	1671 ± 271
20 °C, 14	285 ± 13	338 ± 12*	53.4 ± 5.8	57 ± 7.6	1151 ± 106	1485 ± 92*
4 °C, 1 day	250 ± 33	282 ± 39	53.5 ± 14.5	59.4 ± 16	1353 ± 233	1465 ± 224
4 °C, 14 days	284 ± 30	333 ± 38*	61.8 ± 9.8	65 ± 8.9	1176 ± 66	1380 ± 136*
Formic acid						
20 °C, 1 day	220 ± 40	247 ± 49	74.5 ± 18.7	81 ± 19.2	1211 ± 306	1173 ± 344
20 °C, 14	272 ± 7	308 ± 8*	72.4 ± 7.1	77.1 ± 9	1650 ± 161	1476 ± 66*
4 °C, 1 day	238 ± 17	258 ± 36	76 ± 10.5	82.4 ± 16.2	1415 ± 205	1358 ± 154
4 °C, 14 days	229 ± 34	245 ± 33	72.3 ± 17.1	76.4 ± 23.8	416 ± 42	475 ± 100
Heat Trt.						
20 °C, 1 day	249 ± 18	287 ± 20*	53.6 ± 9.5	58.1 ± 7.4	1198 ± 31	1426 ± 138*
20 °C, 14	269 ± 18	307 ± 17*	54.4 ± 4.7	59.7 ± 4.5*	1247 ± 92	1545 ± 81*
4 °C, 1 day	263 ± 17	291 ± 18*	52.4 ± 8.2	56.4 ± 8.7	1129 ± 12	1489 ± 95*
4 °C, 14 days	245 ± 20	278 ± 19*	54.9 ± 12.5	57.3 ± 11.3	1077 ± 44	1409 ± 104*
No Trt.						
20 °C, 1 day	270 ± 19	310 ± 20*	64.4 ± 3.8	64 ± 4.2	1369 ± 80	1500 ± 91*
20 °C, 14	329 ± 78	388 ± 90	71.6 ± 20.7	76.1 ± 25.9	1138 ± 98	1329 ± 88*
4 °C, 1 day	212 ± 31	232 ± 37	44.6 ± 8.6	47.9 ± 9.4	1223 ± 168	1225 ± 152
4 °C, 14 days	329 ± 13	395 ± 30*	78.6 ± 5.8	80.9 ± 2.9	1342 ± 191	1705 ± 249*

The results indicate a discrepancy in the accuracy as the GC-MS instruments yielded significantly different values for several samples. The GC-MS instrument at Norut estimated in general greater amounts of all three fatty acids than the one at the Department of Pharmacy (IFA); although 20:5 (n-3) in one sample was estimated to be significantly less. The standard curve for 20:5 (n-3) used at Norut ranged from 50-1000 μg/mL, hence the obtained result exceeded the standard curve; while the one used at IFA ranged from 25- 5000 μg/mL. This can possibly explain why the differences were the biggest for this fatty acid. Standard curves flatten out at very high concentrations when the limit of linearity is reached. However, analysis of diluted 5000 μg/mL calibration standards and the R^2 value (see table 13 in the appendix) suggested a linear detector response up to 5000 μg/mL. For the scarce 18:0, only one sample showed a significant difference. The varying results does not necessarily mean that at least one of the instrument is inaccurate. Loss of analyte through degradation or evaporation might influence the results as the samples were analyzed at different times. However, this does not explain how values varied in opposite directions. Furthermore, under the assumption that unsaturated FAMEs are lost faster than the internal standard due to oxidation, and because the time difference in analyzing the calibration standards was greater than the algae samples, obtaining lower values by the GC-MS instrument at Norut would be more intelligible. Puncturing of the GC-MS vial septum leads to evaporation of the solvent before the second analysis and can influence the results as the detector response might not be linear for high concentrations. This can also cause incorrect integration where peak areas are close in retention even though the same software and peak detecting settings are used. However, the inaccuracy is in absolute values, and will most likely not affect the relative values of each fatty acids in a sample to the same extent.

4.3.2 Limitations

There were some separation problems for the FAMEs.18:0 and 18:1; and 24:0 and 24:1 did partly overlap for most concentrations in the calibration standards (see figure 27). The highly polar ZB-FAMEwax column was able to separate these peaks but was not used for quantification as it resulted in overlapping of other FAMEs of different chain lengths. Partly overlapping can yield a false position of the baseline and separation line, which potentially results in inaccurate peak values. However, this problem is of bigger concern when smaller peaks are eluting on the tail of larger ones. The overlapping peaks had relatively similar detector

response, thus the obtained peak values are most likely not significantly affected. The standard curves that were obtained had acceptable R^2 values indicating a linear detector response for the concentration range. Because the calibration standards were stored over a long time (approximately 1 month) prior to analysis, a synthetic antioxidant should have been added to prevent autoxidation and ensure stability. This also applies for the prepared algae samples. It would be important to choose an antioxidant that does not co-elute with any of the FAMEs.

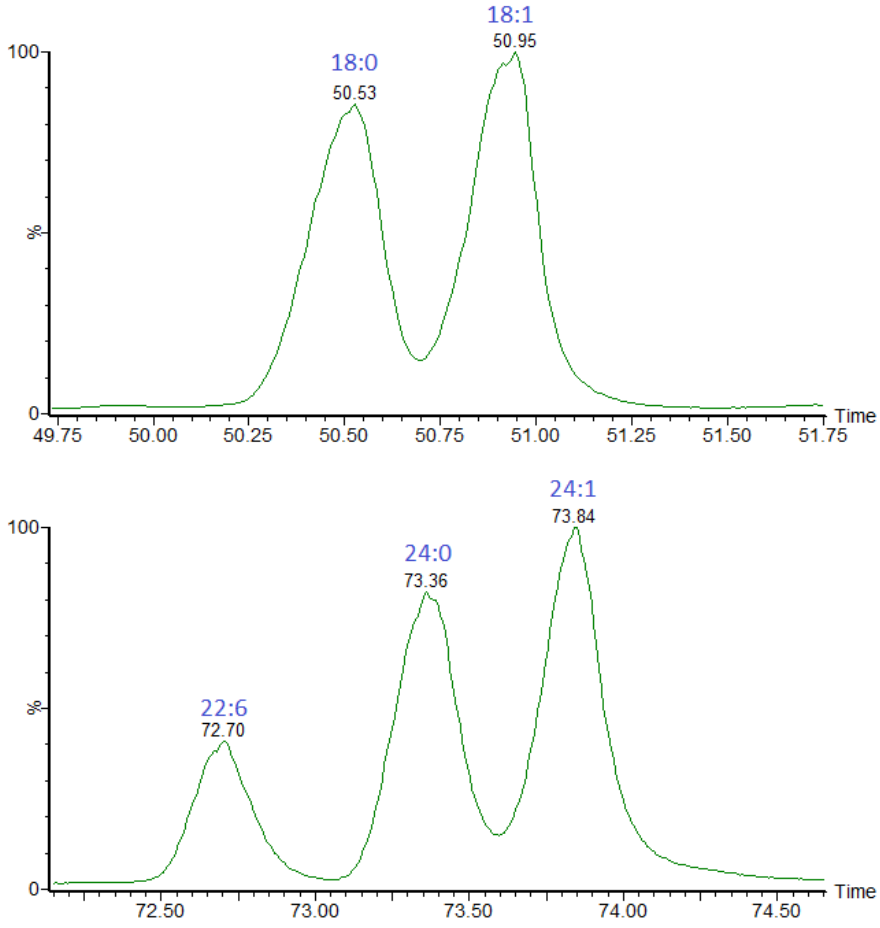


Figure 27: FAME calibration standard (500 µg/mL)

Quantification of the different isomers was done under the assumption that detector sensitivity is similar among isomers. 16:1 (n-5) and 16:2 (n-7) found in the algae co-eluted to give almost completely overlapping peaks. Because the standards (16:1 n-7 and 16:2 n-4) had similar detector response, quantification of the overlapping fatty acids was based on the adjusted molecular ion peak areas and taken as a proportion of the total peak area. 16:2 (n-4) had 2.3 times bigger molecular ion peak area compared to 16:1 (n-7) at the same concentration. Hence the molecular ion peak area of 16:2 (n-7) was divided by 2.3 to give an adjusted proportion to the molecular peak area of 16:1 (n-5). This would yield an approximation of the quantity of the

fatty acids. There is also some uncertainty to the quantity of 16:4 (n-1) as it exceeded the calibration curve range. Several small contaminants eluted almost continuously around the 18-carbon chain length group, making the baseline as well as the FAME peaks very hard to locate (see figure 28). All peaks in this area partly overlap and are likely to cause inaccurate peak values for the low abundant 18:1, 18:2 and 18:3 fatty acids. It might be better to exclude these peaks from the overall fatty acid composition determination. However, these fatty acids only amount to a small percentage of the total fatty acid composition; hence false values will not have a significant impact on the total fatty acid composition.

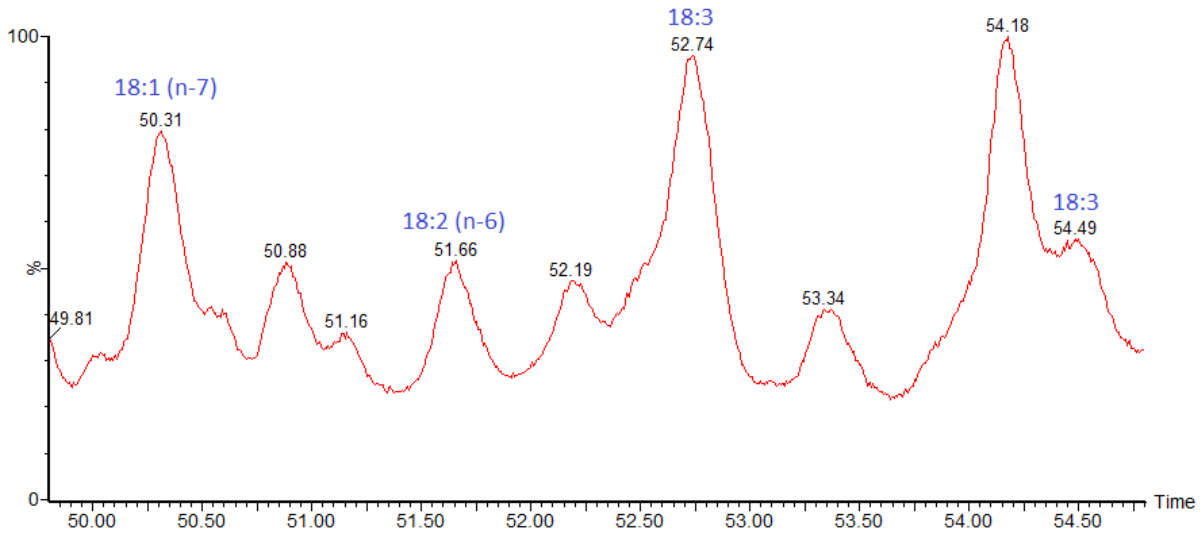


Figure 28: FAME chromatogram of algae sample in the 18-carbon chain length area (full scan)

The preparation of calibration standards was time consuming given the high number of fatty acid present in the microalgae. For some of the fatty acids different nonpolar solvents had to be applied in order to get them dissolved. Furthermore, evaporation of these solvents is a major concern and can yield inaccurate concentrations during the preparation of the calibration standards. In order to minimize technical errors a quantitative standard mix of fatty acids or FAMEs would have been ideal. Finding a commercially available mix that contain all the fatty acids required can however be challenging.

