Master thesis for the degree of Master of Pharmacy

Isolation and characterization of wax esters from *Calanus finmarchicus*

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Preface

This project was performed in collaboration with Calanus AS from October 2009 to May 2010. Thank you to Kurt Tande for giving me an interesting project to work with over the last year.

To my great supervisor Einar Jensen. Thank you for your belief in me, and your amazing encouragement over the last year. You have shown me a true enthusiasm and commitment that has really inspired me.

To Ragnar Olsen and Birthe, thank you for helping me with the TLC analysis and lending me your lab and your time.

Thank you to my amazing friend Meera, I have, over the last five years really appreciated your friendship, it has meant the world to me. I will not say that I will miss you, because I do not ever mean to loose you.

And finally to my class and my friends, for making these five years some of the best in my life. I will really miss you guys.

Toril Andersen
Tromsø, May 2010

Abstract

Calanus finmarchicus is a vast resource that until recently have been exploited to a minimal degree only. In the last few years harvesting and processing methods have been developed. There is a large market and great interest in products derived from marine oils. Most of the products today are derived from organisms at a high trophic level, i.e. products derived from cod liver oil. One disadvantage with these products is high levels of fat-soluble pollutants that require extensive purification before the products can be released to the market. As *C. finmarchicus* exists on the first trophic levels it is not polluted and is therefore very interesting as a substitution for cod liver oil, which is the main source for ω-3 fatty acids. In addition to this studies have shown that the oil extracted from the animal, Calanus oil, have beneficial properties and can reduce the risk for coronary heart disease, cancer and inflammation and this could be attributed to the contents of wax esters, high levels of a natural antioxidant, Astaxantine, and high levels of phytosterols. The aim of this thesis was to isolate and characterize the wax esters of the oil, and elucidate their structure.

The wax esters were first separated from the Calanus oil with silica SPE and then transesterified to FAME and FAL. The two structures types were separated into two fractions with APS SPE. The FAME were analyzed as their picolinyl esters in GC-MS for structure elucidation and the FAL as their nicotinate esters and TMS ethers. The fractions from various SPE columns were tested by use of TLC and/or GC-MS to confirm that they contained the actual compounds.

The FAME found were in a range of 14 to 22 carbon chain length, and there were saturated, monounsaturated and polyunsaturated FAME present. The PUFA were 18:4 (n-5, 7, 9, 11) and 22:5 (n-5, 7, 9, 11, 13). The FAL identified ranged from 16 to 22 carbon atoms in chain length, and were mostly monounsaturated, but one saturated compound was found.

The next step would now be to analyze the wax esters in LC-MS to find the complete structure using the pieces of structure found in the FAME and FAL, as there are limited numbers of combinations of the FAME and FAL in a wax ester.

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Abbreviations

AAME	Arachidic acid myristyl ester
APS	Amino propyl silyl
DCM	Dichloro methane
DHA	Docosahexaenoic acid
EI	Electron impact
EPA	Eicosapentaenoic acid
FAL	Fatty alcohol
FAME	Fatty acid methyl ester
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High preformance liquid chromatography
LC	Liquid chromatography
LDL	Low-dencity lipoproteins
MB	Myristyl behenate
MD	Myristyl dodecanoate
MS	Mass spectrometry
MSTFA	N-methyl-N-trimethylsilylfluoroacetamide
M*t*Bu	Methyl *tert*-buthyl eter
PUFA	Polyunsaturated fatty acids
SPE	Solid phase extraction
THF	Tetra hydrofurane
TIC	Total ion count
TLC	Thin-layer chromatography
TMS	Trimethyl silyl
UV	Ultra violet

1 Introduction:

In recent years there has been an increasing interest for new sources for marine products. Of particular interest are organisms that can provide marine lipids since they often contain fatty acids that have a range of well-documented health benefits, from reduced risk of cardiovascular disease, reduction of risk for inflammatory diseases and of certain cancer diseases and they are also essential in the development of normal fetal development [1-3]. It is widely agreed that the intake of marine fatty acids should increase, either by higher consumption of fish or by using dietary supplements of highly concentrated omega-3 fatty acids (ω-3) or by adding these to different foodstuffs [4-6].

Since many environmental pollutants are fat soluble, rather high concentrations can be found in cod liver oil, which has been the main source for edible marine oils. Extensive purification is necessary before the products can be used. The high concentration of these pollutants in cod liver oil is due to bioaccumulation through the food chain. In contradiction, copepods such as *Calanus finmarchicus*, which belong to a lower trophic level, contain only very low amounts of pollutants, and the oil from such organisms can be used without further purification.

One problem with marine oils is instability towards oxidation. The oil extracted from *C. finmarchicus* consist mainly of wax esters, which are much more stable towards oxidation compared to fatty acids. This represents another advantage of oil from *C. finmarchicus* compared with many other products on the market. Since the health benefits of marine oils probably are due to the structure of the fatty acids these lipids are composed of, it is of great interest to characterize the structure of wax esters from *C. finmarchicus.*

In the northern parts of the Atlantic Ocean and in the North Sea there are vast resources of zooplankton like krill (*Thyasnoessa inermis*) and copepods. In the Norwegian Sea alone the production of zooplankton is roughly 200-400 million tons each year. As only 10-15 % of the energy present here is carried on to the next trophic level, there are vast resources available to be harvested.

Compared to krill, the copepods have been exploited to a very small degree. However, in recent years methods to catch and process *C. finmarchicus* have been developed. The uses for these products are many. One of the products, which is the focus of this project, is the oil that is extracted from the organism. It contains among other things a high level of polyunsaturated fatty acids, wax esters, phytosterols and the natural antioxidant, Astaxantine, all of which can have a beneficial effect in a diet.

1.1 The zooplankton Calanus finmarchicus

Calanus finmarchicus, or Raudåte in Norwegian, is a copepod that belongs to the phylum arthopoda, which means joint; this is from the Greek podos, meaning foot. To this phylum, in addition to the copepods, other animals such as insects, crustaceans, arachnids and sea spiders (pycnogonida) belong. This makes it the most numerous phylum on earth, probably containing more than 80 % of all known species [7]. Arthropod is a generic term for all animals that have a segmented body and an outer shell. This shell makes growth difficult and complicated, necessitating moulting, where the old skin or shell dissolves from within while the new is created [7].

C. finmarchicus is a herbivorous animal in the *calanidae* family, which can grow to a size of up to 3 mm in the fully matured adult stage [8]. This is relatively small compared to other zooplankton, like the krill species *Thyasnoessa inermis* and *T. rascal,* that can reach 6-8 mm in their adult stage [9]. *C. finmarchicus* has a life span of approximately one year, being born after the spawning period in parallel with the spring bloom of their food source, phytoplankton. It develops through 12 stages or moults, first 6 naupliar, or larval, stages (N1-N6) and then 6 copepod stages, (C1-C6, C6 being the adult stage). In the late summer the phytoplankton bloom is over, the plankton has reached the C5 stage and the lipid storage is sufficiently high and all of these factors that together will trigger vertical migration into the deep ocean, where they spend the winter diapausing at depths between 400-1500 m [8, 10]. In springtime they resurface to spawn, and this is triggered by the presence of the food source, phytoplankton, which is necessary for the production of an

average of 600 eggs per female in a well-fed state [11]. The eggs are high in lipid content and functions as a food source to the napulii, which does not begin to feed until the N3 stage [11].

As a major constituent of the zooplankton population, *C. finmarchicus* make up about 70-80 % of the zooplankton biomass in the northern part of the Atlantic Ocean. It is an important part in the food chain, and is a link between the primary producers, like phytoplankton, and the commercially exploited species of fish [8, 10].

One of the characteristics of zooplankton from the northern and polar areas of the sea is a high content of storage lipids compared to other zooplankton from more tropical areas [11]. In which of its life stages the copepod exists, and which species the zooplankton belong to, determine the amount of, and which classes of depot fat that are the most prominent. This is also affected by seasonal changes, like in the early spring when the lipid reserves have been utilized, and the localization of the animal, geographically [11]. Grazing on large amounts of phytoplankton over the summer leads to an accumulation of storage lipids in the fall, as a preparation for a winter spent at great depths with no food [9, 11]. In addition to the fatty acids and fatty alcohols that the copepods gain through dietary uptake, it has also been shown that herbivorous zooplankton such as *Calanus finmarchicus,* are able to synthesize fatty acids and fatty alcohols de novo [11].

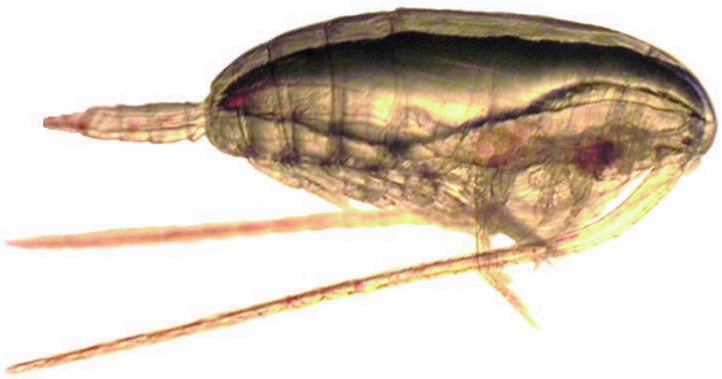

Figure 1: *Calanus finmarchicus*. The picture is used with the permission of K. Tande of Calanus AS

1.1.1 Lipid content of Calanus finmarchicus

As mentioned earlier the lipid composition of copepods and other zooplankton species varies highly with season, life stage, species and geographical location. The main lipids in *C. finmarchicus* are wax esters and triglycerides, which both are neutral lipids. The wax esters of *C. finmarchicus* will, at the end of summer, towards the preparation of the diapause, constitute up to 80-90 % of the total lipid content [10].

The wax esters serve as a long-term energy source and are an addition to triglycerides whose energy is utilized on a more short-term basis [10-12]. The lipids are more advantageous than carbohydrates and proteins as a source of energy, with an energy content of 39 kJ g^{-1} compared to 17-18 kJ g^{-1}, in carbohydrates and proteins [11]. In addition to their usefulness as energy storage, the wax esters are also useful in controlling buoyancy. In surface waters the wax esters provide positive buoyancy, as does all lipids. But during diapause, in the deep, cold water, the compressibility and thermal expansion properties of wax esters are greater than that of seawater and this makes the copepod neutrally buoyant. This minimizes the energy requirement to keep the zooplankton at these depths [10, 11].

1.2 Wax esters

A wax ester is composed of a long chained fatty acid and a long chained fatty alcohol moiety, both of which can be both saturated and unsaturated, and branched or unbranched. The wax esters of *C. finmarchicus* probably consist mainly of unbranched structures of alcohols and acids, where either side contains one or more elements of unsaturation, which is common in the lipids of marine organisms.

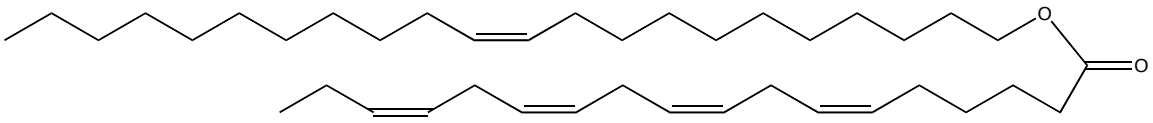

Figure 2: Wax ester composed of 22:1 (n-11) alcohol and 18:4 (n-3) acid

Previous studies show that it can be expected that the major fatty acids are the 20:1(n-11), 20:1(n-9) and 22:1(n-11) and the major fatty alcohols are 20:1(n-9) and 20:1(n-11).

These studies were performed using Gas Chromatogram (GC) equipped with Flame Ionization Detector (FID) [11-14], and the position of double bonds are suggested by comparing retention times of the analytes with the retention time of methyl ester standards with known structure. However, fatty acids with double bonds in different positions might co-elute. Therefore, gas chromatogram-mass spectrometer (GC-MS) should be the preferred method when trying to determine the position of the double bonds.

1.2.1 Benefits of wax esters

Resent studies in rats show that, contrary to prior believes, wax esters can be digested in various terrestrial mammals through bile salt dependent esterases [5, 15]. Several types of wax esters, especially those containing ω-3 fatty acids, can be beneficial as a nutritional- and, to some extent, a medicinal supplement [5, 6, 15, 16]. Studies have shown that it is possible, and can be preferable, to use wax esters containing polyunsaturated fatty acids (PUFA) because of better oxidative stability in storage and in living organisms, as opposed to the fatty acids by themselves [6]. PUFA, such as eicosapentaenoic acid (EPA, 20:5(n-3)) and docosahexaenoic acid (DHA, 22:6(n-3)), has for a long time been acknowledged as an important part of human nutrition and can lower the incidence of atherosclerosis, coronary heart disease, inflammatory disease and cancer [5].

One review article by Hargrove et al. (2003) [15] address the alcohol moiety of the wax ester and suggests that it too could have beneficent qualities, lowering cholesterol- and low-density lipoproteins (LDL) levels in mammalian animals and humans in studies.

1.3 Choice of methods

The Calanus oil, which is very high in wax esters had to be refined so that the wax esters could be studied without interference from the other lipids that are present in the oil. Calanus oil has in previous studies been successfully separated into its individual components of various neutral and polar lipids, and further analysis and processing of the lipids have long been performed.

The first issue was to separate the wax esters from the Calanus oil. A solid phase extraction method (SPE) suited our purpose, and was performed with silica cartridges and an eluent consisting of a mixture of n-hexane and diethyl ether. The purity of the fractions was confirmed with thin layer chromatography (TLC) and gas chromatography-mass spectroscopy. The TLC method was taken, almost unchanged, from another master thesis that deals with the various lipids in *Calanus finmarchicus* [9].

An attempt to study intact wax esters by use of combined Gas Chromatography-Mass Spectrometry was performed. However, the problems with this method were apparent. Due to high molecular weight, most of the wax esters are not volatile enough to be analyzed by GC-MS and even when GC-MS is possible, the resulting MS-spectra are hard to interpret due to absence or very low intense molecular ion. An alternative to studying the whole structure, is to transesterify the ester and study the fatty alcohol moiety and the fatty acid moiety separately in a GC-MS system, and then trying to puzzle together the pieces and bits of information by trying to find the molecular ion in an liquid chromatography-mass spectroscopy system (LC-MS).

For the transesterification of the wax esters two methods was attempted, one obtained from an article [17] and the other based on an established method in another project at this institute [18]. The results obtained from these methods were analyzed with TLC and GC-MS, and as they both were considered adequate, the least complicated one, when considering time and method, was chosen. This method involves the use of acetyl chloride as the reagent.

After the fatty alcohols (FAL) and the fatty acid methyl esters (FAME) were released from the wax esters they had to be separated into distinct fractions in order to study them further. SPE was here considered a good method, but several attempts were needed before the separation was complete. Trying different strategies based on different articles, one method finally achieved good separation of the two substance groups.

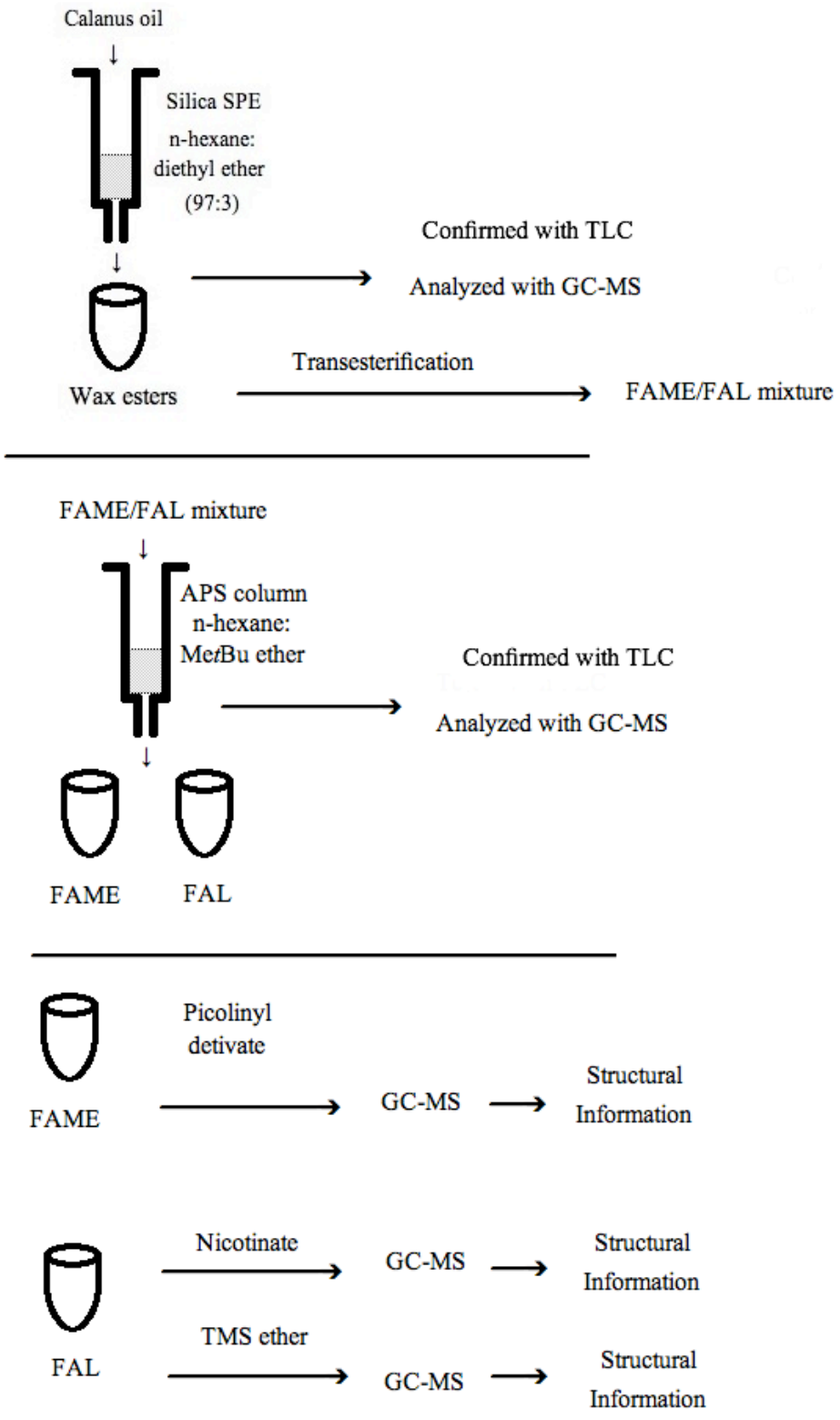

Figure 3: Flow chart of the procedure

The fatty alcohol portion of the wax ester had to be altered chemically in order to obtain good mass spectra, which could help identify the different fatty alcohols with respect to molecular weight, degree of unsaturation and the length of the carbon chain. The alcohols were analyzed as their trimethylsilyl (TMS) ethers and nicotinate esters. The information obtained from these two methods together facilitated full structure elucidation of the FALs.