In terms of analyzing fatty acid content internal standards could have been added before the lipid extraction. In the developed method internal standards were added after; hence possible losses from the extraction is not accounted for. In addition, dissolving the lipid in a small volume of volatile solvents and transferring a proportion of this before internal standard was

added may cause inaccuracy in determining fatty acid content. Assuming the majority of the fatty acids are bound to glycerol backbones in lipids, accounting for potential extraction losses would require the usage of triacylglycerols or complex lipids as internal standard. However, adding the internal standard before extraction is not applicable for the gravimetrical method used for total lipid content determination, as it would add to the total weight. Furthermore, it would not account for the lost lipids that remain unextracted from the algae biomass.

5 Conclusion and future perspectives

A total of 20 different fatty acids were found in *Porocira glacilis*. Double bond position was identified by analyzing 3-pyridylcarbinol esters of the fatty acids on GC-MS. Double bond position of all fatty acids were determined, except the 18:3 isomers which were of low abundancy in the samples. The signal was likely disturbed by co-eluting substances. Product ion scan of selected ions did not yield fragments indicative of double bonds position. SIM in combination with CI yielded relatively high signal for the quasi-molecular ions of the fatty acids and was helpful in distinguishing the fatty acids from other substances.

The fatty acid composition of *Porocira glacilis* is highly unsaturated with roughly 80% PUFAs. The PUFAs essentially consist of 16:4 (n-1) and EPA (20:5 n-3). Furthermore, very low amounts of omega-6 fatty acid (<1%) were found. The low omega-6 to omega-3 ratio is favorable and makes *Porocira glacilis* a candidate for fish feed production. Lipid content is lower than salmon fish feed. Hence the biomass may be applicable as an ingredient rather than whole feed.

Heat treatment seem to be the most promising treatment of the ones tested. 14 days of storage at 4 ºC and 20 ºC did not seem to influence the composition of fatty acids, as the content of the most abundant PUFAs including EPA did not change significantly. Furthermore, the lipid yields from the extraction were consistently higher than for the other treatments. Long term storage of heat treated biomass should be done to assess the effectiveness of heat treatment.

Generally, results from the lipid content analysis were conflicting. Hence the analysis should be repeated. In addition, free fatty acids, peroxides, and antioxidants should be analyzed to verify whether the extracted lipid content is indicative of lipid stability.

Different GC-MS instrument yielded significantly different concentration of EPA. The quantitative determination should be repeated with new calibration standards to assess the precision of the results.

6 Referances:

1. Pauly D, Christensen V, Guenette S, Pitcher TJ, Sumaila UR, Walters CJ, et al. Towards sustainability in world fisheries. Nature. 2002;418(6898):689-95.
2. Salem N, Jr., Eggersdorfer M. Is the world supply of omega-3 fatty acids adequate for optimal human nutrition? Curr Opin Clin Nutr Metab Care. 2015;18(2):147-54.
3. The worlds most energy-efficient ferrosilicon producer [Available from: http://www.finnfjord.no/en/the_worlds_most_energy_efficient_ferrosilicon_producer. (Accessed 2017 December 27)
4. Helga. Finnfjord AS 2011, used under CC-BY-SA [Available from: https://commons.wikimedia.org/wiki/File:IFinnfjord_smelteverk_001.jpg.
5. Torsøe M. Gavepakke på 18 millioner til UiT og Finnfjord AS 2017 [Available from: https://uit.no/om/enhet/aktuelt/nyhet?p_document_id=518275&p_dimension_id=88141.
6. Svenning JB. Personal communication. 2018.
7. Tomas CR. Identifying marine phytoplankton. San Diego: Academic Press; 1997.
8. Skjevik A-T. Porosira glacialis 2012, used under CC-BY-ND [Available from: http://nordicmicroalgae.org/taxon/Porosira%20glacialis?media_id=Porosira%20glacialis_2.jpg&page=1.
9. Naustvoll L. Kiselalger - en nøkkelorganisme i marine økosystemer [Available from: https://www.imr.no/filarkiv/2014/03/kiselalger_en_nokkelorganisme_i_marine_okosystemer.pdf/nb-no.(Accessed 2017 December 27)
10. Christie WW. Lipid analysis : isolation, separation, identification and structural analysis of lipids. 3rd. ed. ed. Bridgewater: P.J. Barnes & Associates; 2003.
11. Chen Y-C. The biomass and total lipid content and composition of twelve species of marine diatoms cultured under various environments. Food chemistry. 2012;131:211-9.
12. Kalisch B, Dormann P, Holzl G. DGDG and Glycolipids in Plants and Algae. Subcell Biochem. 2016;86:51-83.
13. Certik M, Shimizu S. Biosynthesis and regulation of microbial polyunsaturated fatty acid production. J Biosci Bioeng. 1999;87(1):1-14.
14. Mozffarian D. Fish oil and marine omega-3 fatty acids [updated August 24, 2016. Available from: https://www.uptodate.com/contents/fish-oil-and-marine-omega-3-fatty-acids. (Accessed 2017 December 28)
15. Miles EA, Calder PC. Influence of marine n-3 polyunsaturated fatty acids on immune function and a systematic review of their effects on clinical outcomes in rheumatoid arthritis. Br J Nutr. 2012;107 Suppl 2:S171-84.
16. Oken E. Fish consumption and docosahexaenoic acid (DHA) supplementation in pregnancy [updated june 06, 2017. Available from: https://www.uptodate.com/contents/fish-consumption-and-docosahexaenoic-acid-dha-supplementation-in-pregnancy?source=see_link. (Accessed 2017 December 28)
17. Frankel EN. Lipid oxidation. 2nd ed. ed. Bridgewater: Oily Press; 2005.
18. Goh S-H, Yusoff FM, Goh S-P. A comparison of the antioxidant properties and total phenolic content in a diatom *Chatoceros sp.* and a green microalga, *Nannochloropsis sp.* Journal of Agricultural science. 2010;2(3):123-30.
19. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957;226(1):497-509.

20. Cequier-Sanchez E, Rodriguez C, Ravelo AG, Zarate R. Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures. J Agric Food Chem. 2008;56(12):4297-303.
21. Axelsson M, Gentili F. A Single-Step Method for Rapid Extraction of Total Lipids from Green Microalgae. PLoS ONE. 2014;9(2):e89643.
22. Ryckebosch E, Muylaert K, Foubert I. Optimization of an Analytical Procedure for Extraction of Lipids from Microalgae. Journal of the American Oil Chemists' Society. 2012;89(2):189-98.
23. Dubois N, Barthomeuf C, Berge J-P. Convenient preparation of picolinyl derivatives from fatty acid esters. European Journal of lipid science and thechnology. 2006;108(1):28-32.
24. Hansen S, Pedersen-Bjergaard S, Rasmussen KE. Introduction to pharmaceutical chemical analysis. Chichester: Wiley; 2012.
25. Dolan JW. When should an internal standard be used? LCGC North America. 2012:474-80.
26. Holm-Hansen O, Riemann B. Chlorophyll a determination: improvements in methodology. Oikos. 1978;30:438-47.
27. Søltoft-Jensen J, Hansen F. 15 - New Chemical and Biochemical Hurdles A2 - Sun, Da-Wen. Emerging Technologies for Food Processing. London: Academic Press; 2005. p. 387-416.
28. Smith BH. FORMIC ACID AS A PRESERVATIVE.2. Journal of the American Chemical Society. 1907;29(8):1236-41.
29. Thermal Processing of Food: Safefood 360°; 2014 [Available from: http://safefood360.com/free-resources/whitepapers/. (Accessed 2018 May 11)
30. Christie WW. 3-Pyridylcarbinol ('picolinyl') Esters of Fatty Acids. Archive of Mass Spectra [updated July 16th 2017. Available from: http://www.lipidhome.co.uk/ms/pyrcarbinol/pyrcarb-arch/index.htm.(Accessed 2017 December 15)
31. Han D, Peter W, Luisi P. Dependence of Lipase Activity on Water Content and Enzyme Concentration in Reverse Micelles1990. 153-61 p.
32. Boden G. Obesity and Free Fatty Acids. Endocrinology and Metabolism Clinics of North America. 2008;37(3):635-46.
33. Bergé J-P, Gouygou J-P, Dubacq J-P, Durand P. Reassessment of lipid composition of the diatom, Skeletonema costatum1995. 1017-21 p.
34. Ryckebosch E, Muylaert K, Eeckhout M, Ruyssen T, Foubert I. Influence of drying and storage on lipid and carotenoid stability of the microalga Phaeodactylum tricornutum. J Agric Food Chem. 2011;59(20):11063-9.
35. Wichard T, Gerecht A, Boersma M, Poulet SA, Wiltshire K, Pohnert G. Lipid and fatty acid composition of diatoms revisited: rapid wound-activated change of food quality parameters influences herbivorous copepod reproductive success. Chembiochem. 2007;8(10):1146-53.
36. Gao C, Xiong W, Zhang Y, Yuan W, Wu Q. Rapid quantitation of lipid in microalgae by time-domain nuclear magnetic resonance. J Microbiol Methods. 2008;75(3):437-40.
37. Van Pelt CK, Brenna JT. Acetonitrile Chemical Ionization Tandem Mass Spectrometry To Locate Double Bonds in Polyunsaturated Fatty Acid Methyl Esters. Analytical Chemistry. 1999;71(10):1981-9.

38. Lawrence P, Brenna JT. Acetonitrile covalent adduct chemical ionization mass spectrometry for double bond localization in non-methylene-interrupted polyene fatty acid methyl esters. Anal Chem. 2006;78(4):1312-7.
39. Ytrestøyl T, Aas TS, Åsgård T. Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway. Aquaculture. 2015;448:365-74.
40. Gunstone F, Gunstone FD. Vegetable oils in food technology : composition, properties and uses. Hoboken: Wiley-Blackwell; 2011.
41. Montero D, Kalinowski T, Obach A, Robaina L, Tort L, Caballero MJ, et al. Vegetable lipid sources for gilthead seabream (Sparus aurata): effects on fish health. Aquaculture. 2003;225(1):353-70.
42. Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med (Maywood). 2008;233(6):674-88.
43. Sarker PK, Kapuscinski AR, Lanois AJ, Livesey ED, Bernhard KP, Coley ML. Towards Sustainable Aquafeeds: Complete Substitution of Fish Oil with Marine Microalga Schizochytrium sp. Improves Growth and Fatty Acid Deposition in Juvenile Nile Tilapia (Oreochromis niloticus). PLOS ONE. 2016;11(6):e0156684.
44. Kraugerud RL. Algae may replace fish oil in salmon feed2014. Available from: https://nofima.no/en/nyhet/2014/10/algae-may-replace-fish-oil-in-salmon-feed/. (Accessed 2018 May 3)

Appendix

3-pyridylcarbinol esters spectra (EI 70eV)

.

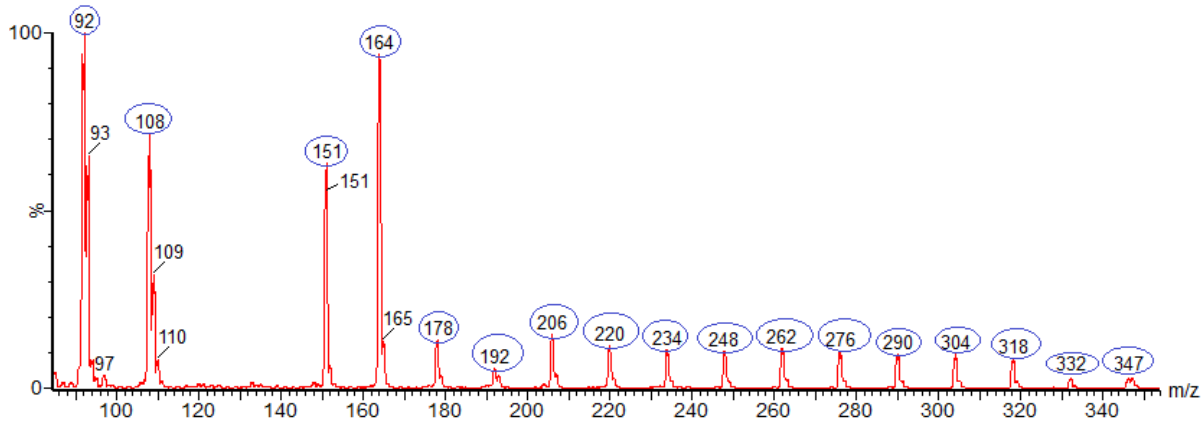


Figure 29: 16:0. The 14 amu gaps is indicative of no double bonds.

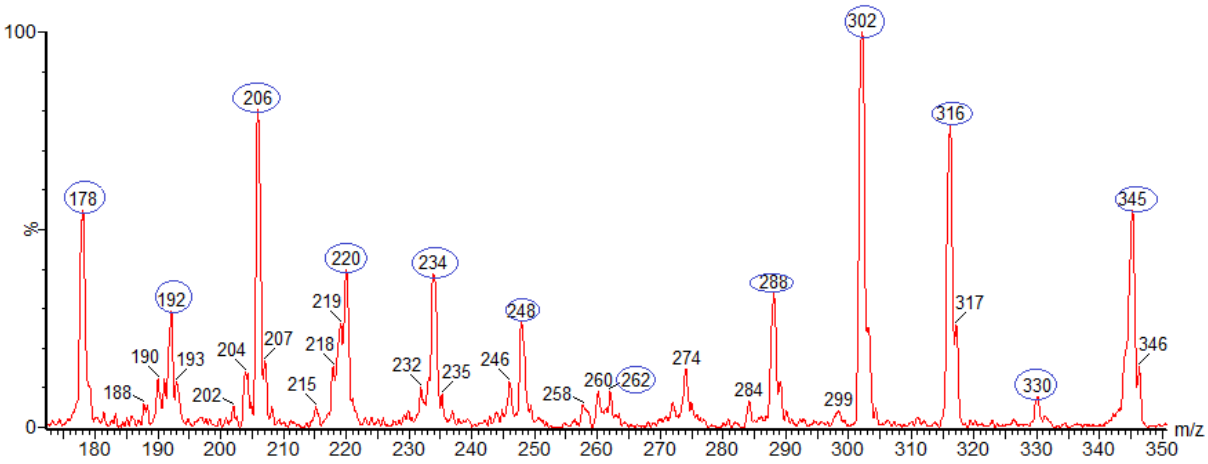


Figure 30: 16:1 (n-5). The double bond can be located by the 26 amu gap between m/z 288 and 262. The abundant doublet at m/z 302 and 316 provide evidence.

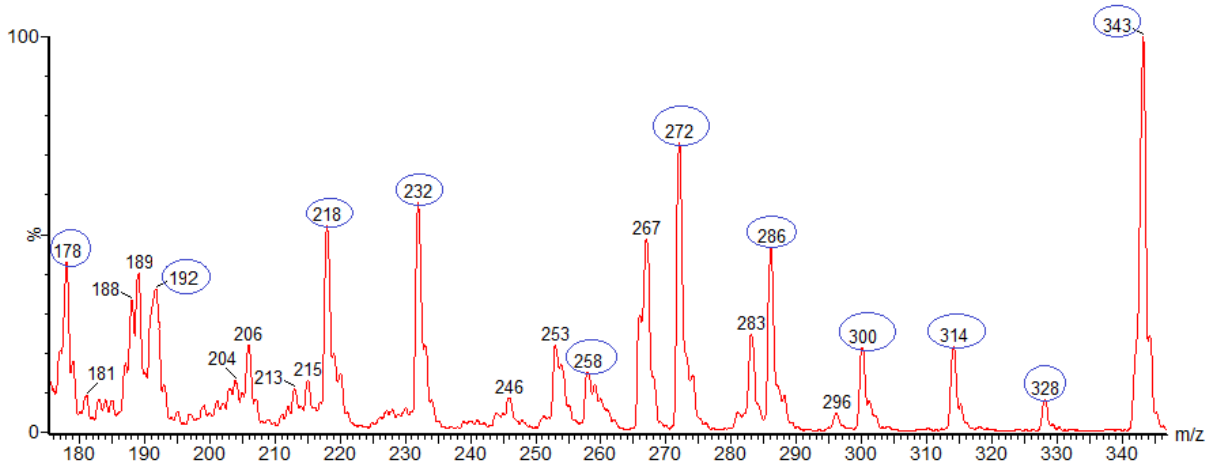


Figure 31: 16:2 (n-7). The double bonds can be located by the 26 amu gaps between m/z 258 and 232; and m/z 218 and 192. The abundant doublet at m/z 272 and 286 provide evidence.

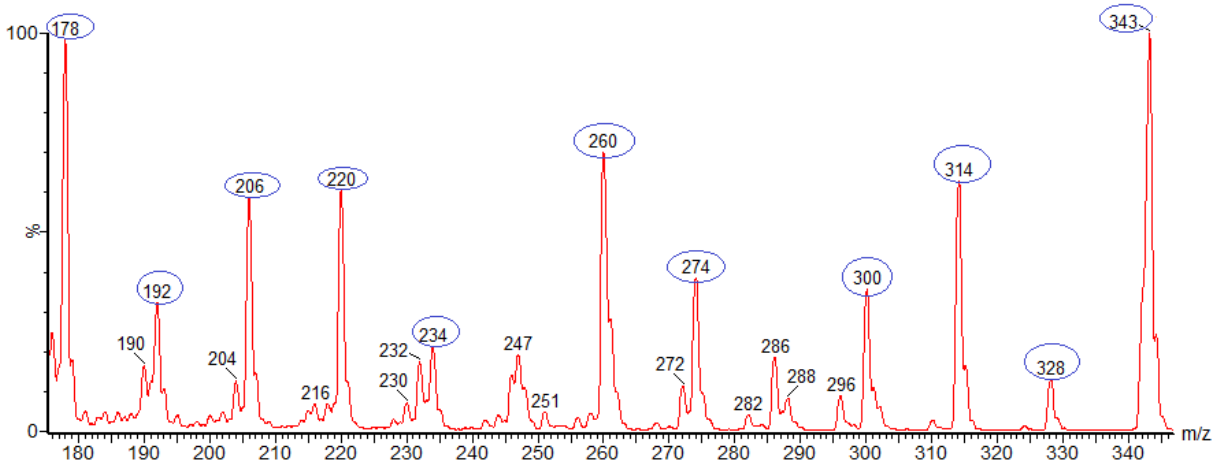


Figure 32: 16:2 (n-4). The double bonds can be located by the 26 amu gaps between m/z 300 and 274; and m/z 260 and 234. Here only the first ion of the doublet is abundant, at m/z 314.

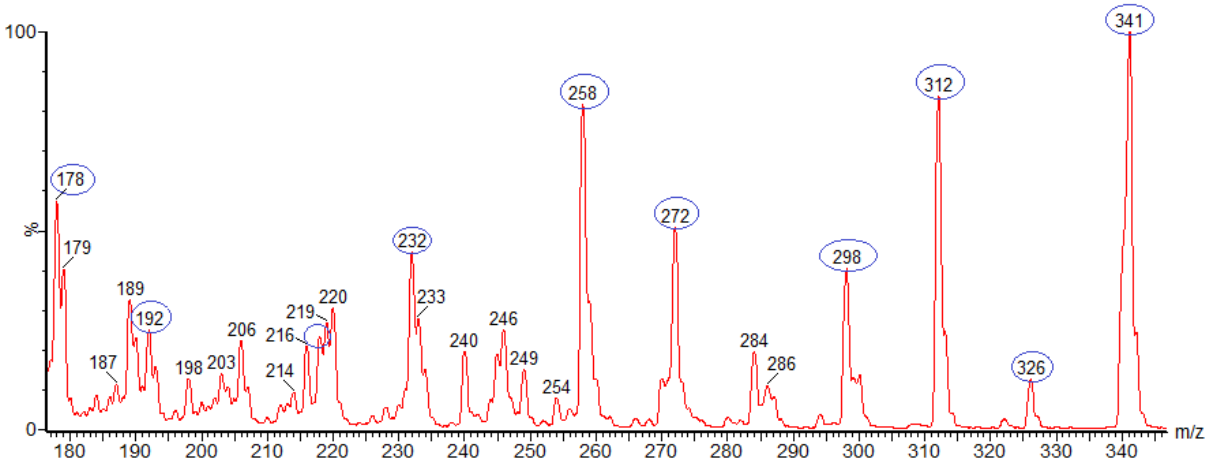


Figure 33: 16:3 (n-4): The double bonds can be located by the 26 amu gaps between m/z 298 and 272; m/z 258 and 232; and m/z 218 and 192. The first double bond is not easily seen but reference spectra of other isomers are distinctly different.

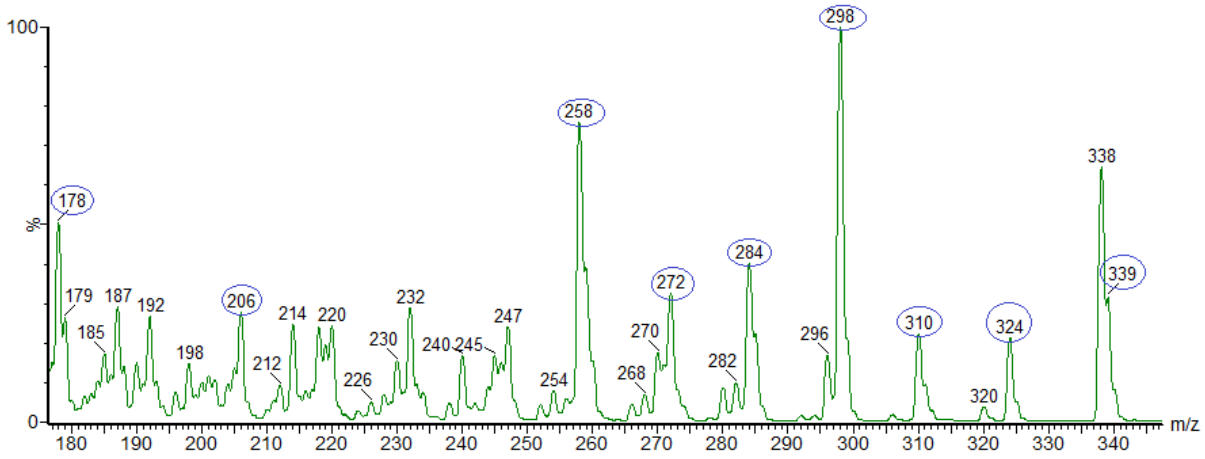


Figure 34:16:4 (n-1). Double bond at the terminal position causes rearrangement. The refernace specrum was essential to be sure of exact position. Marked ions are charactheristic.