The fatty acid methyl ester moiety of the wax ester also had to be modified to identify the different variations of the structure present in the samples. The interesting aspects that had to be addressed were molecular weight, carbon chain length and degree of unsaturation. Picolinyl esters of the fatty acids served this purpose well and the method was found described in several articles on the subject.

The methods for production of both the derivates of the fatty alcohols and the fatty acid derivate were analyzed in the GC-MS system.

1.3.1 Sample preparation and testing

Solid phase extraction is a method used for purifying and cleansing samples in order to isolate the lipid groups that are under interest in one fraction. It is based on the principle that molecules have varying degree of affinity for a solid sorbents compared to a solvent. Samples are loaded onto a cartridge containing a porous sorbent, usually silica or a derivate of silica, and the various analytes and other similar molecules will bind with the active groups of the silica. The cartridge can then be washed of pollutants in the sample before the analyte is eluded with an appropriate eluent.

In developing a method of solid phase extraction it is usual to test the method. This can be done with thin layer chromatography, which is a method based on almost the same principle as SPE. The samples, dissolved in a volatile solvent, are applied on a line parallel to one side of a plate with a thin layer of silica. The plate is placed in a container with a small amount of mobile phase, which will be drawn up along the plate by capillary

forces. The different substances in the sample will, if they have different affinity for the silica, have different degree of retardation on the silica, and in this way the sample can be separated into its different constituents. If, alongside the sample, different known substances are also loaded onto the plate in separate positions, the identity of the different constituents can be determined with a fair degree of certainty.

1.3.2 Analysis of the samples

Gas chromatography is a common method for analyzing most classes of lipids, and combined with a mass spectrometer (MS) it is quite useful for deducing the structures of the lipids present in a sample. Many lipid classes are studied as intact molecules, but some have to be split into smaller components because of either thermal instability or low volatility in the normal temperature range of the GC systems. One such example is wax esters that, as mentioned earlier, require high temperature system [19]. In addition to splitting lipids into its building blocks it can also be necessary to chemically alter some structures to reveal more about their structure.

1.3.2.1 Gas chromatography

Gas chromatography is based on the principle that different substances have different boiling points and different degrees of interaction with the stationary phase. The mobile phase in GC have no influence on the retention time of a substance. Separation of different compounds is influenced by their boiling point and their interaction with the stationary phase. In the temperature program of the system, a gradual increase in the temperature on the column is programmed, and this will also influence the separation of the compounds through their varying boiling point. The substances that are best fitted to be analyzed in this system are volatile compounds with an extent of heat stability, because the temperature are usually within the range of 200-250 °C but can be adjusted to both higher and lower based on the volatility of the compounds [20].

1.3.2.2 Mass spectrometry

A mass spectrometer is an analyzer that uses the mass of a molecule and the mass of the fragments of the molecule, to unravel information about the structure. It uses a process

that ionizes the individual molecules in an ion source that can leave the ions intact or split them up into fragments, depending on the stability of the ions, the ions are then separated in a mass separator and then the intensity of the ions are measured in a detector.

1.3.2.2.1 Ion source

In the MS system in this thesis, an electron ionization (EI) ion source was used. In this ion source, which operates under a vacuum, electrons originating from a heated rhenium or wolfram filament are accelerated towards the anode in an electric field of 70 V, giving them the energy of 70 eV. When neutral molecules enter the ion source, they collide with the electrons and are ionized.

$$M + e^- = M^{+\bullet} + 2\,e^-$$

As these are radicals they are not very stable and will easily undergo fragmentation in a pattern that will give the mass specter we observe. This is a plot of the mass to charge ratio against the intensity of the fragments, the most numerous being denoted as 100 % [20, 21].

1.3.2.2.2 Mass separator

The quadrupol analyzer is a system of four parallel cylindrical or hyperbolic rods. These are interconnected in diagonal pairs with opposite electrical charge, and the conformation of the polarity alternates with high frequency. This electric field makes it possible to guide the ions from the ion source along the axis of the structure through to the detector in an oscillating motion. By changing the voltage on the rods it is possible to choose what ions are allowed to reach the detector. Ions with either too large or too small *m/z* values will be deflected in an unstable trajectory and will be lost before detection. Using a computer it is possible to scan a wide range of mass to charge ratios, or it can be used to look for specific ions of interest [20, 21].

1.3.2.3 Gas chromatography-Mass spectroscopy

A cooperation of the two techniques gas chromatography and mass spectroscopy represent a major advantage. The coupling of the two systems will be an easy solution to the problem of injecting complicated mixtures, as the GC will separate the molecules before the MS will analyze them.

1.3.3 Derivates of the FAME and FAL

Both fatty acid methyl esters and fatty alcohols have major drawbacks in MS analysis. The information that can be derived from the mass spectra is incomplete and faulty. Often it is not possible to obtain proper molecule ions in a detectable intensity compared to the noise levels and this severely inhibits the elucidation of the structures. It is therefore advantageous to make derivates of these structures that will yield better mass spectra.

1.3.3.1 Picolinyl derivates of fatty acid methyl esters

Attempts have been made to unravel the structure of the fatty acids of WE by analyzing them as FAME on GC-MS. However, when the FAME is introduced to the MS, the charge introduced on to the molecule will enable the double bonds to become mobile and migrate along the aliphatic chain faster then the fragmentation process. The result of this is that the original position of the double bond cannot be determined. [19]

There are two main ways of circumventing this problem, substitution of the double bond or making derivatives of the carboxyl group that will stabilize the electrons and hinder the relocation of the double bonds. One way of doing this is to introduce a picolinyl ester group in place of the methyl ester. A picolinyl group contains a nitrogen atom that will carry the charge instead of the aliphatic chain and stabilize the double bonds. The derivatives induce cleavage of the aliphatic chain without double bond migration [17, 22].

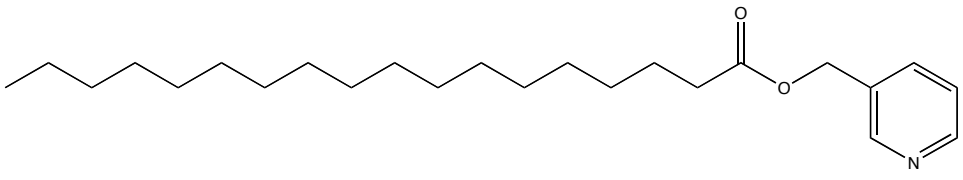

Figure 4: Picolinyl ester of a 18:0 fatty acid

1.3.3.2 Trimethylsilyl derivates of fatty alcohols

One of the drawbacks of analyzing long chained primary aliphatic alcohols is that the molecular ion [$M^{+\cdot}$] is not visible because of large degree of fragmentation in the EI ion source. What can be more visible is the ion produced by a loss of water [M-18]$^{+}$. One way to deduce the molecular ion is to derivatize the hydroxyl group with a trimethylsilyl ester group. The molecular ion is still weak but the [M-15]$^{+}$ ion is abundant, and is a result of the loss of one of the methyl groups [19, 23]. Using this method it is possible to determine the amount of carbon atoms and the degree of saturation in the fatty alcohols, based on the information about the molecular ion.

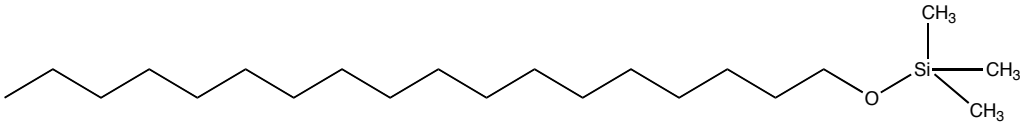

Figure 5: Trimethylsilyl ether a of 18:0 fatty alcohol

1.3.3.3 Nicotinyl derivates of fatty alcohols

Similarly as the problem with the fatty acids, there can be a problem in determining the position of the double bonds in fatty alcohols. Here too it has to do with migration of the double bond and how this happens faster than the fragmentation process. Just as the solution with the fatty acids came from adding an electron-stabilizing group to the carboxyl group, adding a similar group to the hydroxyl group will serve to eliminate this problem.

Nicotinate esters have long been used to obtain information on the location of the double bonds in fatty alcohols, and also other functional groups of interest. As was the case with picolinyl esters, the nicotinates stabilize the double bond and enable radical cleavage of the aliphatic chain without double bond migration [17, 19].

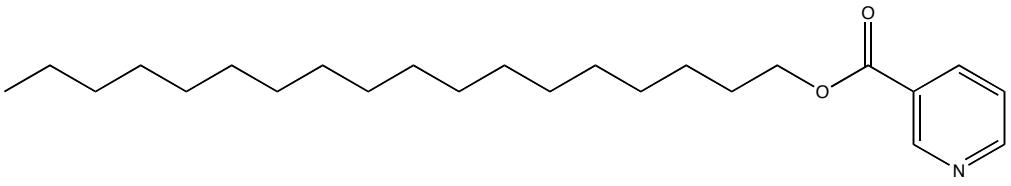

Figure 6: Nicotinyl ester of fatty alcohol 18:0

1.4 The aim of the thesis

The aim of this thesis was to isolate and characterize the wax esters in the Calanus oil extracted from *Calanus finmarchicus* using analytical methods to elucidate their structure.

2 Materials and method:

2.1 Materials

The Calanus oil was kindly provided Calanus AS. The chemicals used were of ACS, Reag. Ph. Eur grade and were mainly from Merck KGaA (Darmstadt, Germany). This include:

n-hexane	(CAS 110-54-3, Lot nr: K39517267)
Diethyl ether	(CAS: 60-29-7, Lot nr: K39633821)
Chloroform	(CAS: 67-66-3, Lot nr: K33904045)
Heptane	(CAS: 142-82-5, Lot nr: K39389879)
Methanol	(CAS: 67-56-1, Lot nr: K39517409)
Toluene	(CAS 108-88-3, Lot nr: K33641025)
Sulfuric acid	(CAS 7664-93-9, Lot nr: K15949231)
Methyl-tert butyl ether	(CAS 1634-04-4, Lot nr: K1289684)
Acetic acid (glacial)	(CAS 64-19-7, Lot nr: K40377663)

There were also chemical bought from other suppliers:

Flucka Chemicals Inc (St. Luis, MO, USA)

Acetyl chloride	CAS 75-36-5, Lot nr: 335614/1
Natriumhydrogensulphate wasserfrei	CAS 7681-38-1, Lot nr: 181844

Sigma Aldrich Inc (St. Luis, MO, USA)

Potassium tert-butoxide	CAS 865-47-4, Lot nr: 1435708
3-pyridine methanol	CAS 100-55-0, Lot nr: S42361-309
Nicotinoylchloride HCl	CAS 20260-53-1, Lot nr: 03511CJ279
Copper(II)sulphate	CAS 7758-98-7, Lot nr: 068K0038

Pierce Chemical (Rockford, IL, USA)

N-methyl-N-trimethylsilylfluoroacetamide	CAS 24589-78-4, Lot nr: AL42573

Dried tetrahydrofurane (THF) and dried dichloromethane (DMC) was dried at the chemistry laboratory and the Drug Chemistry laboratory respectively, both of which are at the University of Tromsø.

In the solid phase extractions a Visiprep vacuum manifold (Supelco, Bellafonte, PA, USA) was used. In the isolation of wax esters from Calanus oil (see chapter 2.2.2) a Sep-Pak® vac 3cc (500 mg) Certified Silica Cartridge SPE column (Waters, Milford, MA, USA, lot. Number: 010139016A) was used and for the separation of FAME and FAL with a SPE method (see chapter 2.2.4) a Bond elut aminopropylsilyl (APS) solid phase extraction column (Varian Inc, Habour city, CA, USA, Lot. 1119301) was used.

The thin layer chromatography was performed on a HP-TLC plate (Silica gel 60, 10 x 10 cm, Merck, Darmstadt, Germany) and the developed plates were imaged using a GelDoc plate reader (BioRad GelDoc XR, Hercules, CA, USA) using UV-light.

The standards that were used to test the different experiments were bought from different suppliers. The WE standards were purchased from Sigma Aldrich Inc (St. Lois, MO, USA):

Myristyl dodecanoate	(CAS: 22412-97-1, Lot nr: 105H1289)
Myristyl behenate	(CAS 42233-09-0, Lot nr: 087F0766)
Aracidic acid mytistyl ester	(CAS 22143-04-3, Lot nr: 46498MJ)

The FAME standards used to confirm the presence of FAME in the TLC runs and to test the applicability of the methods on our oil sample were bought from Supelco (Bellafonte, PA, USA), these were the 37 FAME mixture, GLC-40, GLC-50 and GLC-80. The FAME composition of the latter three is given in table 1 with the percentage of each constituent. The 37 FAME mix contains as the name indicates, a mixture of 37 different FAME ranging from C4:0 to C22:6 n3 with the amount either 2, 4 or 6 %.

The FAL standards Dodecanol, Tetradecanol, Hexadecanol and Octadecanol were all purchased from Larodan (Malmö, Sweden) with the CAS numbers 40-1200, 40-1400, 40-1600 and 40-1800, and the Lot nrs: 8354:9, F219:6, 2123:4, and 1199-5 respectively

Table 1: The content of FAME in the different standards that had been used. The numbers indicate the percentage of each of the FAME

	Weight percent of each component		
Mixture	GLC-40	GLC-50	GLC-80
C13:0 Me.Tridecanoate			20.0
C14:0 Me.Myristate			20.0
C15:0 Me.Pentadecanoate			20.0
C16:0 Me.Palmitate	25.0		20.0
C16:1 Me.Palmitoleate		25.0	
C17:0 Me.Heptadecanoate			20.0
C18:0 Me.Stearate	25.0		
C18:1 Me.Oleate		25.0	
C20:0 Me.Arachidate	25.0		
C20:1 Me.Eicosenate		25.0	
C22:0 Me.Behenate	25.0		
C22:1 Me.Ecurate		25.0	

2.2 Method

2.2.1 The GC-MS analysis

The GC-MS analysis was performed similarly for all the samples. The fractions that were gathered in the solid phase extraction were transferred to GC-MS vials (roughly 1 mL) for analysis on a Quattro micro GC Micromass MS technologies (Waters, Milford, MA, USA) with a 6890N Network GC systems (Agilent Technologies, Santa Clara, CA, USA) and a 7683B Series injector (Agilent Technologies, Santa Clara, CA, USA). The samples were injected with a splitless injector to confirm the identity of the wax esters. The system utilized EI ionization.

The column in the GC was a DB-5MS from J&W Scientific (Folsom, CA, USA). It was 30 meters long, had an inner diameter of 0.25 mm and a film thickness of 0.25 μm. The temperature in the splitless injector was 250 °C, it had a purge time of 2 minutes and a

purge flow of 50 mL min^{-1}, the carrier gas was helium. The initial temperature on the column was 60 °C and it was held for 5 minutes, it then increased to 290 °C by 30 °C min^{-1} and the temperature was held for 23 minutes. The mass range in the in the MS was 40-450 *m/z*.

2.2.2 Solid phase extraction of wax esters

Wax esters were isolated from the Calanus oil using solid phase extraction, the method used were loosely based on the method described by Vaghela et al. [24] and the method described in the master thesis of A. Pedersen [9]. A Sep-Pak® Silica Cartridge SPE column was placed on a vacuum manifold and conditioned with 2 mL of n-hexane p.a grade. The solutions were pulled through the column at a speed of 1-2 drops per minute, using negative pressure. The oil sample (30 mg) was dissolved in n-hexane (1 mL) and loaded onto the column. The eluent of the sample was collected for analysis with the different elution fractions. The wax esters were eluted with 9 mL of n-hexane:diethyl ether (97:3) divided into fractions of 3 mL. All the fractions, including sample eluate, were collected for further analysis.

2.2.2.1 Analysis of wax esters using gas chromatography- mass spectroscopy

The different fractions of eluate from the SPE were evaporated to dryness under a stream of nitrogen. They were dissolved in n-hexane and transferred to GC vials from Waters (Milford, MA, USA). Each fraction was analyzed on the GC-MS system described above.