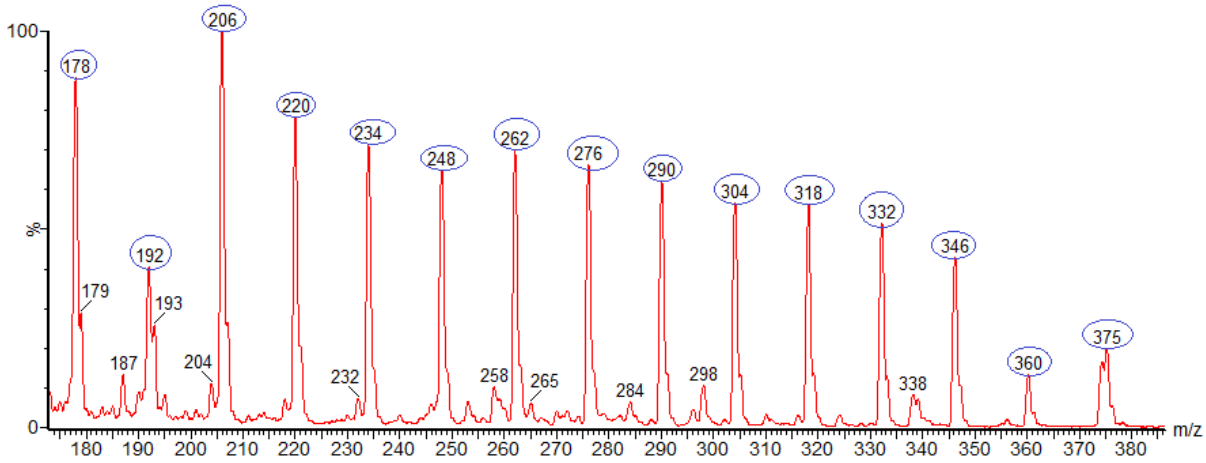


Figure 35: 18:0. The 14 amu gaps is indicative of no double bonds.

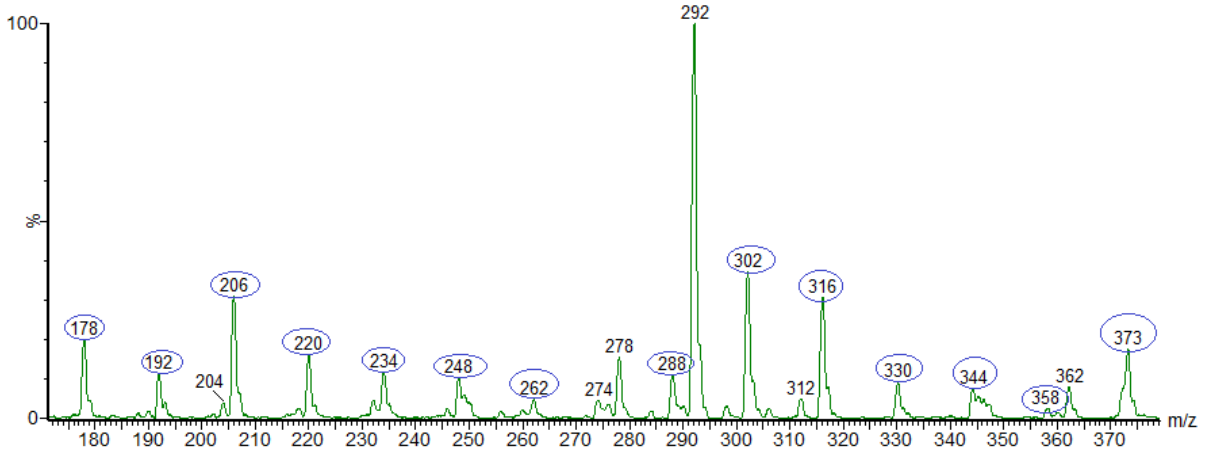


Figure 36: 18:1 (n-7). The double bond can be located by the 26 amu gap between m/z 302 and 288. The abundant doublet at m/z 302 and 316 provide evidence.

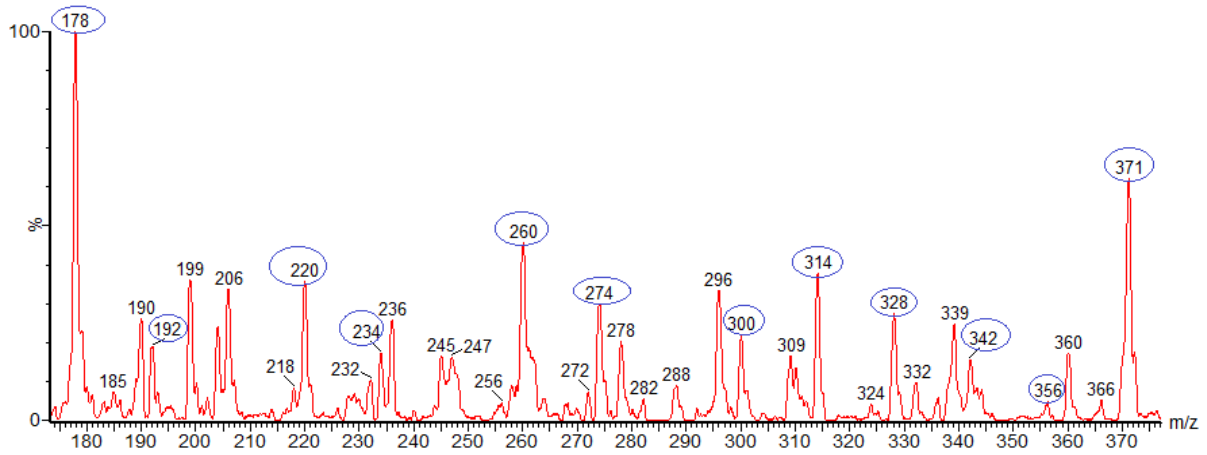


Figure 37: 18:2 (n-6). The double bonds can be located by the 26 amu gaps between m/z 300 and 274; and m/z 260 and 234. The doublet at m/z 314 and 328 are slightly elevated and provide evidence.

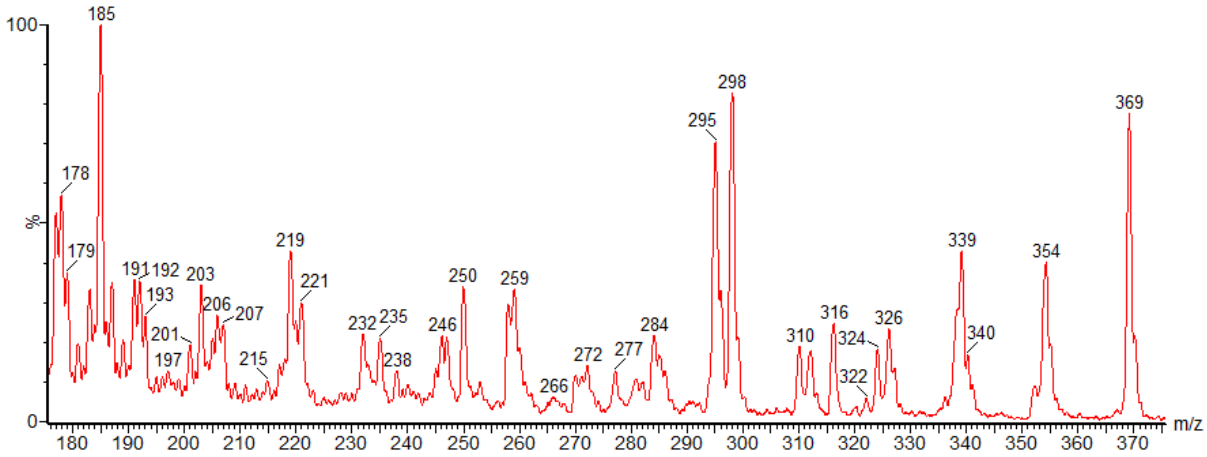


Figure 38: 18:3. The molecular ion at m/z 369 and the fragment ion corresponding to cleavage of the terminal methyl group at m/z 354 is visible. Most other peaks did not match with the reference spectra of different 18:3 isomers.

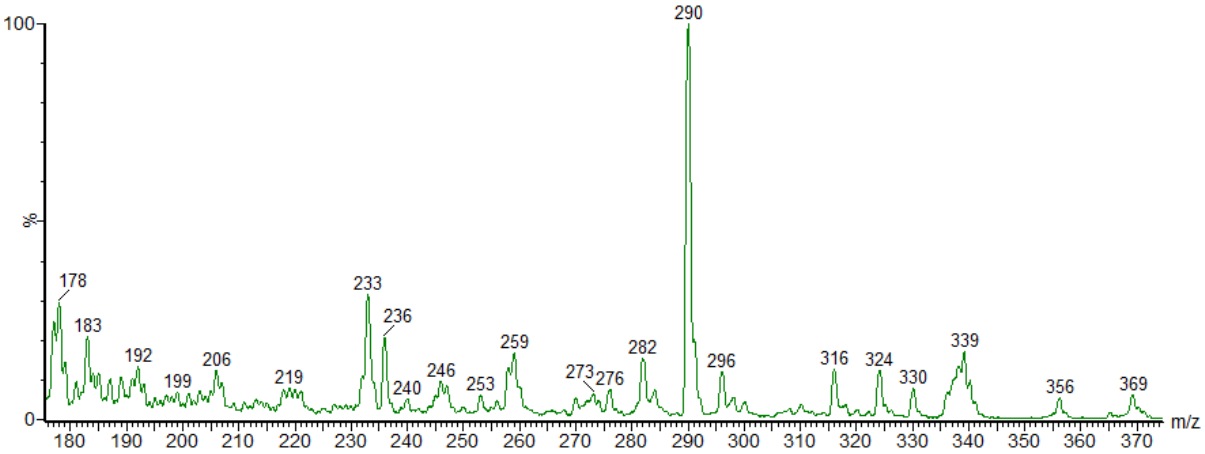


Figure 39: 18:3 (2). The molecular ion at m/z 369. Most other peaks did not match with the reference spectra of different 18:3 isomers.

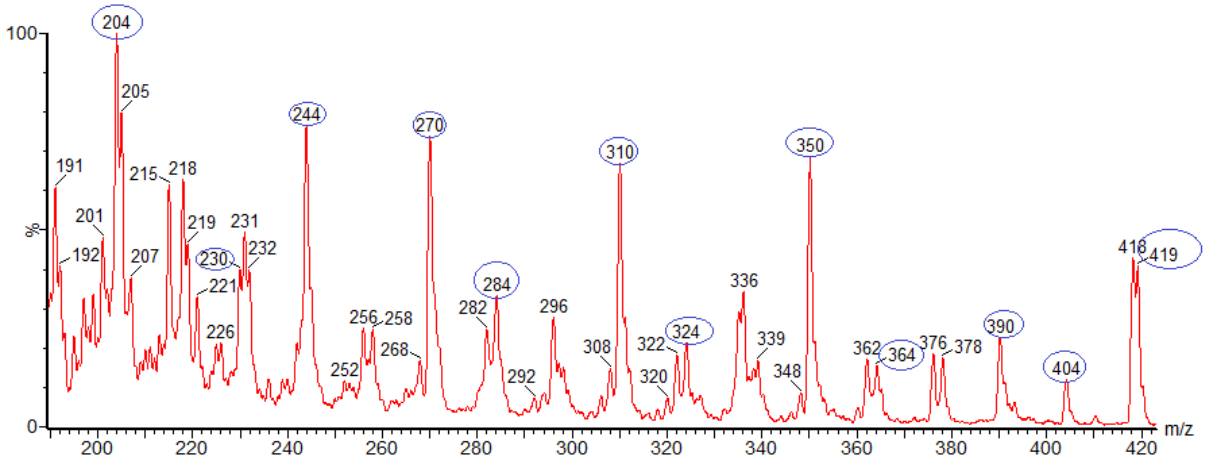


Figure 40: 22:6 (n-3). The last five double bonds (closest to the methyl end) can be located by the 26 amu gaps between m/z 390 and 364; m/z 350 and 324; m/z 310 and 284; m/z 270 and 244, and m/z 230 and 204. Reference spectra was used to be sure of exact position.

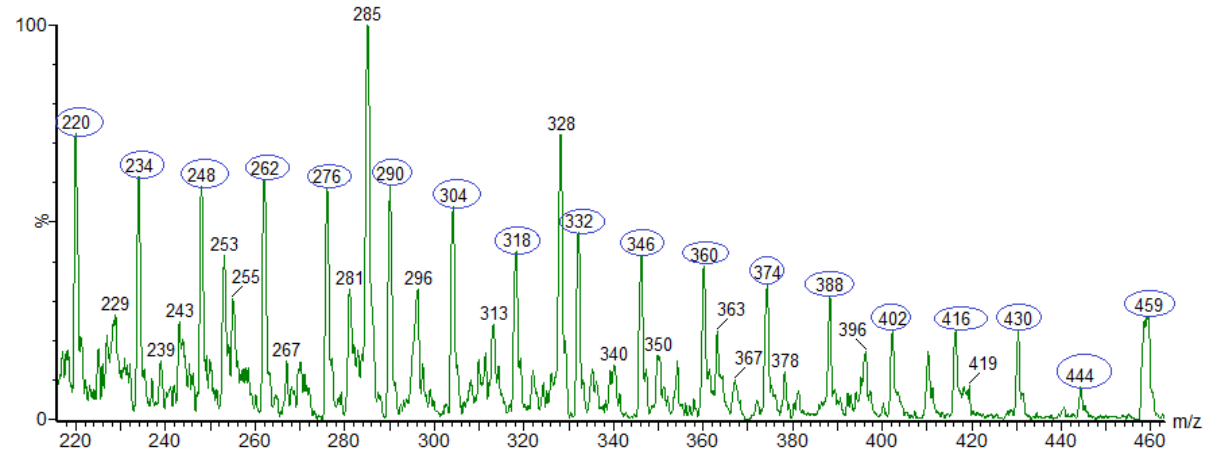


Figure 41: 24:0. The 14 amu gaps is indicative of no double bonds.

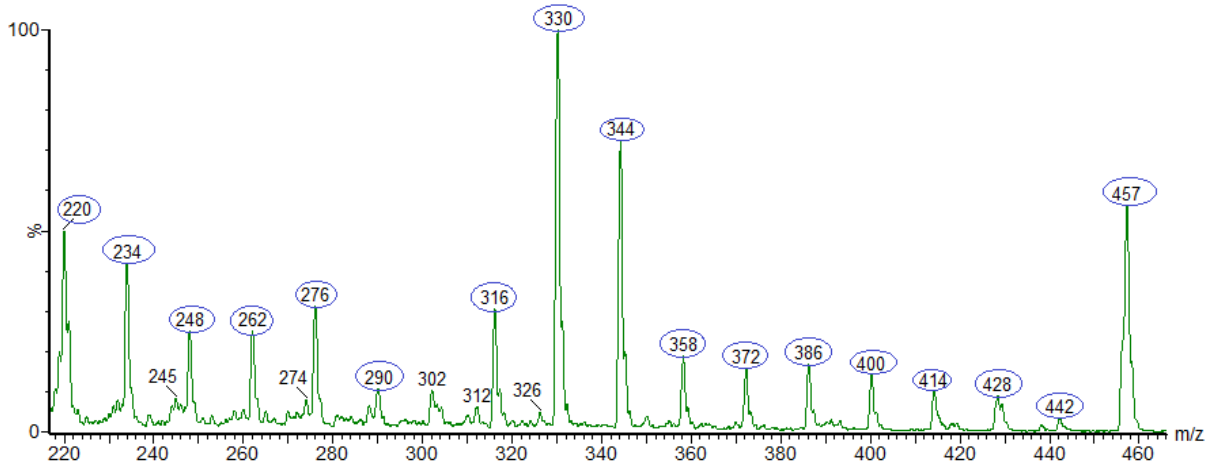


Figure 42:24:1 (n-11). The double bond can be located by the 26 amu gap between m/z 316 and 290. The abundant doublet at m/z 330 and 344 provide evidence.

Lipid content

Table 11: Lipid content given in percentage of the algal sample dry weight with standard deviation.

	1 day	3 days	7 days	14 days
Benzoic acid				
20 °C	7.6 ± 0.8	7.3 ± 0.1	7 ± 0.3	8 ± 0.3
4° C	7.7 ± 0.2	8.3 ± 1.2	8.8 ± 2	7 ± 0.4
No treatment				
20 °C	7.4 ± 1.5	7.4 ± 0.2	7 ± 0.9	6.9 ± 0.7
4° C	8.6 ± 1.4	6.9 ± 0.5	7.6 ± 0.9	6.6 ± 0.6
Formic acid				
20 °C	6.4 ± 0.5	6.4 ± 0.6	7.2 ± 0.6	6.5 ± 0.4
4° C	4.7 ± 1.3	6.3 ± 0.3	5.8 ± 0.2	5.1 ± 0.2
Heat treatment				
20 °C	8.5 ± 0.3	7.6 ± 0.8	8.8 ± 0.9	8.1 ± 0.7
4° C	8.2 ± 0.7	8.7 ± 0.5	8.1 ± 0.6	8.8 ± 0.5

Calibration standard curves

Table 12: Calibration standards

10µg/ml	25µg/ml	50µg/ml	100µg/ml	250µg/ml	500µg/ml	1000µg/ml	2500µg/ml	5000µg/ml
10µL stock 2	25µL stock 2	50µL stock 2	100µL stock 2	25µL stock 1	50µL stock 1	100µL stock 1	250µL stock 3	500µL stock 3
100µL stock IS	100µL stock IS	100µL stock IS	100µL stock IS	100µL stock IS	100µL stock IS	100µL stock IS	100µL stock IS	100µL stock IS
890µL DCM	875µL DCM	850µL DCM	800µL DCM	875µL DCM	850µL DCM	800µL DCM	650µL DCM	400µL DCM

Table 13: Calibration curves. a is the slope and b is the intercept

	a	b	R^2	CONC. Range
14:0	0.0136	-0.4956	0.9749	10-1000
16:0	0.0125	-0.1576	0.9875	10-1000
16:1	0.0123	-0.2477	0.9853	10-1000
16:2	0.0127	-0.3325	0.982	10-1000
16:3	0.0092	-0.2544	0.9798	10-1000
16:4	0.0071	-0.2063	0.9788	10-1000
18:0	0.0062	0.0123	0.9952	10-250
18:1	0.007	-0.0337	0.9963	10-250
18:2	0.0132	-0.1883	0.9922	10-250
18:3	0.0141	-0.2023	0.9922	10-250
18:4	0.0151	-0.184	0.9908	10-1000
20:0	0.0137	0.0089	0.9905	10-1000
20:4	0.008	-0.113	0.9858	25-1000
20:5	0.0037	0.5013	0.9809	25-5000
22:0	0.0109	-0.0293	0.9884	10-1000
22:6	0.0035	-0.0601	0.9605	25-1000
24:0	0.0079	-0.0459	0.9729	10-1000
24:1	0.0113	-0.1382	0.9816	10-250