2.2.2.2 Thin layer chromatography of isolated wax esters

The different fractions that were collected from the solid phase extraction of the wax esters were analyzed using thin layer chromatography based on the method described by A. Pedersen in her master thesis [9]. The solvent was evaporated from the samples and they were dissolved in 1 mL of chloroform, the standards that were to be used was dissolved in the same solute to a concentration of roughly 25 mg/mL.

The samples were placed on a HP-TLC plate in volumes of 1 µL using a finnpipette on a line one centimeter from the bottom of the plate. The plates were placed in a

chromatography tank containing a solution of heptane:diethyl ether:acetic acid (70:30:1 v/v), which reached less than one centimeter up along the walls of the container, and which had been allowed to saturate the atmosphere of the closed vessel.

The TLC plate was removed when the mobile phase had run up to about one centimeter from the top of the plate. The plate was dried and then sprayed evenly with a solution of copper sulphate (10 %) and phosphoric acid (8 %) in heptane. The plate was then left to dry for 10 minutes before the plate was placed in an oven at room temperature, and was heated to about 170 ºC. The samples were then visible as dark spots on the plate, and the plate was developed under UV light on a GelDoc plate reader. The samples were identified using known standards containing wax esters and compared to unprocessed Calanus oil.

After the analysis of the different fractions with thin-layer chromatography it was clear that the only fraction containing any interesting substances, was the one from the first three milliliters of the eluent. Subsequent to a confirmative TLC run, with wax esters standards it was therefore decided to only include this fraction in the further sample preparations

2.2.3 Transesterification of wax esters

2.2.3.1 Transesterification of wax esters using Acetyl chloride

The solvents in the first fraction of the SPE run containing the wax esters, was evaporated under a stream of nitrogen. From the oily residue 20 µL was transferred to another screw-capped vial and dissolved in 1 mL of a solution of methanol:toluene (3:2 v/v). When the lipids were fully dissolved, 1 mL of methanol: acetyl chloride (20:1 v/v) was added, before the solution was heated for one hour at 100 °C.

After cooling 1 mL of n-hexane was added and 200 µL of the organic phase (the upper one) was transferred to a GC-MS vial for analysis.

2.2.3.2 Transesterification of wax esters using sulfuric acid

Another method for transesterification of the wax esters was tried. It was based on the method described by Y.-G. Joh et al. [17]. A sample of the first three milliliters of the eluation liquid form the SPE isolation of wax esters was evaporated under a stream of nitrogen. From the oily residue of the sample 20 μL was removed and transferred to a screw-capped vial and dissolved in 0,50 mL of toluene. To the vials 2 mL of a solution of 2 % sulfuric acid in methanol was added. The solution was heated to 60 ºC for 36 hours.

At the end of the 36 hours a mixture of n-hexan:diethyl ether (1:1) was added to the cooled liquid. The sample was partially dried under a stream of nitrogen until only a residual liquid was left, 1 mL of n-hexane was added and a two-phase system was created. The upper, organic phase was transferred to a GC-MS vial and analyzed on the GC-MS as described above.

2.2.3.3 Confirmation of the transesterification methods by TLC

Both of the transesterification methods used were tested by TLC, using the same method as described in "thin layer chromatography of isolated wax esters" (2.2.2.2) the transesterified wax esters were compared to standards of fatty acid methyl esters, GLC-80, a fatty alcohol standard, octadodecanol, and a wax ester standard on a HP-TLC plate to confirm the transformation. Both methods were compared on the same plate, so it was clear that the first method, using acetyl chloride as the reagent, was preferable, but only in convenience of the method.

Both methods were also compared using the GC-MS with the production of equal results.

Because analysis of these two methods, both by GC-MS and TLC showed that they were equal in results, the first method was chosen because of its ease and convenience, time wise and in method.

2.2.4 Separation of fatty acid methyl esters and fatty alcohols

The method that in the end was used for further experiments was based on a method described by Y-G. Joh [17]. At first the method described in this article was used to its full extent.

The samples containing fatty acid methyl esters (FAME) and fatty alcohols (FAL) were separated on a Bond elut aminopropylsilyl solid phase extraction column. A sample of transesterified wax esters was evaporated to dryness under a stream of nitrogen and dissolved in 1 mL of n-hexane. The solution was loaded onto a column that had been prepared with 3 mL of n-hexane.

As described in the article the fraction that should contain FAME were eluted with 10 mL of n-hexane:methyl-tert-butyl ether (M*t*Bu ether) (98:2 v/v), the column was then washed with 5 mL of n-hexane:M*t*Bu ether (90:10 v/v), and finally the fatty alcohols were eluted with 10 mL of n-hexane:M*t*Bu ether (75:25 v/v).

Each of the fractions, including the sample eluate, were collected and dried under a stream of nitrogen, before they were dissolved in n-hexane and analyzed on the GS-MS.

The result of this was that all the peaks were collected in the first fraction and the separation was incomplete. Because of this we tried to alter the method.

The first alteration to the method was to separate the first fraction, 10 mL of n-hexane:M*t*Bu ether (98:2 v/v), into 10 different 1 mL fractions because all of the peaks in the GC-MS analysis were collected in this fraction. But because of the amount of the resulting fractions and the low concentration of FAME and FAL in the fractions where they were present, this method was also deemed inconvenient and unusable.

The next thing that was attempted was to disregard the method described in the article entirely and base our next experiment on the fact that on a silica based TLC-plate, the two different substance groups are separated with the solvent used in those experiments,

heptane:diethyl ether:acetic acid (70:30:1 v/v). In the first attempt this solvent was used as an eluent on silica SPE columns in 5 fractions of 2 mL each.

Using TLC plates and the appropriate standards of FAME and FAL to test this method similarly to the previously described thin-layer chromatography methods, it was revealed that the result again was all the substances were gathered in the first fraction. In order to test this method again the first fraction of 2 mL heptane:diethyl ether:acetic acid (70:30:1 v/v) was separated into two equal 1 mL fractions, but to no end, the result was the same.

Finally it was decided to return to, and to try to modify the first method further. By trial and testing various amounts of the three original solvents, n-hexane:M*t*Bu ether (98:2 v/v), n-hexane:M*t*Bu ether (90:10 v/v) and n-hexane:M*t*Bu ether (75:25 v/v), a method that worked was determined. This final successful method, used in all further sample preparations of isolated FAME and isolated FAL, is as follows.

Firstly an APS column was placed on a Visiprep vacuum manifold and conditioned with 2 mL of n-hexane. The sample, a portion of the isolated wax esters, dried and redissolved in 0.5 mL n-hexane, was loaded onto the column. The column was washed with 1 mL of n-hexane:M*t*Bu ether (98:2 v/v). The FAME was eluted with 2 mL of n-hexane:M*t*Bu ether (98:2 v/v), and the FAL was eluted with 5 mL of n-hexane:M*t*Bu ether (75:25 v/v). The fractions were dried under a stream of nitrogen and the method was confirmed in TLC, using the same method as described above.

2.2.5 Derivates of FAME and FAL

2.2.5.1 Derivatization of fatty acid methyl esters (FAME)

Using the method described by N. Dubois [22] the FAME was transferred to a picolinyl esters derivative so that the double bond pattern could be determined on a GC-MS system. A 1 M solution of potassium tert-butoxide in dried tetrahydrofurane was mixed with 3-pyridine methanol in a ratio of 1:2 (v/v). The isolated FAME fraction of the

transesterified wax esters was dried under a stream of nitrogen, and the residual oily substance was dissolved in dried dichloromethane. After the FAME had been fully dissolved, 0.5 mL of the reagent mixture was added and the solution was heated at 45 ºC for 45 minutes.

After a period of cooling 2 mL of water and 2 mL of n-hexane was added and the mixture was shaken lightly. The organic phase, in the upper layer, was collected and another 2 mL of water was added to wash the mixture further. The organic phase was collected, dried over anhydrous sodium sulphate and evaporated under a stream of nitrogen. The dry sample was dissolved in 1 mL of n-hexane and analyzed on the GC-MS.

In the chromatogram of the result there was no change from the underivatized fraction. Therefore it was decided to attempt a different stoichiometry, the method was applied to two more samples, one containing only 10 % and the other 1 % of the original fraction of FAME. In the 1 % sample there was not many signals, but the 10 % test was successful and the underivatized peaks had disappeared altogether.

2.2.5.2 Derivatization of fatty alcohols as trimethylsilyl ethers

In order to learn more about the structures of the fatty alcohols, the purified fractions of fatty alcohols were derivatized to their trimethylsilyl esters. These products give a more intense molecular ion and a $[M-15]^+$ and the spectra also give more structural information. The fraction containing the FAL isolated from the transesterified wax esters was evaporated to dryness under a stream of nitrogen. The oily residue left over was mixed with 200 μL of N-methyl-N-trimethylsilylfluoroacetamide (MSTFA) and 20 μL of methanol in a screw-capped vial. The vial was sealed securely; the mixture was mixed well and heated at 60 °C for 60 minutes on a heating block. After the vial was cooled, the solution was transferred to a GC-MS vial and analyzed directly by use of GC-MS.

2.2.5.3 Derivatization of fatty alcohols as nicotinyl esters

In order to obtain even further information about the structures of the fatty alcohols they were derivatized to nicotinyl esters, using the method described in the book “Lipid

analysis: a practical approach" [19]. This would enable us to determine the double bond pattern of the FAL, similarly to what the picolinyl derivates of the FAME enabled us to.

A fraction of FAL isolated from the transesterified wax esters was dried under a stream of nitrogen in a screw-capped vial and 400 µL of a saturated solution of nicotinyl chloride hydrochloride in dry acetonitrile (a solution of roughly 5%) was added. The solution was heated on a heating block for 10 minutes at 100º C in the sealed vial. After the solution had been allowed to cool, water and n-hexane was added (1 mL of each). The mixture was shaken lightly. The hexane layer was decanted (the upper, clear layer) and analyzed directly on the GC-MS [19].

3 Results:

3.1 Isolating wax esters from Calanus oil

Before further analysis, the wax esters had to be isolated from the Calanus oil. This was done with a SPE method using silica cartridges and n-hexane: diethyl ether (97:3 v/v) in 3 fractions of 3 mL each. All the fractions and the sample eluate were collected and analyzed with TLC and GC-MS.

The method was first tested on two wax ester standards with known structure, dissolved in hexane. These two, myristyl dodecanoate (MD) and arachidic acid myristyl ester (AAME) had molecule weights of 396 amu and 508 amu respectively. In the chromatogram resulting from the analysis of the different eluates there was only one peak present, and this was in the first fraction (0-3 mL). In the mass spectrum of this chromatographic peak, the highest *m/z* value observed was 396. This clearly indicate that this peak represent myristyl dodecanoate. It was suspected that AAME was not volatile enough at this temperature to enter the column. Even after increasing the maximum temperature in the temperature program to 300 °C there was no peak that could be assigned to the larger of the two standards, AAME.

In the thin-layer chromatography plate of the different fractions this was confirmed too. One spot showed up in the first fraction (0-3 mL) and it was compared to standards that had not been through the process. After testing the whole process with only AAME it was confirmed that it too was located in the first fraction (0-3 mL), even though it did not appear in the chromatogram it was apparent on the TLC plate.

The method was then applied to the Calanus oil. When the eluates from each fraction and the sample loading was dried under a stream of nitrogen there was an oily residue in the 0-3 mL fraction, in the other fractions there was no noticeable residue. The fractions were analyzed in the GC-MS system and as expected after the analysis of the standards there

was not much to see. The wax esters of *C. finmarchicus* are expected to be larger than the biggest wax ester standard, AAME, which contains 34 carbon atoms. In the second and the third fraction there were just noise, but in the sample eluate and in the first fraction there were one peak in each, both of which had the same retention time. This was an indication that the two peaks arised from the same compound, in addition to this the spectra were very similar, giving an even stronger indication of there being only one compound. Using the intensity of what probably was the molecular ion, *m/z* 268, and the intensity of the C^{13} M+1 at *m/z* 269 the number of carbon atoms was calculated to be 19 using the equation:

$$n = \frac{\left(100\% \times A_{M^{\bullet+}}\right)}{\left(A_{M+1} \times 1{,}1\%\right)}$$

Where n is the number of carbon atoms, A_M is the intensity of the molecular ion, A_{M+1} is the intensity of the molecular ion containing one carbon of the C^{13} isotope. As the latter constitutes 1.1 % of all carbon atoms this will help us calculate the number of carbon atoms, given that the intensities are known. The mass spectrum contained elements that pointed to the structure of the molecule. The low mass part of the spectrum contained the fragment pattern 43, 57, 71, 85 and 99, which is typical in the mass spectra of aliphatic compounds. In addition, high intense *m/z* values 113 and 185 were observed an indication of there being a branched structure, and the structure arrived at had a methyl group in the 7-position.

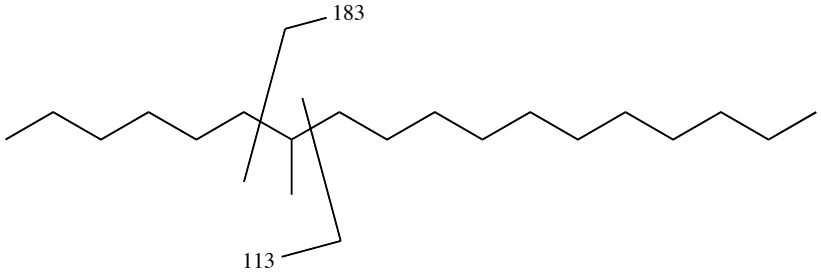

Figure 7: 7-methyloctadecane, the hydrocarbon identified in Calanus oil

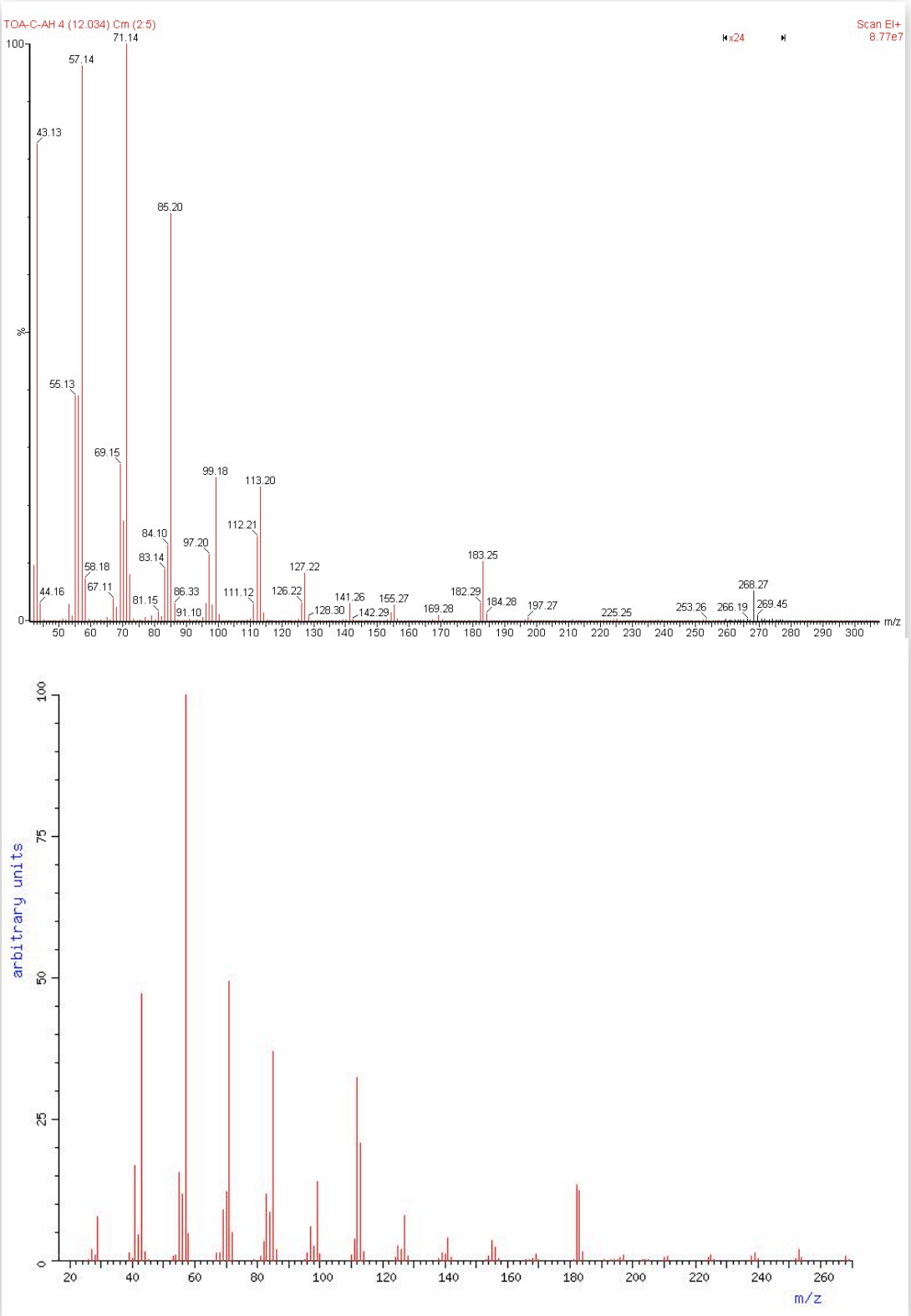

Figure 8: The upper spectrum is the MS-spectrum of the hydrocarbon, 7-methyloctadecan, found in the sample. The lower spectrum is the MS-spectrum of the same substance taken from literature. Both of the spectra have intense peaks at *m/z* values 113 and 185. The molecular ion, which can just be made out in both spectra is 268 amu.

The structure was confirmed by searching the SciFinder Scholar database where the mass spectrum of this structure was found and was very similar to the mass spectrum that was obtained experimentally.

This molecule, 7-methyloctadecane, which has the CAS number 26741-16-2 has been seen in mosquito larva and, as one among other similar hydrocarbons, works as a response to overcrowding in the population. This overcrowding factor is toxic to the different stages of the mosquito larva and will give growth retardation in the surviving larval population [25].

The analysis of the SPE fractions and the sample eluate, along with known standards, by TLC resulted in a plate (figure 9) that confirmed that the WE of Calanus oil behaved similarly to the WE standards. Aliquots of the fractions were applied on a plate together

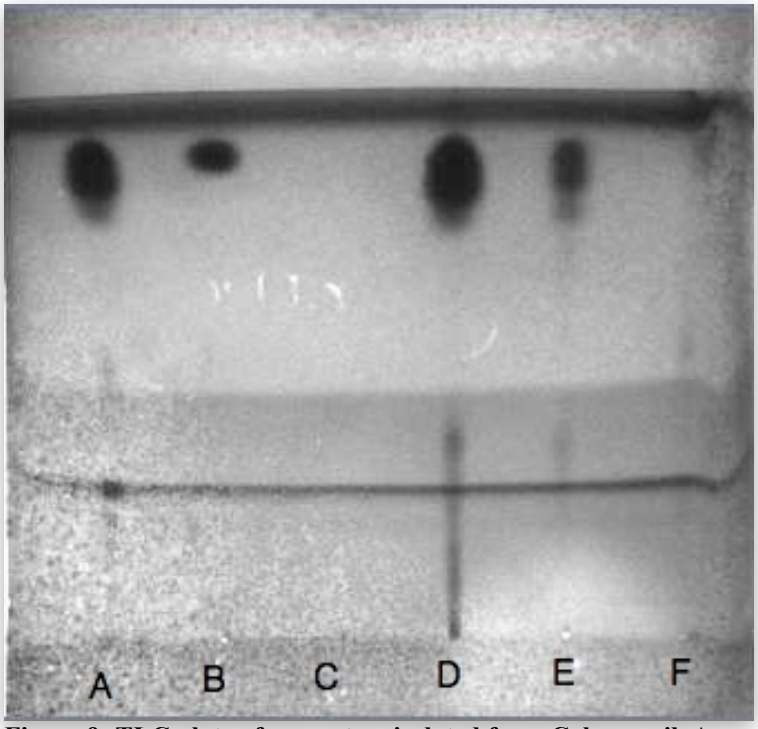

Figure 9: TLC plate of wax esters isolated from Calanus oil. A: Calanus oil, B: WE standard MD and AAME, C: sample eluate, D: fraction 1 (0-3 mL), E: fraction 2 (3-6 mL), F: fraction 3 (6-9 mL). Both fractions 2 and 3 are products of 6 parallels added together

with wax ester standard and Calanus oil that had not been processed. Because it was assumed that the second fraction (3-6 mL) and the third fraction (6-9 mL) would not contain any WE or only low amounts of WE, six parallel fractions were pooled together before the analysis by TLC, so that it would be completely certain that there was nothing of interest. There was a faint spot in the second fraction with the same R_f value as the wax esters in the second fraction, but it was very small and faint. The method was not altered based on these results.

3.2 Transesterification of wax esters

The wax esters isolated from Calanus oil had to be transesterified in order to be analyzed properly. This was initially tested with two different methods, one using acetyl chloride as the reagent and the other using sulphuric acid as the reagent. The former was a quick process that required about an hour to perform, and the latter was a more extensive process that had to be left to work for a-day-and-a-half. Aside from this inconvenience in one of the methods, both worked adequately.

The products obtained after transesterification, was analyzed by TLC and standards of a wax ester, Myristyl dodecanoate, a FAL, Octadecanol, and a mixture of FAMEs, 37 FAME mix were analyzed on the same plate. In both the methods there where spots that aligned with both the FAME and the FAL spots, so it was clear that the processes were successful methods, there were also spots in close proximity to the spot correlating to the FAME standard, the identity of this has not been determined, but one suspicion was that it is a result of the dual spots of the wax esters from Calanus oil which arises from two types of saturations [26], meaning that the duality is because of the FAME moiety and that the FAME has the most varying degree of saturation and unsaturation. On both plates there were also faint traces of spots that aligned with the WE standards, this indicates that neither of the two methods had complete transformation of the wax esters. However since the spots were very faint the method was not altered as a response to this.

As the two methods had been proved adequate and equal, either of the methods could be chosen for further use. The method where acetyl chloride was the reagent was by far the easier method; both in time consumption and the method in it self was less complicated to perform, and was consequently selected.

The analysis of this fraction resulted in a chromatogram where there was several peaks, all but one of which where new, the hydrocarbon peak in the wax ester fraction of the silica SPE run was still present. The mass spectra of these peaks indicated that they were a mixture of both FAME and FAL, which was expected. The mass spectra of those peaks that were assumed to be FAME generally had a 74 *m/z* peak that is a highly intense peak and corresponds to the McLafferty rearrangement of the methyl ester. This generally is the case for FAME spectra, but increasing degree of unsaturation will decrease the intensity of this peak [19]. In addition to this they also contain generally one or two peaks in the *m/z* range of 100-300 that are higher than the noise ratio, but these cannot give any indication of the structure of the substances. The molecule ion is not detectable or if there are patterns of peaks present that can indicate $M^{\bullet+}$, there are often several candidates in the same mass spectrum. For the sake of comparison FAME standards GLC-40 was also analyzed in the GC-MS system. This standard contained the saturated FAME C16:0, C18:0, C20:0 and C22:0. The spectra of these standards had a pattern similar to those for the suspected FAME peaks.

In the mass spectra of was presumably FAL the *m/z* value 74 was not present as a prominent peak and this difference was used as a vague indication that it was not a FAME as a quick indication in later analysis. The molecular ion was hard to determine here as well but there were a number of peaks in the upper parts of the spectrum that could be candidates.

3.3 Separation of fatty acid methyl esters and fatty alcohols

The FAME and the FAL were successfully separated into two fractions after several failed attempts. The method finally used was based on the use of an APS-SPE column.

Three fractions were collected, the first two eluted with 1 mL and 2 mL of n-hexane:M*t*Bu ether (98:2 v/v) respectively and the third with 5 mL n-hexane:M*t*Bu ether (75:25 v/v).

After the GC-MS analysis showed that the sample eluate and the first fraction of 1 mL n-hexane:M*t*Bu ether (98:2 v/v) only contained the hydrocarbon, 7-methyloctadecane. This is beneficial as it will no longer be present in the following analyzes as a disrupting factor. The GC-MS analysis of the second and third fraction resulted in chromatograms with several peaks, these fractions contained the second and third milliliters of n-hexane:M*t*Bu ether (98:2 v/v), there where a number of peaks and all of them contained a high intensity of the 74 *m/z* ion that indicate that the substances are FAME. They were also similar to the mass spectra of the peaks suspected to be FAME in the chromatogram of the FAME/FAL mixture and similar to the mass spectra of FAME standards. The fourth 1 mL fraction of n-hexane:M*t*Bu ether (98:2 v/v) did not contain any peaks.

The chromatogram of the fifth fraction, where the eluent was 5 mL of n-hexane:M*t*Bu ether (75:25 v/v), also contained a number of peaks that, presumably, corresponds to the FAL stemming from the wax esters of Calanus oil. The mass spectra of these peaks were very similar to the mass spectra presumed to be FAL in the FAME/FAL mixture. The absence of a high intense 74 *m/z* peak was used as crude means of differentiating between the FAME and the FAL. The sixth fraction, the second 5 mL of n-hexane:M*t*Bu ether 75:25 v/v), did not contain any peaks.

For a more certain determination of whether the separation of FAME from FAL was complete or not, the fractions and the sample eluate was analyzed with TLC. Alongside the samples a FAL standard, Octadecanol, and a FAME standard, GLC-80, was included. As expected the sample eluate and the first fraction did not contain any spots as the hydrocarbon do not show as a spot. The second and the third fraction contained two spots both of which correlated to the two spots of the FAME standard. Although there was used a different FAME standard this does not explain the sudden appearance of a second spot in both the FAME standards band and the bands of the fractions that should contain

FAME. As the new spot do not show up in the other bands it is not a contamination either. The fourth fraction did not contain any spots, which was expected and supports the findings of the GC-MS analysis. The fifth fraction, the first eluted with 5 mL of n-hexane:M*t*Bu ether (75:25 v/v), contained a spot that correlated to the FAL standard and the sixth fraction, the last 5 mL of n-hexane:M*t*Bu ether (75:25 v/v) did not contain any spots.

3.4 Derivates of FAME and FAL

3.4.1 Picolinyl derivates of FAME

To obtain experience with picolinyl derivatization, the method was applied to a FAME standard, GLC-50. This contained the mono unsaturated FAME C16:1, C18:1, C20:1 and C22:1. The resulting solution was analyzed in the GC-MS system. In the resulting chromatogram there was four peaks present. According to literature ions at *m/z* values 92, 108, 151 and 164 are supposed to be prominent, 151 is the McLafferty rearrangement and the other are fragments containing the pyridine ring. The molecular ion is odd numbered because of the presence of the nitrogen atom, and is therefore easily distinguished from the nitrogen containing fragment ions, which are even numbered [27]. The loss of increasing lengths of the aliphatic chain was visible as clear peaks with generally 14 amu between them at the loss of another methylen group. By counting backwards from the molecular ion, double bonds can be located by a 12 amu gap between ions, but it can be difficult to decide exactly where the double bond is based on this alone. Generally the double bond is located between the two ions that have a 26 amu gap, not a 28. Using the molecular ion it can be determined the amount of carbon atoms in the aliphatic chain and the amount of double bonds that needs to be located [17, 27].

Using these techniques the position of the double bonds in the monounsaturated FAME was determined and after consulting literature for the actual position and realizing that it was correct, it was determined that the technique was successful.

The method was then applied to the second fraction (2-3 mL of n-hexane:M*t*Bu ether (98:2 v/v)) from the isolated FAME/FAL mixture. The reagent in this reaction was 3-pyridine methanol in THF. The gas chromatogram resulting from this sample was dissimilar to that of the sample before the reaction, the retention times were longer and the peaks resembled those in the chromatogram of the derivatized standards.

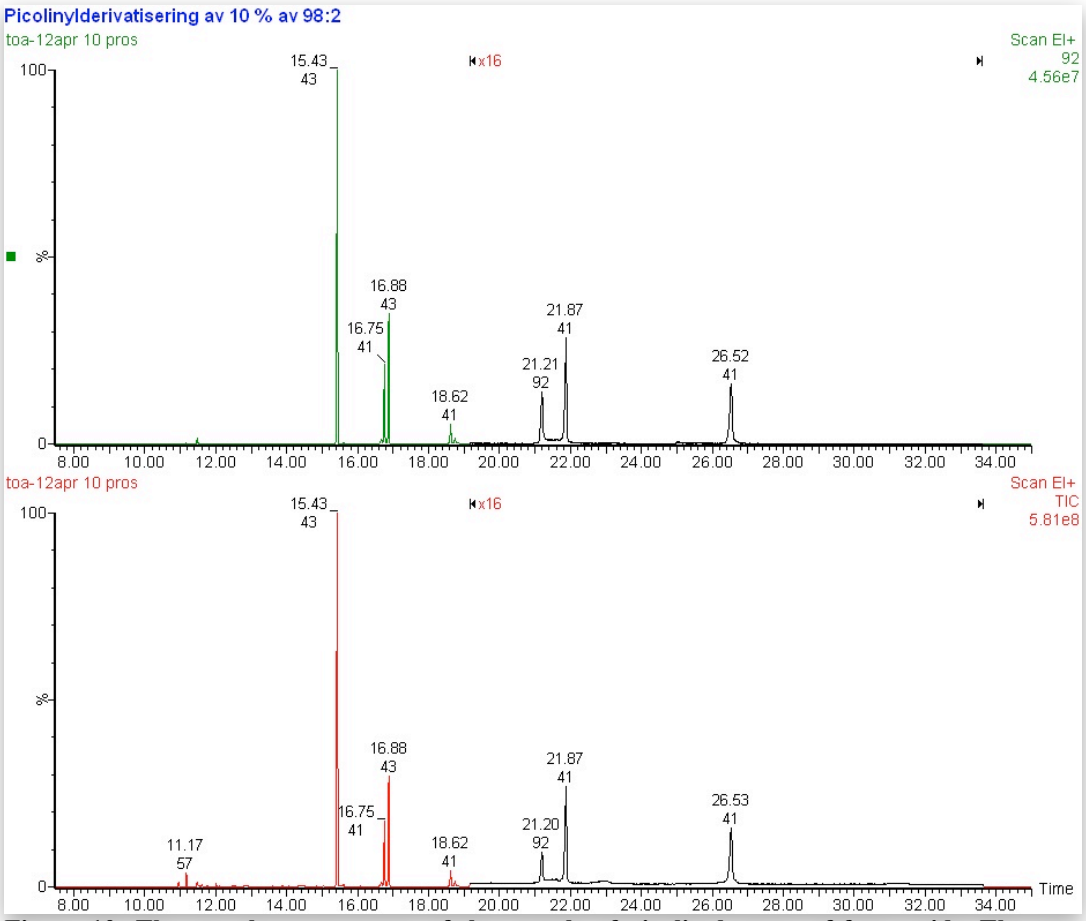

Figure 10: The gas chromatograms of the sample of picolinyl esters of fatty acids. The upper shows the peaks that contain the *m/z* value 92, which is a diagnostic ion for picolinyl esters, and the lower shows the total ion count (TIC)

The mass spectra of the peaks also resembled those of the picolinyl ester standards, with the same collection of peaks that were present as in the standards. In the larger chromatographic peaks it was easy to determine all the peaks in the mass spectrum that were essential in elucidating the structure of the esters. In some of the later eluting peaks

in the chromatogram, which were quite small in size, the signal-to-noise ratio were lower so in some cases the structure was harder to elucidate.

The mass spectra showed that a collection of fatty acids were present in the sample. Both saturated, monounsaturated and polyunsaturated fatty acid were located, ranging from 14-carbon chain length to chains of 22 carbons.

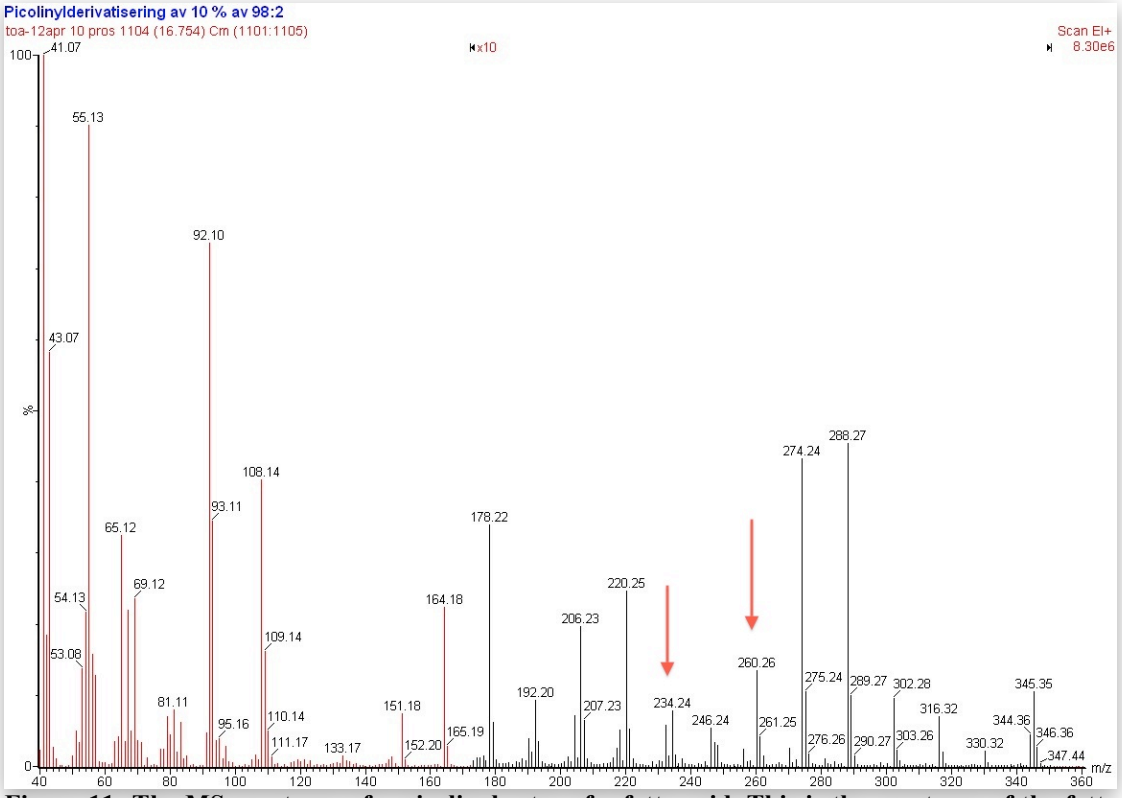

Figure 11: The MS-spectrum of a picolinyl ester of a fatty acid. This is the spectrum of the fatty acid 16:1 (n-7).