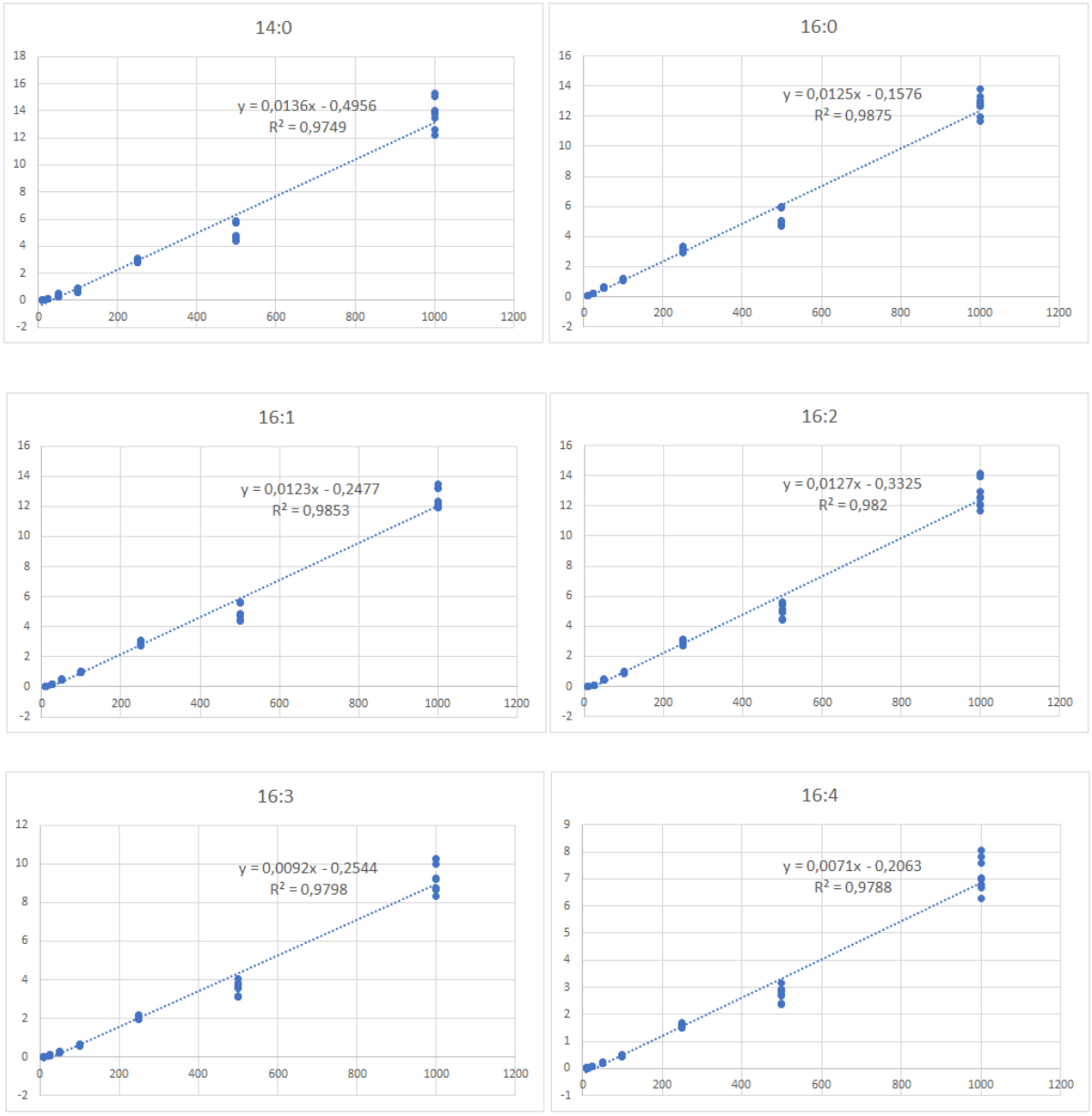


Figure 43: Standard curves

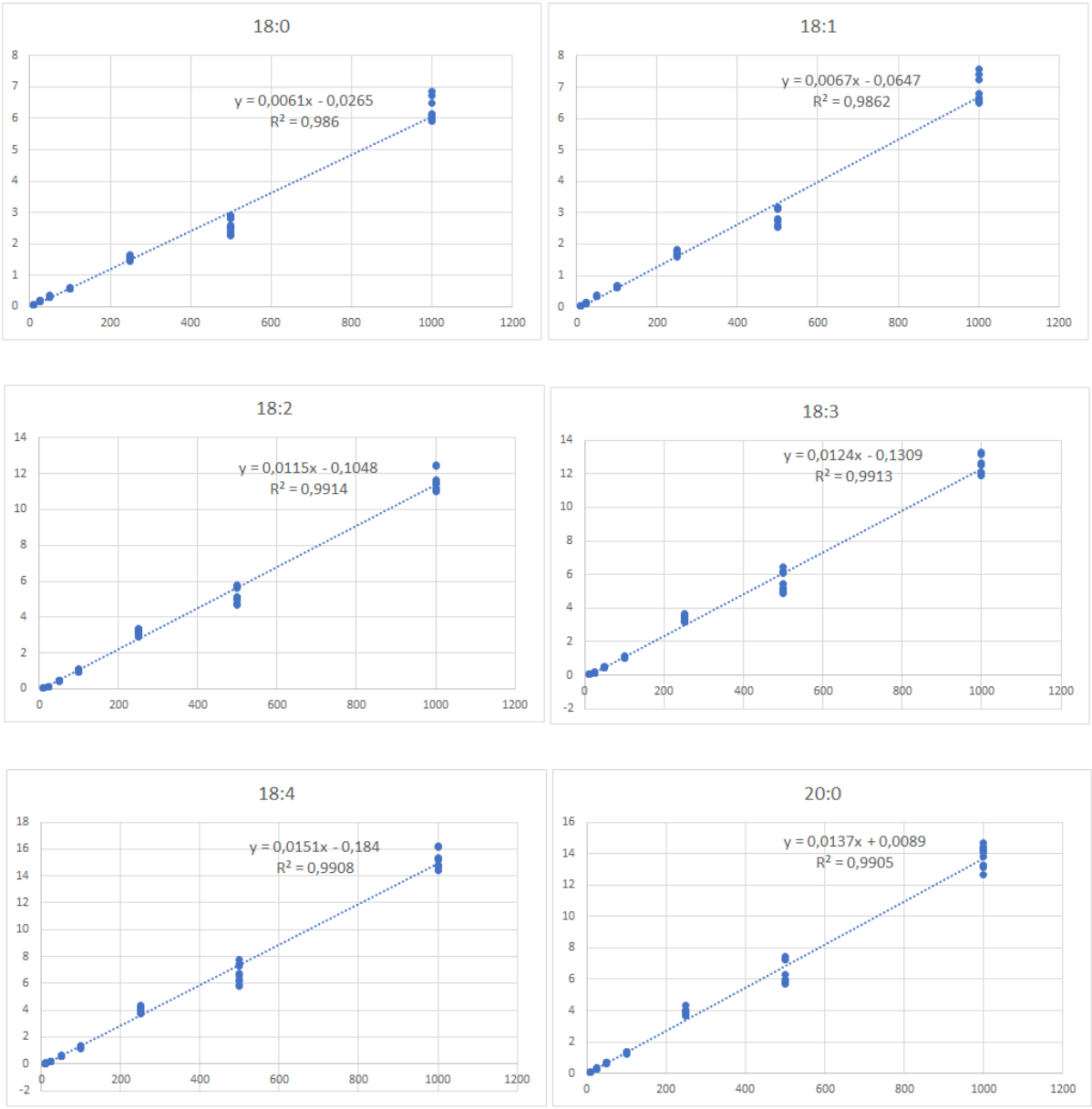


Figure 44: Standard curves

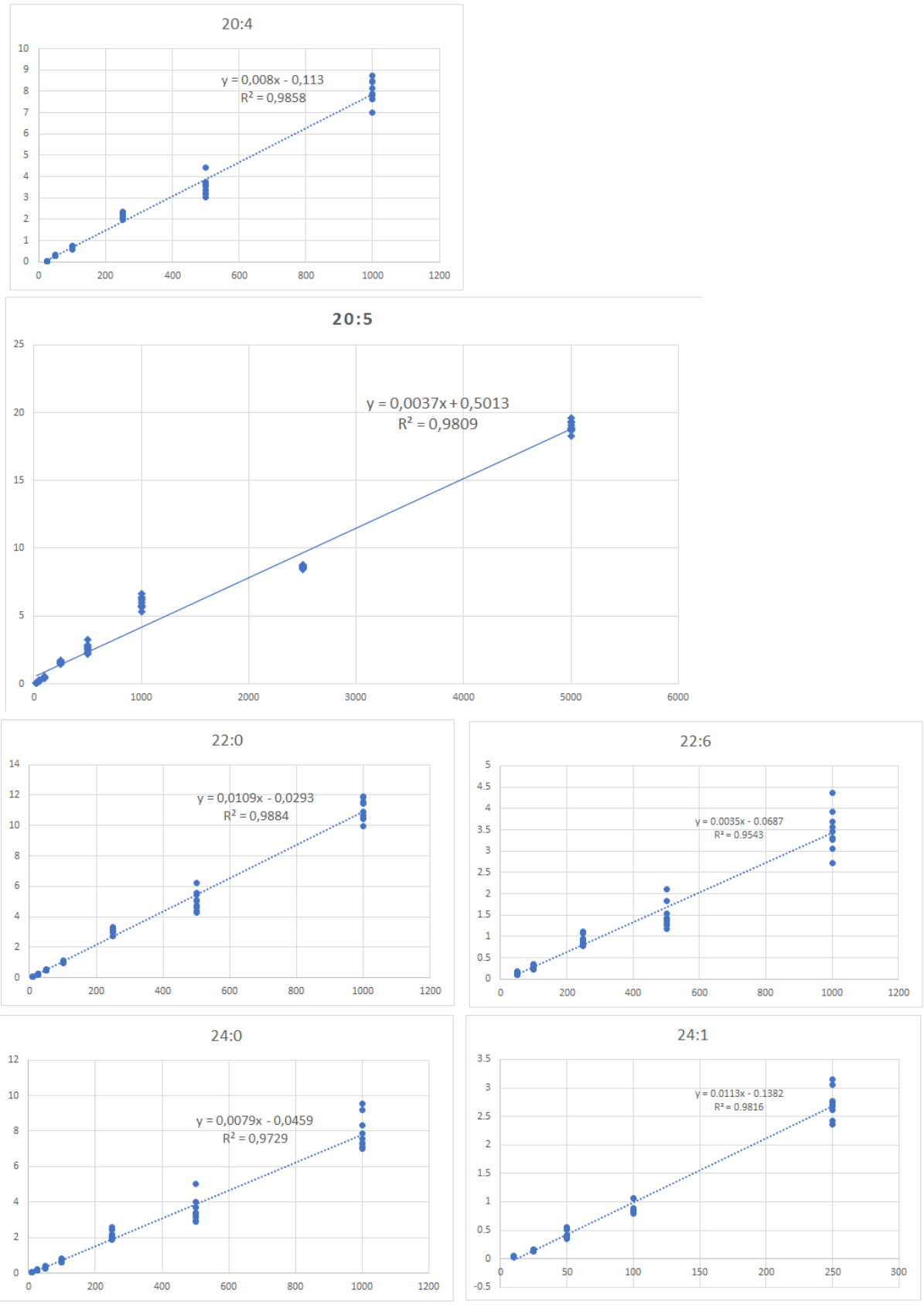


Figure 45: Standard curves

Fatty acid composition analysis

Table 14: Consentration (µg/mL) for samples treated with formic acid and stored 1 day at 4 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	136.69	136.60	160.88	161.25	164.61	164.64
16:0	129.91	127.71	161.51	161.75	164.19	165.01
16:1 (n-7)	222.12	211.33	248.36	247.00	249.23	248.89
16:1(n-5)	67.16	65.68	73.46	73.20	79.03	78.77
16:2 (n-7)	49.61	48.87	52.75	52.62	55.53	55.40
16:2 (n-4)	80.73	79.67	90.77	90.58	96.50	96.74
16: (n-4)	181.94	181.26	215.69	216.80	230.06	231.14
16:4 (n-1)	1331.76	1315.38	1546.60	1533.60	1717.23	1713.23
18:0	65.06	61.21	78.93	79.47	84.32	87.15
18:1 (n-7)	26.25	24.09	28.42	28.68	30.08	32.37
18:2 (n-6)	18.74	18.69	20.23	20.23	18.82	19.29
18:3	33.05	32.68	39.62	40.54	37.38	38.05
18:3	25.87	25.10	30.57	29.97	21.82	22.88
18:4 (n-3)	176.48	184.55	204.41	204.56	233.59	222.27
20:5 (n-3)	1220.34	1252.68	1343.23	1335.12	1719.52	1618.70
22:6 (n-3)	111.08	116.42	122.36	123.00	165.77	158.74
24:0	12.91	13.43	14.51	14.56	17.35	17.36
24:1 (n-11)	17.03	17.28	17.97	18.27	18.55	18.74