The mass spectrum in figure 11 is the spectrum of the fatty acid 16:1 (n-7), which is one of the smaller fatty acids in the sample, the structure of this is shown in figure 12. In the spectrum there are the diagnostic *m/z* values 92, 108, 151 and 164, which is a clear indication of it being a picolinyl ester. In addition the pattern of peaks that are 14 amu apart is characteristic. The high intense peaks 274 and 288 are typical of the two ions that are cleaved on the distal side of the double bond in monounsaturated fatty acids.

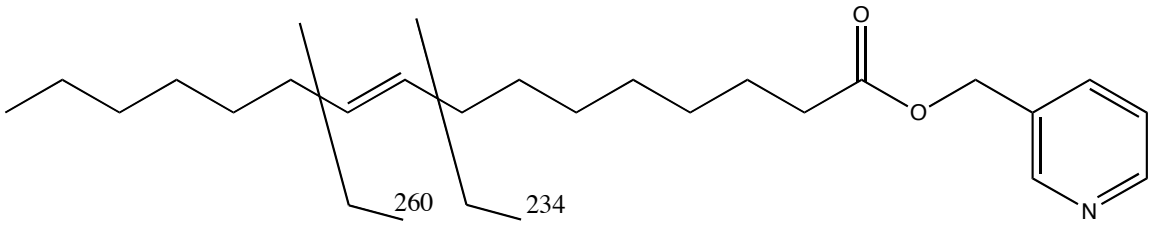

Figure 12: The structure of the 16:1 (n-7) picolinyl ester. The fragmentation around the double bond that identifies the position is marked.

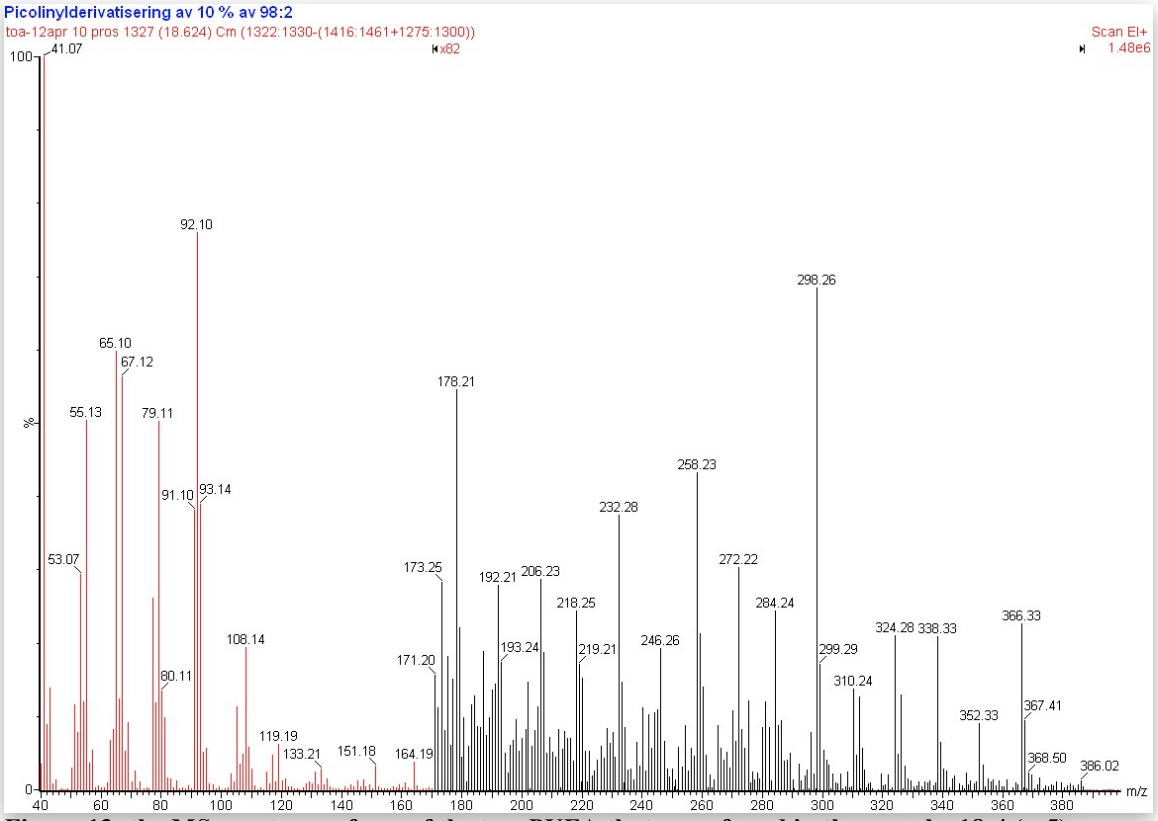

Figure 13: the MS-spectrum of one of the two PUFA that were found in the sample, 18:4 (n-5)

The obtained results indicated that the sample contained at least two polyunsaturated fatty acids. Figure 13 shows the spectrum of one of them, which had a retention time of 18.6 minutes, the structure of this is shown in figure 14. As this is a conjugated system of double bonds there are a series of 26 amu gaps present. Because this was one of the smallest peaks in the chromatogram the signal-to-noise ratio is quite high, but the ions that mark the loss of increasing amounts of methylen groups are still easily distinguished. In figure 14 the fragmentation around the double bonds are marked and these shows the gaps of 26 amu that identifies the position of the double bonds.

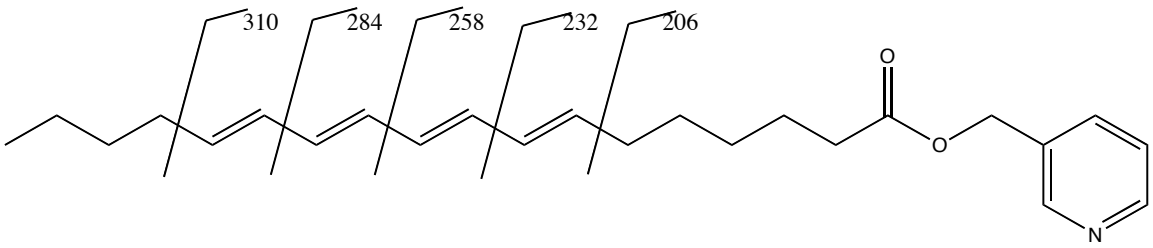

Figure 14: The structure of the shorter of the two PUFA in the sample, 18:4 (n-5)

Table 2: The fatty acid composition of the sample. DB is the double bonds.

		Double bond position from methyl end					Retention
	M_w (amu)	DB 1	DB 2	DB 3	DB 4	DB 5	time (min)
C 14:0	319						15.4
C 16:0	347						16.9
C 16:1	345	7					16.7
C 18:1	373	9					18.7
C 18:4	367	5	7	9	11		18.6
C 20:1	401	9					21.9
C 20:5	393	5	7	9	11	13	21.1
C 22:1	429	11					26.5

Table 2 shows the composition of the fatty acids in the sample. In the smaller mass range there are mostly saturated or monounsaturated and in the higher mass ranges there are polyunsaturated fatty acids and some monounsaturated. The retention time spans from 15 to 27 minutes.

3.4.2 Trimethylsilyl derivates of FAL

The method was first tested of some FAL standard in order to determined whether it would work satisfactorily on this type of molecules. The expected outcome are mass spectra where the $[M-15]^+$ is clearly visible as the molecular ion loses a methyl group, either from the end of the aliphatic chain or one of the methyl groups of the trimethylsilyl group. The $M^{\bullet+}$ can be expected to be barely visible, but can be determined by the $[M-15]^+$. An *m/z* value of 73 should also be a high intense peak and this is the trimethylsilyl group. Another *m/z* value with an expected high intensity peak is 103, which is the trimethylsilyl ether with one methylen group, CH_2-O-Si$(CH_3)_3$ [19].

The chromatogram of the fatty alcohol standard, Octadecanol, contained one peak that had a longer retention time than the underivatized compound, and because of this the reaction was presumed to be successful. The mass spectra of this peak closely resembled what the spectrum was expected to look like based on the literature and this was another fair indication that the reaction was successful. It was clear that the peak was not the molecular ion, but it was the $[M-15]^+$, because it was an odd numbered *m/z* value and the structure contained no nitrogen. The $[M-15]^+$ was used to determine the molecular ion and this indicated that the structure was a 18 carbon chain with no double bonds, which is correct so the method was deemed a success.

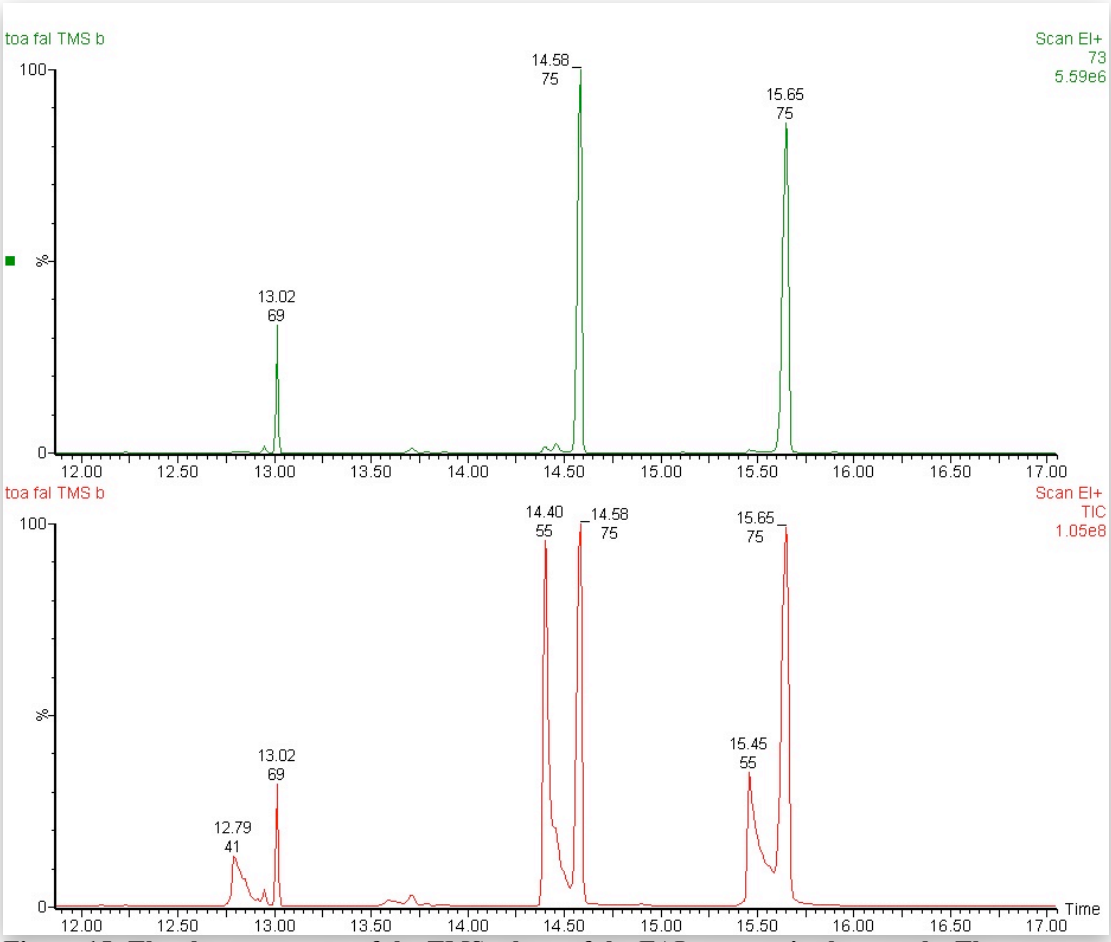

Figure 15: The chromatogram of the TMS ethers of the FAL present in the sample. The upper one shows the peaks containing the diagnostic ion 73 amu, which is the $Si(CH_3)_3$ group. The lower one shows the TIC, it is clear from the difference between the two that there are some FAL that had not been converted in the reaction.

The method was then applied to the FAL fraction from the second SPE run and the results were that the molecular weight and the degree of unsaturation of the FAL derived from the wax esters of Calanus oil.

Figure 15 shows the chromatogram of the TMS ethers, in the tree larger peaks in the chromatogram that shows the peaks that contain the diagnostic ion 73 amu (the upper chromatogram in figure 15), the molecular ion and the $[M-15]^+$ was easily identified. The smaller peaks between the first and second eluting of the large peaks were more difficult to elucidate because of a low signal-to-noise ratio.

In the mass spectra of the substances it is not possible to say anything about the placement of the double bonds but the molecular ion and the $[M-15]^+$ ion determines the amount of double bonds and the chain length of the hydrocarbon chain. Figure 16 is the spectrum of a TMS ether of a FAL 22:1 and figure 17 shows the structure of it.

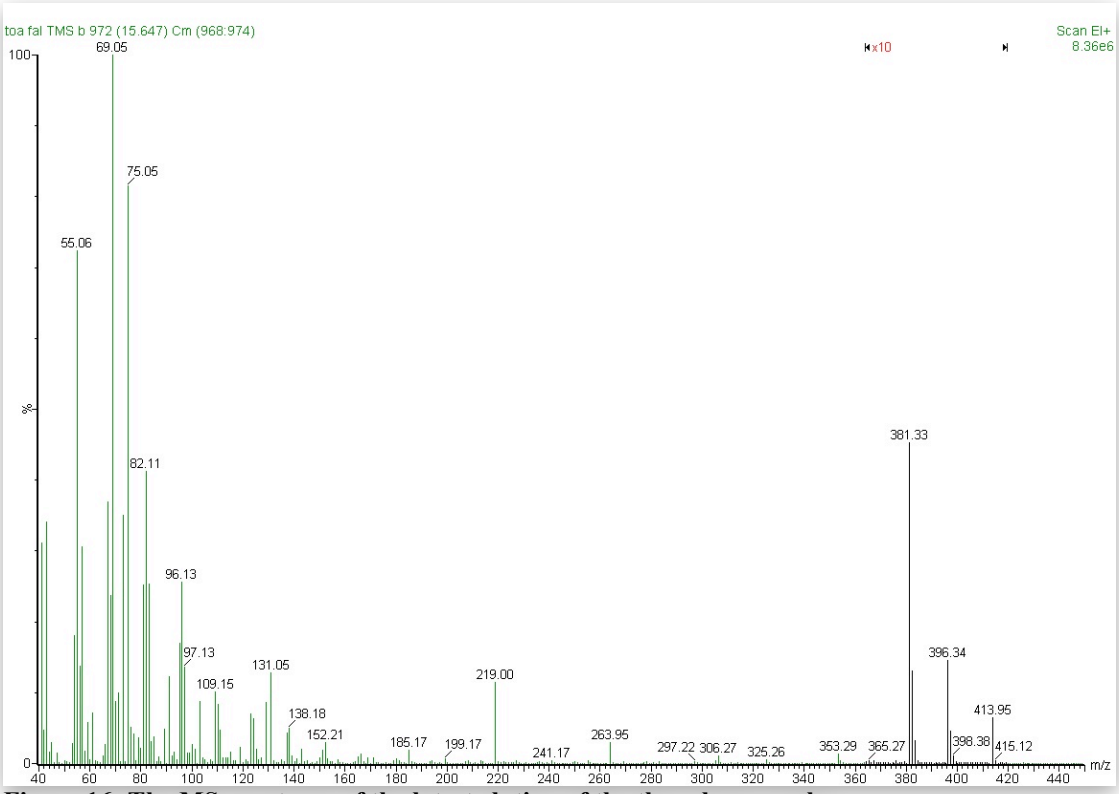

Figure 16: The MS-spectrum of the latest eluting of the three large peaks.

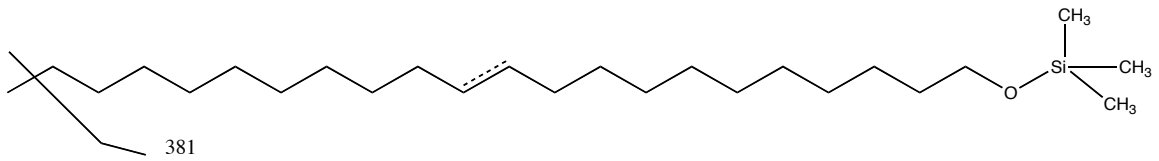

Figure 17: The general structure of a 22:1 FAL TMS ether. The double bond could be positioned anywhere along the length of the chain.

The content of the TMS ethers in the sample is shown in table 3. Because it is not possible to determine the position of the double bonds based on this method it is also not possible to say that each type of FAL consists of one FAL or several isomeric versions of the FAL with different positions of the double bonds.

Table 3: Contents of FAL in the sample based on the TMS ether method. M_w is the molecular weight, No DB is the number of double bonds and R_t is the retention time of each peak.

	M_w (amu)	$[M-15]^+$	No DB	R_t (min)
C 16:1	314	299	1	13.0
C 20:1	368	353	1	14.5
C 22:1	396	381	1	15.6

3.4.3 Nicotinate derivates of FAL

The second type of derivates that was used to determine the structure of the FAL originating from the wax esters of Calanus oil was nicotinyl esters. These were made with the reagent Nicotinylchloride HCl. The method was tested on all four of the FAL standards, Dodecanol, Tetradecanol, Hexadecanol and Octadecanol, all of which are saturated. These derivates are very similar to the picolinyl derivates of the FAME and interpreted in much the same way. According to the literature the major peaks in the mass spectra are supposed to be 79, 106 and 124 *m/z* units, all of which are centred on the pyridine ring and some being a result of rearrangements of the ring and associated structures. The identification of the molecular ion is facilitated by the fact that it must be an odd number since the molecule contains one nitrogen atom. As with the picolinyl derivates of FAME the best way to interpret the spectra is to start at the molecular ion and count losses of 14 amu. At gaps of 12 amu the double bond is located and it is placed between the fragments that have a 26 amu gap [17].

As all the FAL standards were saturated they could not testify to whether the method could be used to identify the position of the double bond, but they did prove that the method worked and alcohols were derivatized. However there still remained some unchanged FAL in the sample so the stoichiometry had to be further investigated. Due to time restrains this was not optimized but it is possible to determine which peaks in the gas chromatogram are FAL and which are derivatized FAL, both by the longer retention time of the nicotinate derivatives and by looking for certain diagnostic ions in the mass spectra that are not present in the mass spectra of FAL. The molecular ion is abundant in the nicotinate spectra and in addition *m/z* values such as 79 and 124 amu are visible as high intense peaks.

The method was applied to the third fraction of the APS SPE run, 5 mL of n-hexane:M*t*Bu ether (75:25 v/v). As the samples contained lower concentration of FAL than what was in the standard run, it became clear after a first attempt that it was necessary to combine two parallels from the SPE run in order to get the same peak size, but unfortunately there was still not a complete transformation to nicotinate derivatives. The unchanged FAL was still easy to distinguish from the nicotinate esters so this did not prove a big problem however.

The resulting chromatogram of the analysis of the FAL nicotinates, is shown in two versions in figure 18. The difference between the TIC chromatogram, (the lower one in figure 18) and the chromatogram showing only the peaks that contains the diagnostic ion 124 amu (the upper one in figure 18) indicate that the reaction was not complete. The earlier eluting peaks does not contain this ion, and the two peaks with retention time 14.40 and 15.46 minutes only contained it to a very small degree, indicating that they are not nicotinates of FAL, but underivatized FAL.

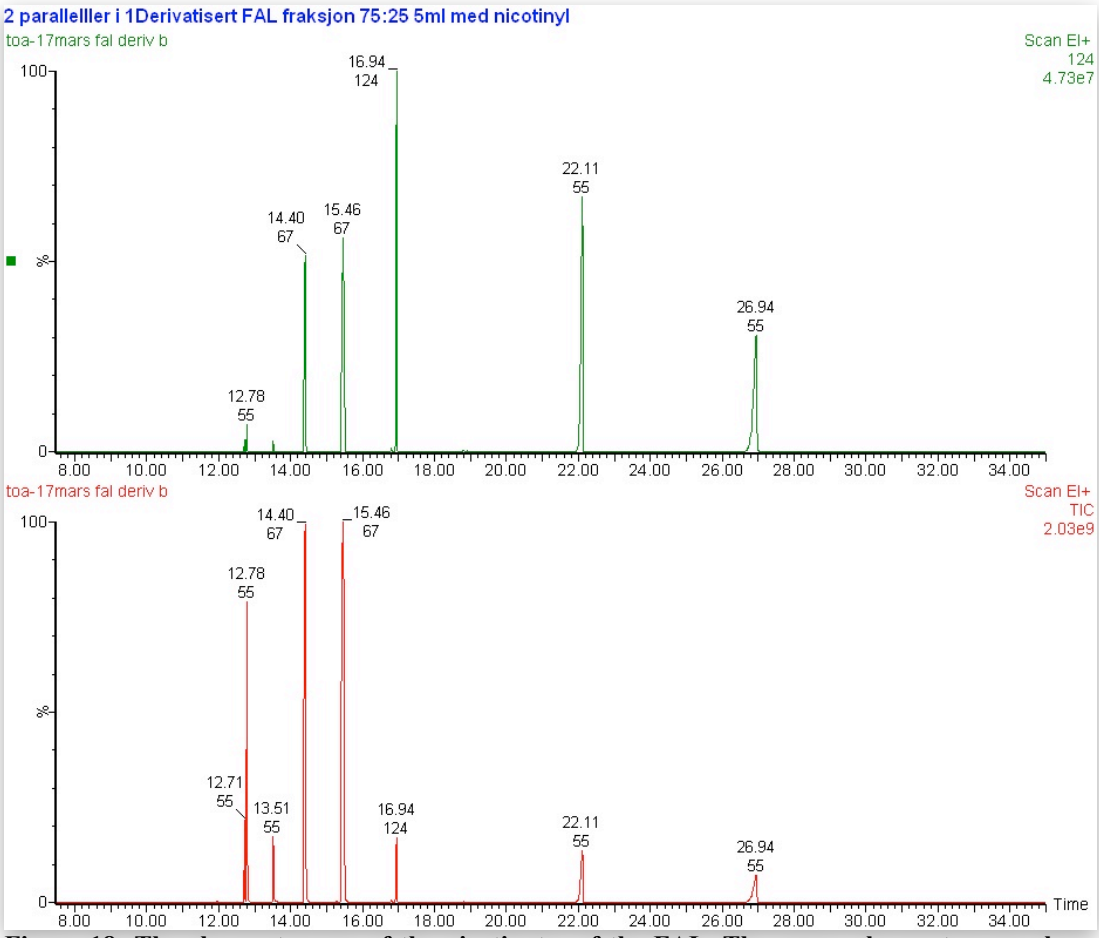

Figure 18: The chromatogram of the nicotinates of the FAL. The upper chromatogram shows the peaks containing the diagnostic ion 124 amu, and the lower one shows the TIC chromatogram.

The MS-spectra of these two peaks did not resemble the spectra of the other, later eluting peaks, which contain clearer indication that they have been derivatized. The spectrum of the peak with the retention time 14.40 is the one in figure 19, and if this was a nicotinate ester of an alcohol it would contain an 11-carbon aliphatic chain and the presumed molecular ion is 2 amu larger what is possible given the structure of a nicotinate ester of a FAL. The same is true of the other chromatographic peak containing only low amounts of the diagnostic ion, 124 amu. This has a retention time of 15.46 minutes. If this was a nicotinate an alcohol it would be a 13-carbon FAL and the molecular ion would be 2 amu greater than what is possible for a nicotinate of a FAL

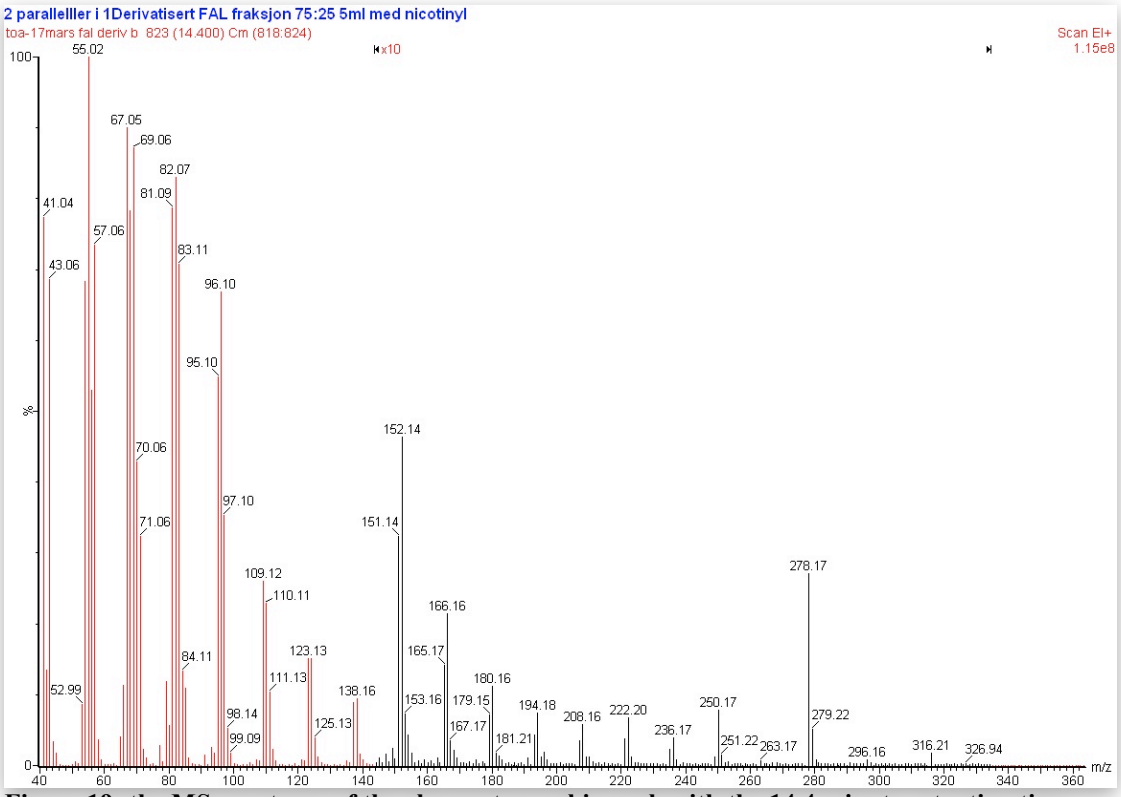

Figure 19: the MS-spectrum of the chromatographic peak with the 14.4 minute retention time.

The chromatogram in figure 21 shows the mass spectrum of the nicotinate of the FAL identified as 22:1 (n-11), which had a retention time of 26.94 minutes. The 26 amu gap lies between the *m/z* values 288 and 262. As in the picolinyl spectra the two ions distal to the double bond, which here is 302 and 316 amu are high intense peaks, marking the double bond and making it easier to locate. The spectrum also contains the diagnostic ions 79, 106 and 124 amu making it even clearer that the spectrum belong to a nicotinyl ester of a FAL. Figure 20 shows the structure of this FAL and the fragmentation around the double bond.

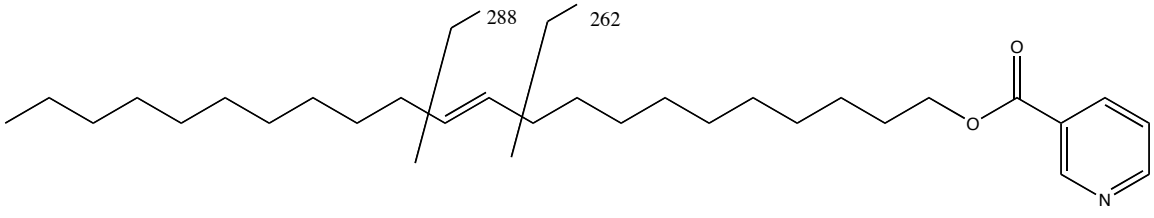

Figure 20: The structure of the FAL nicotinate 22:1 (n-11) and the fragmentation around the double bond.

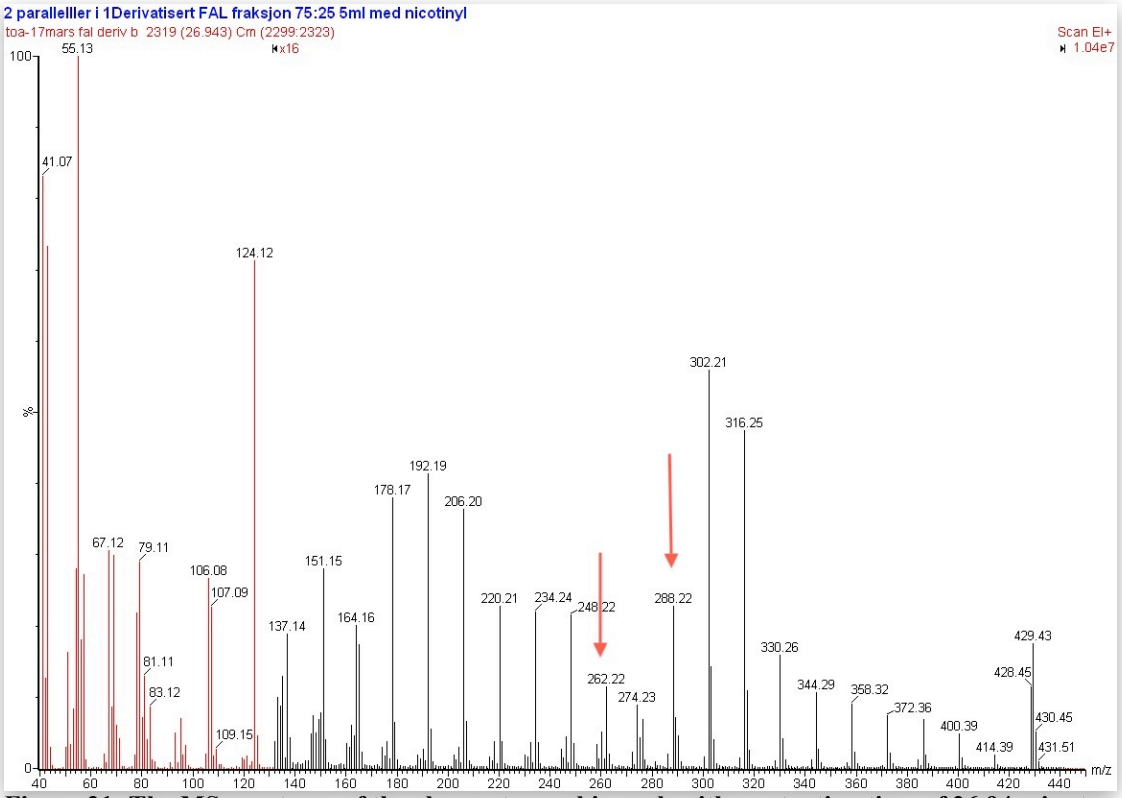

Figure 21: The MS-spectrum of the chromatographic peak with a retention time of 26.94 minutes. This is the 22:1 (n-11) alcohol.

In the sample four FAL were located and these are described in table 4. Most of these were monounsaturated and ranged from 16-carbon to 22-carbon chains. One 16-carbon chain was found that was saturated, but there was not found any polyunsaturated fatty alcohols.

Table 4: The contents of FAL found in the sample. M_w is the molecular weight of the FAL and DB indicates the position of the double bond from the terminal methyl of the carbon chain. R_t is the retention time of the peak.

	M_w (amu)	DB	R_t (min)
C 16:0	347		16.9
C 16:1	345	7	16.8
C 20:1	401	9	22.1
C 22:1	429	11	26.9

Overall the result of both nicotinate derivates and the TMS ether derivates of FAL resulted in the same three monounsaturated FAL. Both methods also contained no polyunsaturated FAL, but the nicotinate method resulted in the discovery of a saturated

FAL that was not located by the TMS ether method. However there were some small peaks in the chromatogram of the TMS ethers where the signal-to-noise ratio was so low that the molecular ion and the $[M-15]^+$ was not possible to distinguish from the noise.

4 Discussion:

Calanus oil is a product that contains large amounts of wax esters, the natural antioxidant, Astaxantine, and PUFA. In preclinical studies in animals, the oil has displayed beneficial health characteristics beyond what can be explained by the polyunsaturated fatty acid, like EPA and DHA, content alone. These PUFA have a well-documented effect on the reduction of cardiovascular disease, inflammation and cancer, and is used extensively as a food supplement to diets that are low in marine products [28]. Astaxantine, a carotenoid that gives the oil its vibrant red colour, also has a beneficial effect on the processes that lead to cardiovascular disease, such as oxidative stress and inflammation [29]. Because many of the beneficial fatty acids in Calanus oil exists as a part of fatty acid monoesters or wax esters, they are more stable against oxidation and the elucidation of these structures will therefore be interesting.

Early on, in the separation of the wax esters from the Calanus oil for further study, a novel and unexpected compound was discovered. The branched hydrocarbon, with a methyl side chain, 7-methyloctadecane, has never been found before in the oil or similar products. Searching in several databases, such as SciFinder, Dictionary of Marine natural products and ISI Web of Knowledge, resulted in one article that described the substance as only having been found in nature once before. In mosquito larva it is produced as a response to overcrowding and will have a toxic or inhibitory effect on the larva under these conditions, along with other methyl octadecanes and methyl nonadecanes [25]. As mosquitoes also belong to the phylum Arthropoda, although it belongs to a different branch, they are still related so it is not illogical that they should contain similar substances. Other than this there was not found any indications that the substance can be found naturally in any other organisms.

The wax esters that were to be investigated structurally had to be split into their two separate components, fatty alcohols and the fatty acids, using a transesterification method that left the fatty acids as fatty acid methyl esters. These two components were to be characterised separately using derivates of both to obtain information about the structures by GC-MS, and this information was to be puzzled together using LC-MS.

The FAME portion was derivatized and analyzed as a picolinyl ester, as these was thought to give the structural information that was required. The GC-MS analysis resulted in both chromatograms and spectra of the substances that were used to elucidate the structure. The FAME found ranged from 14-carbon to 22-carbon chain length, and from saturated to polyunsaturated. Searches in literature gave the expectation that the fatty acid composition of copepod wax esters would mainly consist of 20:1 and 22:1, based upon the composition in other copepods and krill [11-14]. Although these were found, so were others, both saturated and polyunsaturated, and of shorter chain length. The main difference between those studies and this were the method, while they only used a GC system, our analyses were performed using a GC-MS system. This gives a much more reliable structural result as does not relay on the retention time as the main indication of the structure.

The PUFA found in the sample is not one of the more common PUFA like EPA or DHA, they are not ω-3 fatty acids and they are not any of the more common conjugated PUFA. Searching databases did not result in any findings of the PUFA present in wax esters of Calanus oil. The monounsaturated fatty acids located in the sample consisted of ω-9, ω-11 and ω-7 of various lengths. These are more common fatty acids than the PUFA found, but maybe not quite as expected in marine lipids, as ω-3 or ω-6 fatty acid that are quite common in the marine organisms. According to studies on other copepods and krill both the ω-9 and the ω-11 was expected, but these studies were, as previously mentioned, only performed with a GC-FID system that does not give the accurate results that a GC-MS system does.