Table 15: Consentration (µg/mL) for samples treated with formic acid and stored 14 days at 4 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	212.72	207.75	134.98	131.76	165.79	164.88
16:0	163.67	159.08	153.02	152.69	138.40	138.38
16:1 (n-7)	274.40	264.10	193.77	193.70	225.09	224.59
16:1(n-5)	68.87	67.53	48.34	48.60	57.10	56.88
16:2 (n-7)	47.01	46.43	38.23	38.34	41.97	41.88
16:2 (n-4)	72.01	69.91	58.88	59.12	63.78	63.34
16: (n-4)	115.27	113.62	96.29	99.24	101.98	100.90
16:4 (n-1)	648.13	632.61	497.16	501.95	508.78	503.02
18:0	62.61	59.05	93.59	95.16	62.19	61.31
18:1 (n-7)	21.55	19.95	16.82	17.35	17.96	17.46
18:2 (n-6)	20.02	20.16	19.45	19.57	18.57	18.59
18:3	26.27	26.28	24.28	24.66	24.50	23.62
18:3	21.84	21.76	20.64	20.48	21.11	21.16
18:4 (n-3)	98.16	99.02	84.56	85.46	88.52	89.21
20:5 (n-3)	466.46	470.46	377.99	383.99	397.80	397.42
22:6 (n-3)	54.65	52.65	46.04	47.30	46.30	46.00
24:0	11.02	10.13	11.06	11.39	10.62	10.69
24:1 (n-11)	18.22	19.04	17.88	18.05	18.44	18.69

Table 16: Consentration (µg/mL) for samples treated with formic acid and stored 1 day at 20 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	151.75	151.67	207.81	210.17	120.13	119.85
16:0	134.72	134.65	178.70	180.21	116.71	117.39
16:1 (n-7)	215.38	213.99	263.90	268.79	178.17	178.30
16:1(n-5)	72.12	72.47	89.24	92.94	66.12	58.10
16:2 (n-7)	51.02	51.19	59.20	60.97	48.15	44.32
16:2 (n-4)	84.18	84.15	111.32	115.89	70.22	69.56
16: (n-4)	179.66	180.74	239.71	250.38	143.30	141.61
16:4 (n-1)	1218.75	1237.23	1654.92	1717.82	850.90	853.05
18:0	63.61	63.18	95.19	101.78	61.40	61.80
18:1 (n-7)	25.54	24.67	32.49	34.14	19.77	18.96
18:2 (n-6)	18.32	18.30	19.16	18.92	18.29	18.32
18:3	31.92	31.63	34.83	34.51	28.57	28.16
18:3	35.34	35.11	35.73	35.65	33.49	33.40
18:4 (n-3)	179.42	182.76	206.85	203.01	137.10	135.95
20:5 (n-3)	1244.70	1288.67	1537.75	1506.26	849.54	841.89
22:6 (n-3)	115.84	123.20	156.93	161.77	84.40	87.48
24:0	12.95	12.26	14.73	16.63	11.32	12.00
24:1 (n-11)	17.13	16.92	18.39	18.27	16.69	16.57

Table 17: Consentration (µg/mL) for samples treated with formic acid and stored 14 days at 20 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	205.64	208.93	207.40	206.80	183.40	183.20
16:0	155.99	154.36	145.51	146.37	149.47	148.95
16:1 (n-7)	282.88	278.77	267.41	268.55	267.13	267.37
16:1(n-5)	76.77	76.60	76.11	76.38	70.08	69.35
16:2 (n-7)	54.00	53.92	53.68	53.81	50.72	50.35
16:2 (n-4)	95.50	94.72	92.26	92.35	94.19	94.92
16: (n-4)	200.51	200.42	189.05	190.31	205.95	208.65
16:4 (n-1)	1333.64	1323.89	1231.99	1240.73	1392.15	1390.46
18:0	73.09	72.17	64.25	64.67	79.55	80.87
18:1 (n-7)	29.20	28.99	27.82	26.30	29.30	30.02
18:2 (n-6)	20.28	19.70	19.44	19.22	19.48	19.25
18:3	39.28	38.32	35.83	35.80	37.97	38.04
18:3	30.71	29.21	26.02	26.49	41.52	41.55
18:4 (n-3)	201.23	196.77	188.89	188.21	210.22	210.11
20:5 (n-3)	1631.01	1585.78	1498.50	1491.37	1847.64	1843.28
22:6 (n-3)	138.28	137.48	132.75	133.73	146.36	149.56
24:0	15.42	15.10	13.96	13.83	14.50	16.26
24:1 (n-11)	18.38	18.76	18.32	18.51	17.76	18.40

Table 18: Consentration (µg/mL) for samples treated with benzoic acid and stored 1 day at 4 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	177.04	178.25	224.68	225.30	185.70	183.04
16:0	144.64	144.04	189.65	191.82	134.38	133.79
16:1 (n-7)	233.71	228.82	291.03	293.72	225.39	225.44
16:1(n-5)	83.57	83.32	105.66	107.50	85.78	85.57
16:2 (n-7)	53.97	53.86	63.64	64.45	54.94	54.84
16:2 (n-4)	86.81	86.85	103.23	105.09	84.05	83.81
16: (n-4)	169.40	172.10	46.44	219.03	167.58	166.12
16:4 (n-1)	1190.41	1192.49	1513.49	1563.79	1093.89	1101.56
18:0	53.14	52.49	68.51	71.62	37.58	37.81
18:1 (n-7)	25.39	24.68	29.97	31.47	23.74	22.60
18:2 (n-6)	18.41	18.31	19.52	19.38	18.15	18.31
18:3	29.93	30.24	35.05	34.52	30.83	30.75
18:3	33.66	33.60	27.18	26.68	23.96	24.00
18:4 (n-3)	181.44	184.94	211.44	208.72	165.32	166.95
20:5 (n-3)	1287.23	1330.01	1528.41	1718.66	1120.70	1135.49
22:6 (n-3)	129.63	139.29	165.85	168.48	126.25	126.22
24:0	13.70	14.68	15.69	15.46	12.72	12.45
24:1 (n-11)	22.73	23.35	25.66	25.97	23.12	22.83

Table 19: Consentration (µg/mL) for samples treated with benzoic acid and stored 14 days at 4 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	250.24	249.97	184.41	193.99	260.89	256.54
16:0	182.76	180.80	148.62	157.22	197.93	196.90
16:1 (n-7)	299.38	293.83	238.62	253.48	309.52	308.43
16:1(n-5)	107.76	111.02	93.49	97.28	114.68	117.33
16:2 (n-7)	60.64	61.93	55.03	56.52	63.37	64.41
16:2 (n-4)	101.33	103.22	88.39	92.37	106.78	107.89
16: (n-4)	193.37	197.96	166.36	175.33	205.17	207.07
16:4 (n-1)	1349.49	1361.05	1066.61	1140.17	1401.81	1413.55
18:0	62.11	65.16	48.30	52.01	70.73	72.28
18:1 (n-7)	31.84	33.43	24.92	26.55	34.96	35.62
18:2 (n-6)	19.26	19.40	18.36	18.11	19.13	19.21
18:3	30.18	31.01	28.96	28.06	30.38	30.35
18:3	34.84	34.98	34.56	34.43	34.65	34.94
18:4 (n-3)	178.18	178.27	162.74	161.94	171.63	173.40
20:5 (n-3)	1236.36	1236.87	1096.46	1094.24	1187.52	1207.52
22:6 (n-3)	133.25	136.48	120.10	119.22	131.97	135.52
24:0	14.13	14.71	13.56	13.36	14.89	15.32
24:1 (n-11)	23.78	24.32	22.60	22.82	24.82	24.87

Table 20: Consentration (µg/mL) for samples treated with benzoic acid and stored 1 day at 20 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	216.56	216.32	243.58	255.84	284.55	288.07
16:0	148.66	149.02	164.81	173.37	201.06	203.56
16:1 (n-7)	241.73	240.92	279.31	291.44	331.54	336.34
16:1(n-5)	96.03	97.35	109.58	114.57	127.63	130.85
16:2 (n-7)	55.83	56.35	61.13	63.08	68.18	69.44
16:2 (n-4)	86.73	87.50	97.90	100.80	114.02	115.26
16: (n-4)	167.81	170.51	196.68	205.47	232.32	235.19
16:4 (n-1)	1133.79	1148.08	1358.16	1425.47	1622.45	1629.59
18:0	51.42	50.69	47.71	51.41	65.88	66.78
18:1 (n-7)	25.27	24.03	30.61	32.34	35.90	36.07
18:2 (n-6)	18.77	18.89	19.45	19.66	19.87	19.52
18:3	31.12	31.52	34.22	34.19	35.65	35.77
18:3	25.52	25.47	26.06	25.91	26.32	26.39
18:4 (n-3)	180.83	180.63	204.10	201.40	222.81	222.57
20:5 (n-3)	1241.73	1240.93	1431.76	1414.45	1595.91	1591.34
22:6 (n-3)	138.31	142.32	161.00	160.20	177.71	177.68
24:0	14.20	14.96	15.51	14.88	16.93	17.34
24:1 (n-11)	23.60	24.37	27.50	25.45	26.76	27.02

Table 21: Consentration (µg/mL) for samples treated with benzoic acid and stored 14 days at 20 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	239.90	233.53	254.03	261.07	271.74	269.79
16:0	170.23	169.17	184.46	184.62	183.83	182.79
16:1 (n-7)	270.72	268.02	290.67	290.34	296.88	295.88
16:1(n-5)	102.17	100.67	110.71	112.34	114.23	113.11
16:2 (n-7)	57.86	57.28	61.16	61.79	62.52	62.08
16:2 (n-4)	91.68	91.74	100.23	100.78	94.16	93.68
16: (n-4)	170.80	171.78	189.19	190.24	174.96	174.37
16:4 (n-1)	1169.17	1159.57	1308.15	1313.54	1121.42	1128.47
18:0	51.51	51.54	60.50	60.83	47.46	48.77
18:1 (n-7)	33.46	33.60	35.90	36.05	38.20	39.43
18:2 (n-6)	18.16	18.15	18.79	18.73	19.47	19.76
18:3	28.96	28.80	30.06	15.93	30.16	30.12
18:3	30.86	30.78	31.66	31.44	23.59	23.34
18:4 (n-3)	176.48	176.43	178.39	177.40	162.03	161.86
20:5 (n-3)	1208.21	1206.02	1233.55	1229.72	1014.31	1014.81
22:6 (n-3)	135.97	137.97	148.22	148.27	123.03	122.24
24:0	15.03	15.33	15.21	14.77	13.19	13.12
24:1 (n-11)	21.05	21.63	22.54	21.81	24.04	23.51

Table 22: Consentration (µg/mL) for heat treated samples stored 1 day at 4 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	268.85	271.73	250.07	250.01	236.62	234.52
16:0	181.02	180.71	163.37	165.34	143.34	143.25
16:1 (n-7)	286.07	280.25	257.84	259.78	246.32	245.44
16:1(n-5)	110.04	110.06	102.83	104.20	97.92	98.73
16:2 (n-7)	43.88	46.49	58.98	58.97	87.01	87.45
16:2 (n-4)	96.84	97.25	92.05	93.13	89.44	89.42
16: (n-4)	185.45	188.38	178.29	181.32	172.81	172.87
16:4 (n-1)	1334.70	1334.43	1238.07	1257.01	1178.74	1174.43
18:0	60.44	60.38	53.96	54.98	42.79	42.06
18:1 (n-7)	24.91	25.66	25.16	25.65	25.39	24.62
18:2 (n-6)	18.62	18.80	18.47	18.47	18.44	18.06
18:3	29.02	30.03	29.72	30.03	29.92	30.15
18:3	23.64	23.54	23.95	23.71	23.01	23.14
18:4 (n-3)	167.19	169.44	168.53	170.04	171.01	169.76
20:5 (n-3)	1108.61	1130.32	1122.59	1136.90	1141.21	1133.98
22:6 (n-3)	116.68	123.62	123.83	123.66	123.81	124.29
24:0	12.55	13.10	13.27	12.64	11.91	12.59
24:1 (n-11)	21.98	22.07	21.84	21.68	21.77	22.29