The FAL portion of the wax esters were derivatized using two methods, nicotinyl esters and TMS ethers, where the nicotinates gave the most complete structural information. Both of these methods are useful however, because in later experiments the TMS ether derivates can be used to give a quick idea of what is in a sample and the nicotinates can be used if a more complete structure is necessary. As with the fatty acids an idea of what would be present was found in literature, in studies based on other copepods and krill [11-14]. The fatty alcohols found in those studies were 20:1 (n-9) and 20:1 (n-11). Using

the TMS ethers there was located 3 different types of fatty alcohols, 16:1, 20:1 and 22:1. Because this is not a method that will indicate the location of the double bond, it is not possible to determine if the three types of alcohols found are all of one type or if they consist of two or more isomeric structure with different double bond position. The nicotinate derivates resulted in the finding of four types of FAL, 16:0, 16:1 (n-7), 20:1 (n-9) and 22:1 (n-11). The 16:0 was found in the chromatogram that resulted from the analysis of the sample as a very small peak and this could be the reason that it was not found as the TMS ether. In the chromatogram of the TMS derivates there were a few small peaks where the signal-to-noise ratio was too high to detect the $[M-15]^+$ or the molecular ion. It is therefore possible that, in addition to the 16:0 that was not located, there could be others too that was not found, in either method.

In the chromatograms of both types FAL derivates there were peaks that were clearly not derivatized. In spite of this, because the two methods gave the same results, it is suspected that the major FAL has been located. By improving the stoichiometry of both methods it is possible that more FAL can be found if, in both methods, one type of FAL has been left completely underivatized. The best chance of finding more FAL structures, if there were any, would be to focus on the smaller peaks and trying to better the signal-to-noise ratio, so that the structure of these can be elucidated. In order to do this the concentration of the sample needs to be increased and the stoichiometry needs to be improved. It is also possible that it could be necessary to separate the sample into smaller fractions, by use of for instance HPLC fractionation with a column and a mobile phase that will give good separation of each peak. If this is done before the derivatisation to the FAL and the derivatisation methods was performed on each fraction in high enough concentration, maybe the peaks would be more defined and the structure would be easier to elucidate. This could improve the structure elucidation of the FAME as well, because in the chromatogram of the FAME there are also small peaks where the structure is not possible to elucidate because of the noise level, but the stoichiometry of the FAME is satisfactory so it is only necessary to split the FAME sample into smaller fractions before the derivatisation took place.

Another possible improvement to the method used is the exchange of the GC-column for a different column. The column used was one that separates mainly based on the boiling point of the different substances, and the boiling points of different fatty alcohols and fatty acids are not very different, especially when the difference between them is the degree of unsaturation. In this column there is the possibility of two substances co-eluting. A better choice would be a column that separates the substances based on boiling point and the structure of the molecule, one that could give good separation of, for instance, two different FAME with the same chain length but different degree of unsaturation. An even better option would be a column that could separate two structures were the only difference was the position of the double bond, this would be useful to see if there are isomeric differences in the placement of the double bond.

Because of time restraints it was not possible to attempt to puzzle together the information found about the wax esters in Calanus oil. The idea was that information found by analysing the complete structure of the wax esters, in a LC-MS system, could put the correct FAME and FAL together. If the molecular weight of the wax esters and maybe a fragmentation pattern around the carboxyl group could be identified there are limited numbers of combinations of the FAL and FAME that would make a wax ester.

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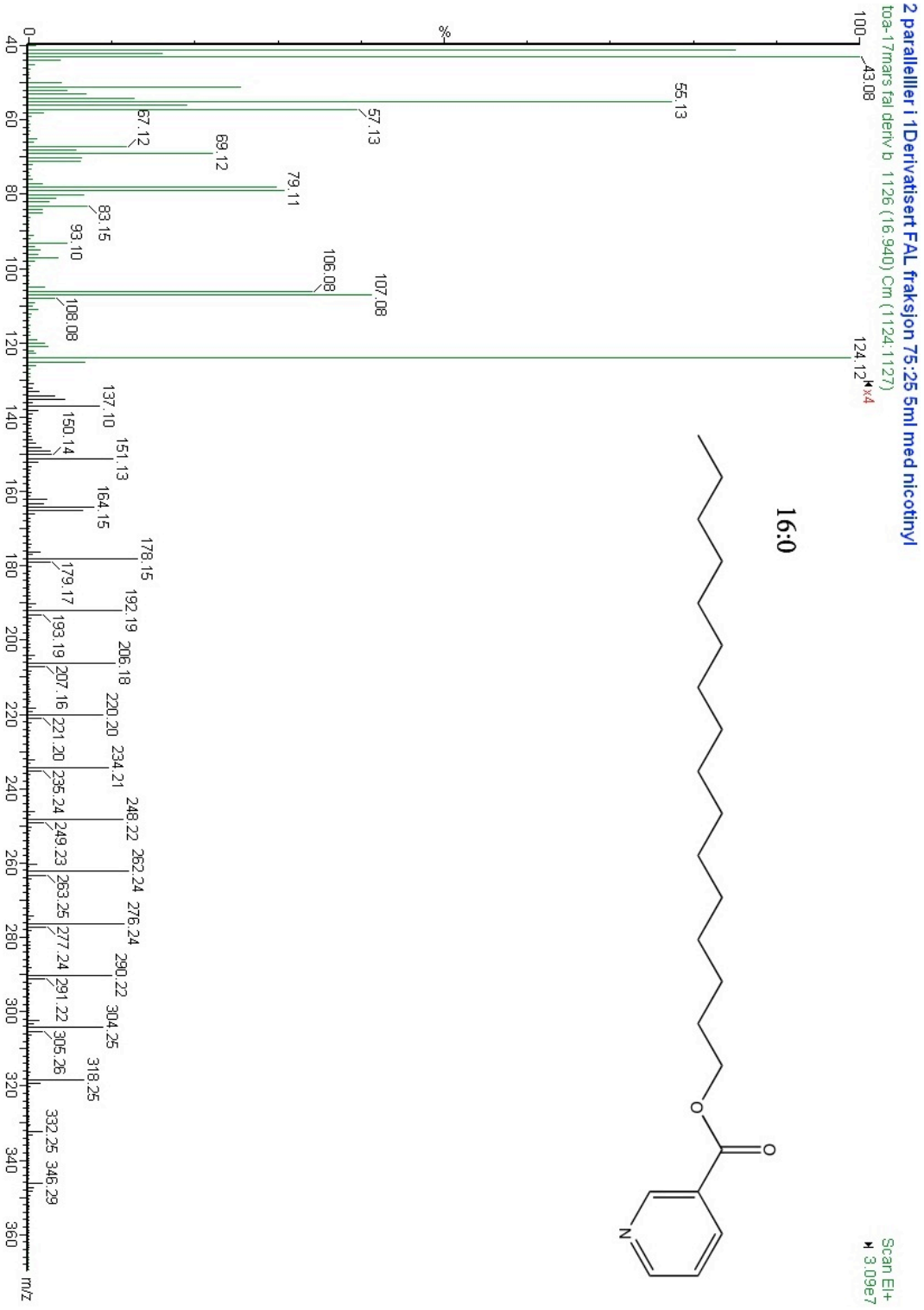
6 Appendix:

6.1 Nicotinate spectra

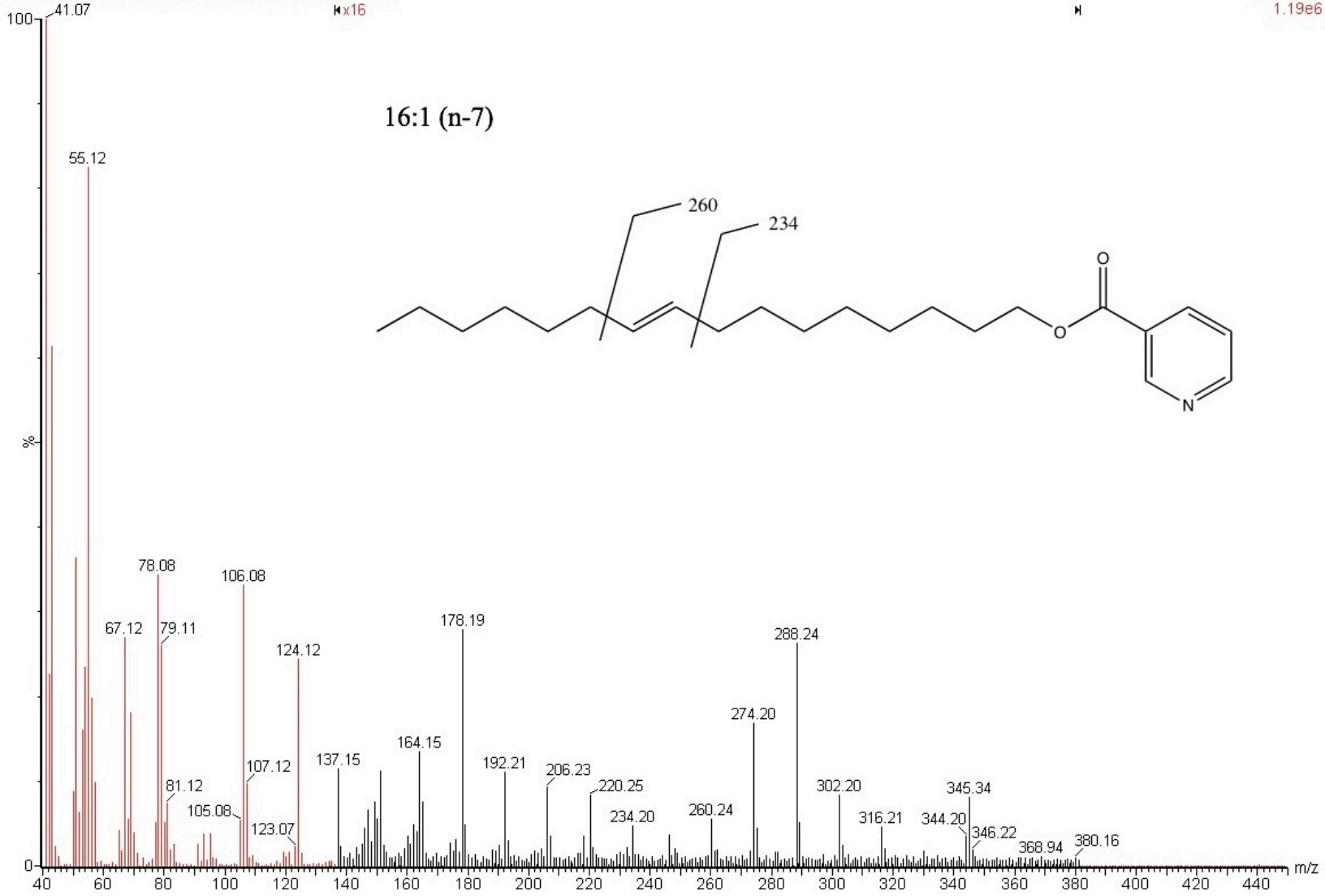


2 parallellIler i 1Derivatisert FAL fraksjon 75:25 5ml med nicotinyl

toa-17mars fal deriv b 1109 (16.798) Cm (1107:1111)

Scan EI+
1.19e6

x16



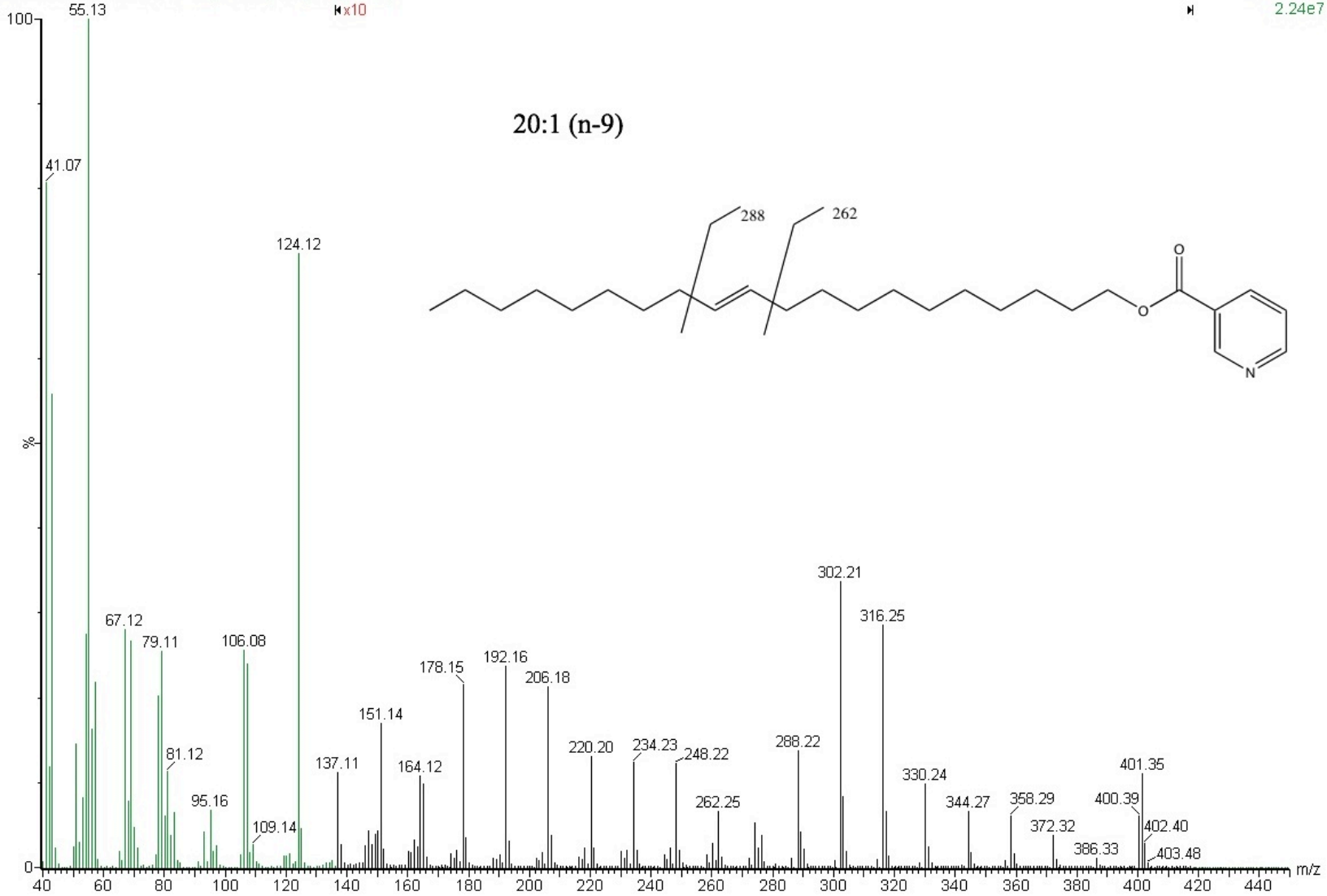
16:1 (n-7)

2 parallellIler i 1Derivatisert FAL fraksjon 75:25 5ml med nicotinyl

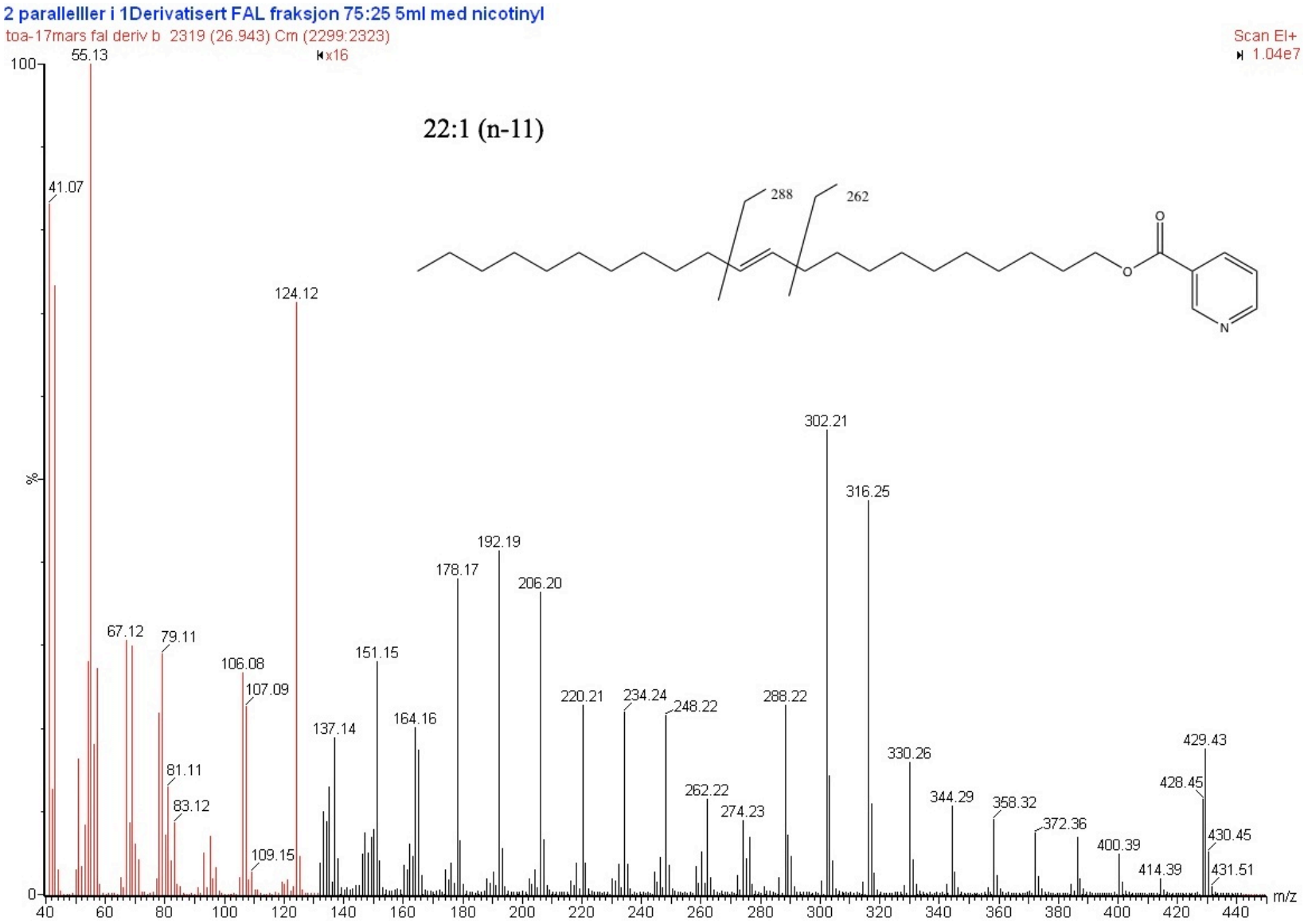
toa-17mars fal deriv b 1743 (22.113) Cm (1731:1745)