Table 23: Consentration (µg/mL) for heat treated samples stored 14 days at 4 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	206.16	204.74	244.58	242.91	245.75	244.27
16:0	133.86	133.54	167.60	167.53	167.53	166.90
16:1 (n-7)	221.58	219.01	255.08	256.19	260.50	260.00
16:1(n-5)	86.97	86.04	102.55	103.21	105.19	104.54
16:2 (n-7)	41.16	41.47	57.37	59.96	33.34	33.14
16:2 (n-4)	84.29	84.26	92.74	93.25	94.38	94.99
16: (n-4)	159.28	161.30	179.32	181.15	182.96	184.11
16:4 (n-1)	1115.84	1121.35	1227.63	1231.65	1291.09	1289.01
18:0	38.78	39.41	64.70	65.79	59.99	60.85
18:1 (n-7)	24.28	24.62	26.75	28.04	28.99	29.72
18:2 (n-6)	17.90	18.04	18.07	18.51	18.57	18.60
18:3	28.00	28.22	29.48	30.03	30.47	30.46
18:3	34.04	33.96	22.86	22.87	22.34	22.58
18:4 (n-3)	162.64	161.96	156.51	159.09	162.63	164.15
20:5 (n-3)	1123.58	1117.12	1016.00	1032.88	1082.73	1087.01
22:6 (n-3)	121.27	124.21	116.26	117.07	126.22	127.89
24:0	12.78	12.91	13.92	14.17	14.64	15.01
24:1 (n-11)	21.08	21.32	20.94	21.28	21.96	22.00

Table 24: Consentration (µg/mL) for heat treated samples stored 1 day at 20 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	209.43	208.22	233.10	234.29	252.04	251.02
16:0	132.25	132.23	156.61	157.72	169.51	169.59
16:1 (n-7)	229.59	225.69	251.14	253.29	267.99	267.82
16:1(n-5)	89.43	90.17	100.66	100.54	107.16	108.15
16:2 (n-7)	53.14	53.43	57.51	57.47	60.04	60.43
16:2 (n-4)	85.87	86.64	94.97	95.52	97.73	98.35
16: (n-4)	163.96	167.63	185.98	187.36	192.66	193.61
16:4 (n-1)	1159.78	1172.45	1305.31	1308.97	1346.89	1344.83
18:0	40.34	42.37	58.62	59.32	59.87	60.91
18:1 (n-7)	24.06	25.19	27.69	27.96	28.76	29.26
18:2 (n-6)	18.12	18.22	18.18	18.13	18.43	18.21
18:3	28.57	29.39	29.94	29.68	30.54	30.75
18:3	30.82	31.22	31.73	31.77	23.12	23.04
18:4 (n-3)	172.81	174.75	174.80	175.73	172.42	171.09
20:5 (n-3)	1203.65	1223.25	1219.96	1220.98	1166.19	1151.69
22:6 (n-3)	132.65	137.43	137.75	139.15	136.13	135.79
24:0	13.23	14.11	13.62	13.82	15.12	15.16
24:1 (n-11)	22.27	22.15	22.40	22.65	23.06	22.88

Table 25: Consentration (µg/mL) for heat treated samples stored 14 days at 20 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	206.93	203.24	178.30	184.10	166.50	169.67
16:0	187.74	184.97	168.10	175.27	158.93	163.37
16:1 (n-7)	288.46	281.84	269.08	279.99	244.86	251.11
16:1(n-5)	117.41	115.13	112.57	116.33	101.85	104.21
16:2 (n-7)	62.47	61.62	60.66	62.07	56.66	57.55
16:2 (n-4)	99.24	97.57	98.36	101.44	88.87	90.89
16: (n-4)	194.59	191.75	197.38	204.71	174.29	179.90
16:4 (n-1)	1312.87	1284.35	1348.18	1398.91	1172.20	1207.26
18:0	60.62	60.04	49.86	51.34	51.92	52.51
18:1 (n-7)	27.34	27.90	30.00	29.44	26.67	26.40
18:2 (n-6)	18.65	18.55	18.48	18.06	18.11	17.87
18:3	29.56	29.46	29.04	28.87	28.21	27.90
18:3	23.14	23.33	31.82	31.78	30.46	30.44
18:4 (n-3)	163.09	162.06	170.53	170.31	156.95	153.75
20:5 (n-3)	1199.22	1176.32	1365.09	1365.73	1199.15	1175.88
22:6 (n-3)	134.01	134.92	151.86	151.46	137.00	135.49
24:0	12.34	12.86	11.87	11.83	13.53	13.46
24:1 (n-11)	19.28	19.54	18.48	18.86	18.62	18.46

Table 26: Consentration (µg/mL) for untreated samples stored 1 day at 4 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	140.74	141.63	173.66	171.23	197.94	196.83
16:0	107.58	106.63	134.07	132.38	152.27	151.68
16:1 (n-7)	177.83	175.48	214.89	212.21	245.69	244.59
16:1(n-5)	70.24	69.54	83.18	82.19	96.85	97.09
16:2 (n-7)	46.88	46.60	52.23	51.82	57.88	57.98
16:2 (n-4)	71.43	70.34	81.53	80.80	93.49	93.67
16: (n-4)	137.20	135.99	160.78	159.16	186.09	187.25
16:4 (n-1)	863.18	859.46	1046.04	1036.53	1298.35	1306.13
18:0	34.52	33.14	48.19	47.32	51.85	52.33
18:1 (n-7)	20.05	19.90	21.98	21.46	27.76	28.60
18:2 (n-6)	17.92	17.98	18.46	18.77	18.23	18.72
18:3	27.99	27.92	30.10	30.42	31.81	32.35
18:3	23.93	23.76	24.69	24.46	35.56	35.93
18:4 (n-3)	157.23	152.73	169.59	169.37	192.94	193.89
20:5 (n-3)	1081.59	1047.30	1178.19	1173.37	1424.29	1435.45
22:6 (n-3)	120.74	118.94	134.77	134.85	159.25	163.31
24:0	12.36	11.73	13.64	14.39	17.04	17.37
24:1 (n-11)	22.22	21.86	23.97	23.96	26.18	26.10

Table 27: Consentration (µg/mL) for untreated samples stored 14 days at 4 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	249.21	261.59	260.57	259.28	259.82	249.21
16:0	203.21	206.68	211.30	217.78	218.09	203.21
16:1 (n-7)	318.05	318.03	324.51	345.73	346.84	318.05
16:1(n-5)	119.95	121.68	125.68	132.96	132.23	119.95
16:2 (n-7)	64.86	65.54	67.09	69.91	69.62	64.86
16:2 (n-4)	109.23	110.50	125.08	122.35	122.33	109.23
16: (n-4)	209.65	223.70	231.50	247.95	246.29	209.65
16:4 (n-1)	1421.23	1466.71	1506.08	1707.32	1691.17	1421.23
18:0	74.21	84.10	87.50	75.32	76.85	74.21
18:1 (n-7)	36.39	37.92	38.63	40.58	41.67	36.39
18:2 (n-6)	19.27	18.99	18.94	19.59	19.48	19.27
18:3	30.92	30.29	30.38	33.57	34.07	30.92
18:3	36.01	34.27	34.32	38.92	39.50	36.01
18:4 (n-3)	184.71	169.15	168.53	216.38	214.78	184.71
20:5 (n-3)	1271.18	1175.98	1169.51	1589.30	1575.28	1271.18
22:6 (n-3)	144.66	138.30	137.69	178.53	176.42	144.66
24:0	16.80	16.48	16.11	16.72	18.26	16.80
24:1 (n-11)	26.35	25.59	27.64	25.99	26.83	26.35

Table 28: Consentration (µg/mL) for untreated samples stored 1 day at 20 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	238.80	243.61	218.03	227.15	250.08	264.65
16:0	170.33	169.56	157.22	164.72	187.88	199.76
16:1 (n-7)	271.49	268.16	244.19	255.65	280.86	298.34
16:1(n-5)	107.77	109.69	101.06	105.43	114.48	121.36
16:2 (n-7)	60.76	61.51	58.11	59.83	63.40	66.12
16:2 (n-4)	99.60	100.73	90.83	93.86	99.82	105.04
16: (n-4)	201.35	204.43	178.66	185.45	197.15	209.47
16:4 (n-1)	1424.86	1415.80	1160.14	1214.74	1326.78	1418.91
18:0	63.52	64.11	59.72	62.14	66.17	70.85
18:1 (n-7)	34.09	34.40	29.37	30.72	34.13	35.89
18:2 (n-6)	18.52	18.67	18.82	18.59	19.66	19.86
18:3	31.84	32.00	32.28	32.41	34.65	34.80
18:3	34.26	34.58	24.13	24.16	26.27	26.25
18:4 (n-3)	196.36	194.47	184.71	184.28	199.52	202.99
20:5 (n-3)	1452.37	1436.55	1273.96	1268.89	1371.96	1407.77
22:6 (n-3)	169.64	173.72	150.08	151.65	162.47	166.67
24:0	16.58	17.32	14.36	14.91	16.61	16.39
24:1 (n-11)	25.54	25.64	23.89	24.80	26.31	26.47

Table 29: Consentration (µg/mL) for untreated samples stored 14 days at 20 °C

Paralell	**1**		**1**		**1**	
Injection	1	2	1	2	1	2
14:0	382.08	364.91	237.14	231.66	280.71	274.25
16:0	298.53	294.93	168.86	162.48	203.28	201.23
16:1 (n-7)	431.85	420.06	258.73	254.48	306.82	303.29
16:1(n-5)	150.49	150.61	108.04	104.65	124.31	123.57
16:2 (n-7)	71.41	71.45	56.68	55.50	62.32	62.07
16:2 (n-4)	107.47	107.83	92.84	91.05	101.01	100.83
16: (n-4)	201.19	202.96	181.32	176.72	202.90	202.47
16:4 (n-1)	1125.75	1118.01	1116.58	1085.05	1284.98	1276.57
18:0	97.25	97.63	53.45	51.73	64.94	64.77
18:1 (n-7)	36.40	36.35	32.98	29.73	48.90	48.68
18:2 (n-6)	22.97	22.73	18.37	18.34	19.07	18.87
18:3	32.02	31.25	28.42	28.42	29.68	29.38
18:3	36.16	35.85	30.76	30.74	31.76	31.61
18:4 (n-3)	189.26	186.58	152.46	153.40	163.16	162.34
20:5 (n-3)	1254.73	1237.14	1026.37	1026.08	1144.50	1140.05
22:6 (n-3)	154.32	155.00	135.35	135.23	152.10	149.12
24:0	17.99	17.17	13.90	14.33	14.73	14.06
24:1 (n-11)	31.40	30.29	22.74	22.46	24.12	23.34