Scan EI+
2.24e7

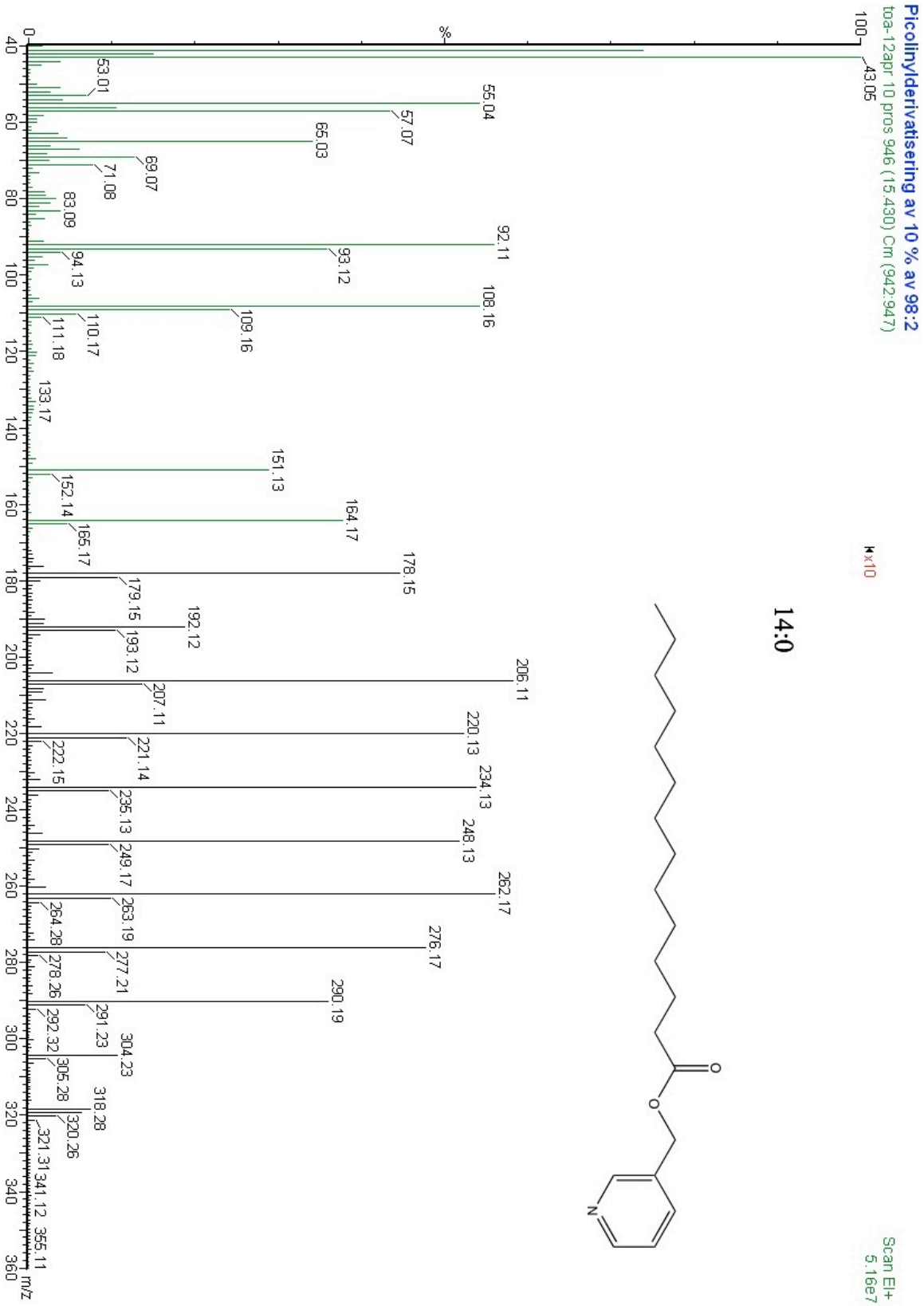
x10

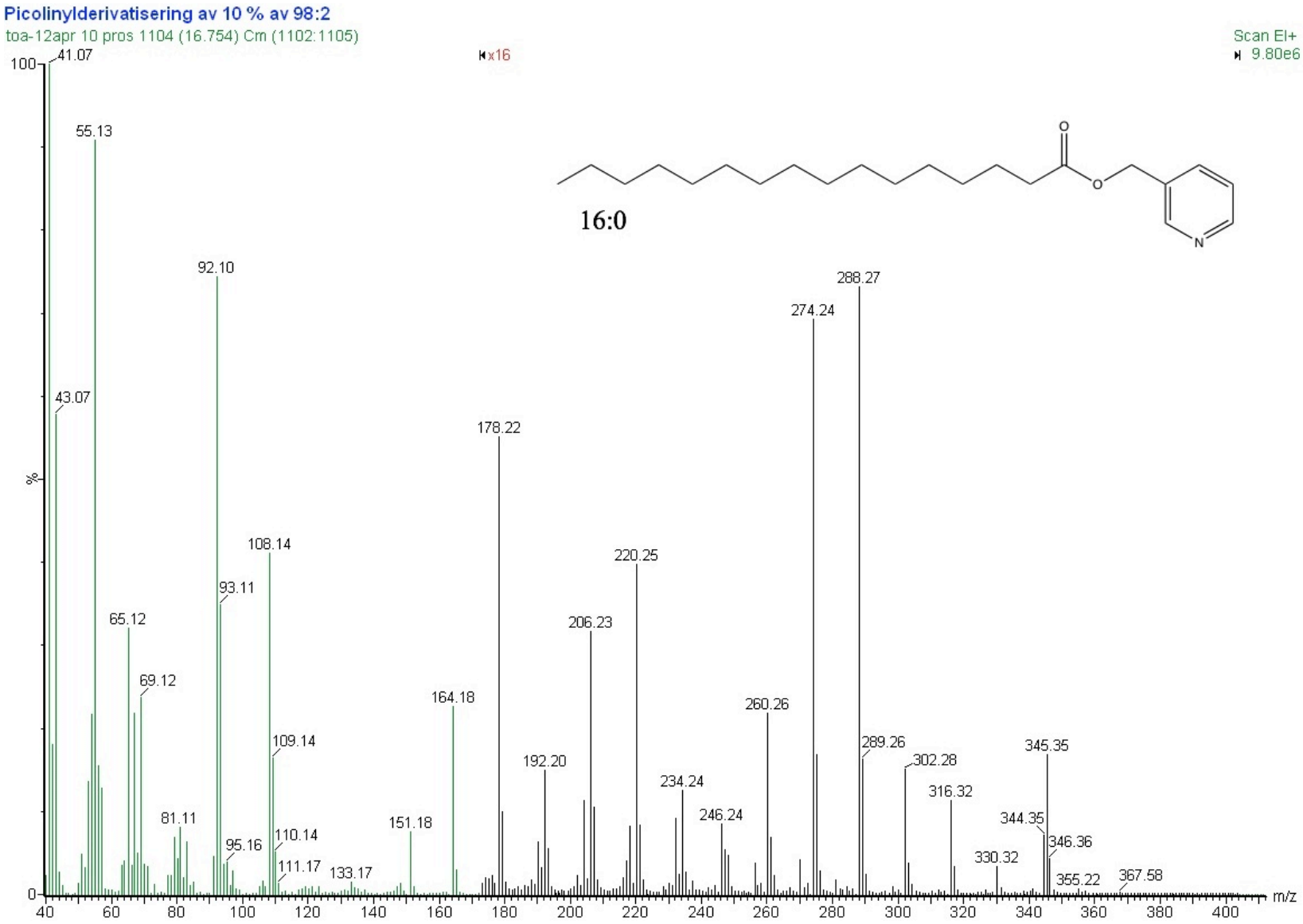


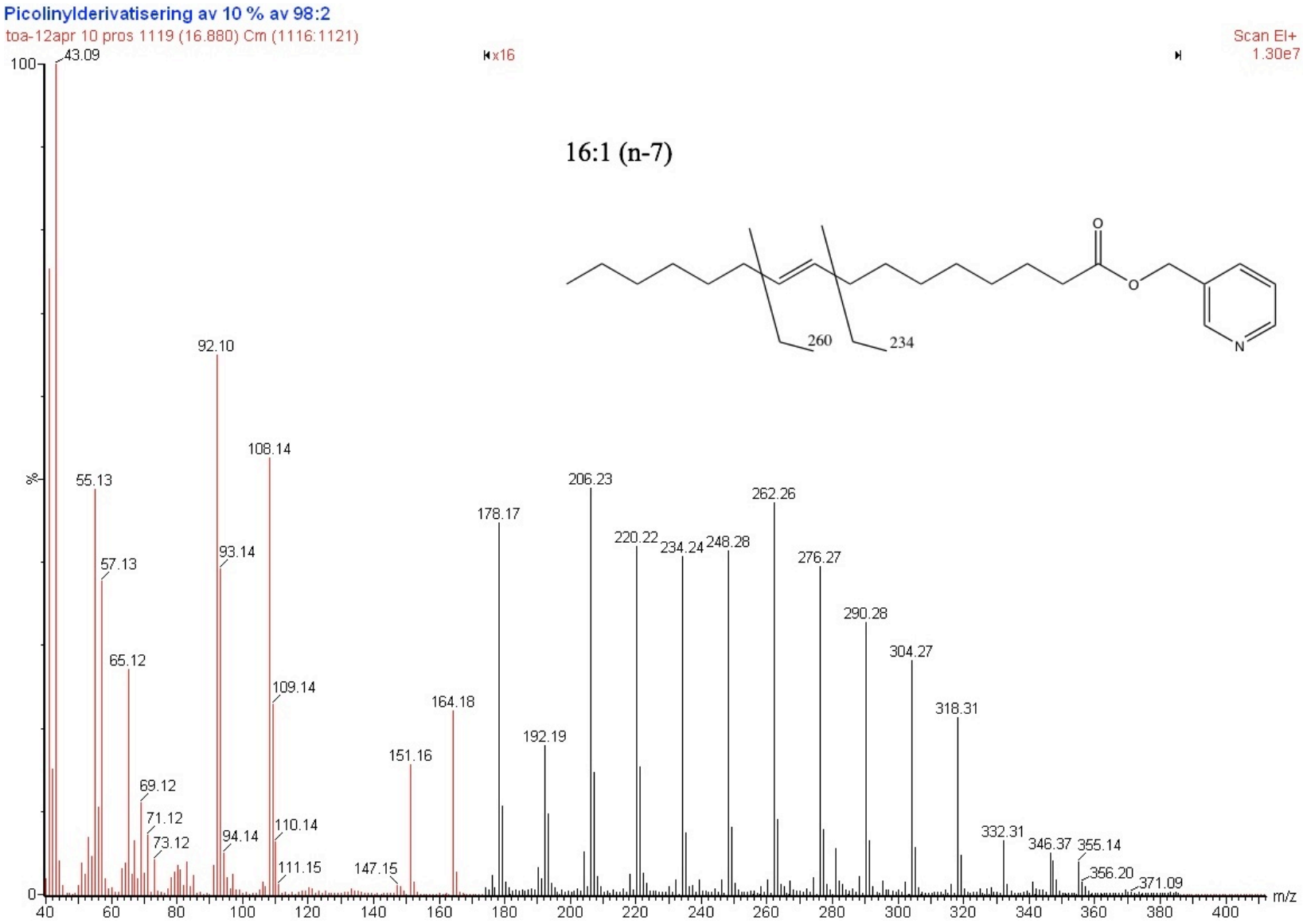
20:1 (n-9)

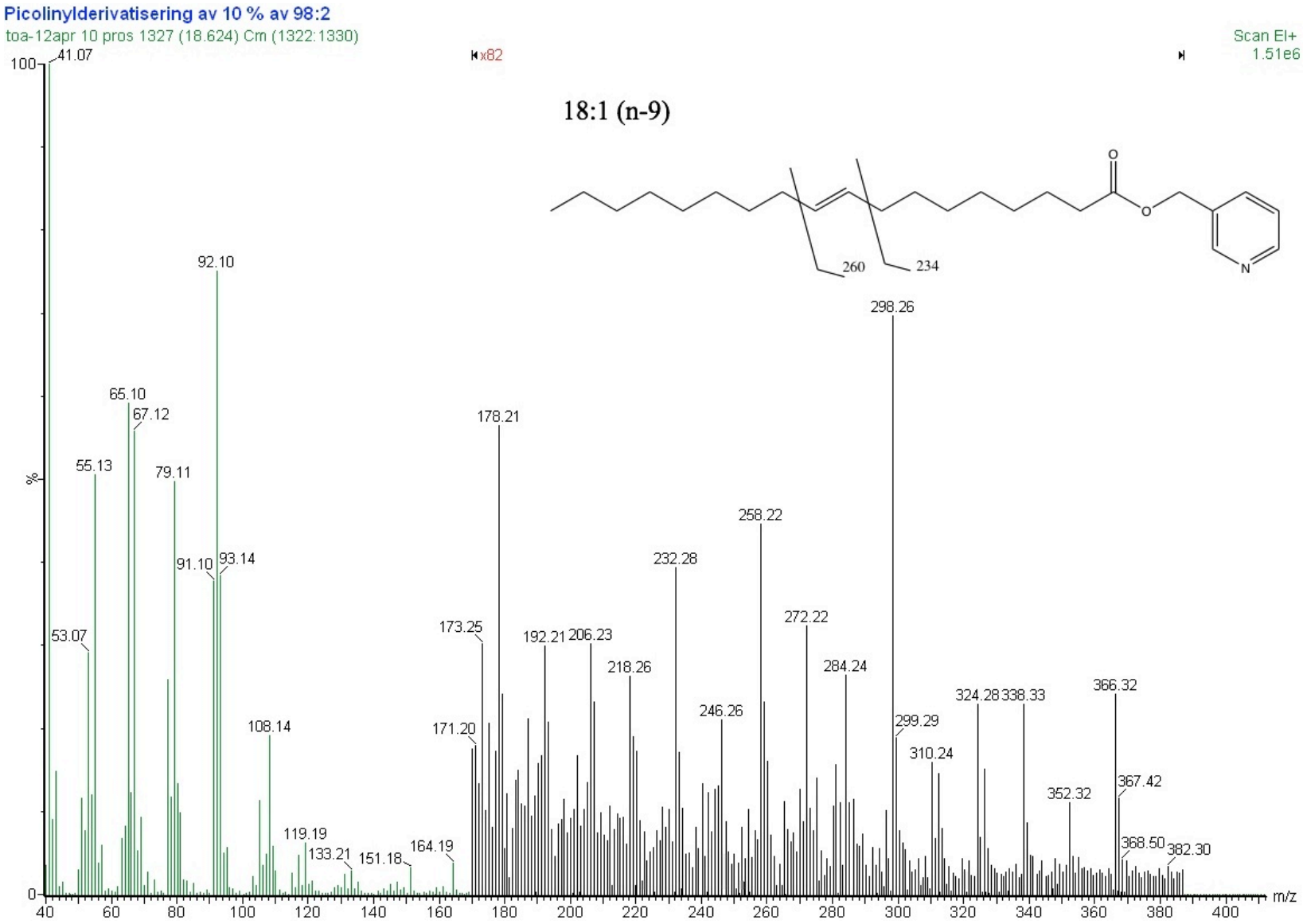

2 parallellIler i 1Derivatisert FAL fraksjon 75:25 5ml med nicotinyl
toa-17mars fal deriv b 2319 (26.943) Cm (2299:2323)
Scan EI+
1.04e7
x16
22:1 (n-11)
288
262
O
O
N
55.13
41.07
124.12
302.21
316.25
192.19
178.17
206.20
67.12
79.11
151.15
106.08
107.09
220.21
234.24
248.22
288.22
137.14
164.16
330.26
429.43
428.45
262.22
274.23
344.29
358.32
372.36
430.45
400.39
414.39
431.51
81.11
83.12
109.15
100
%
0
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
m/z

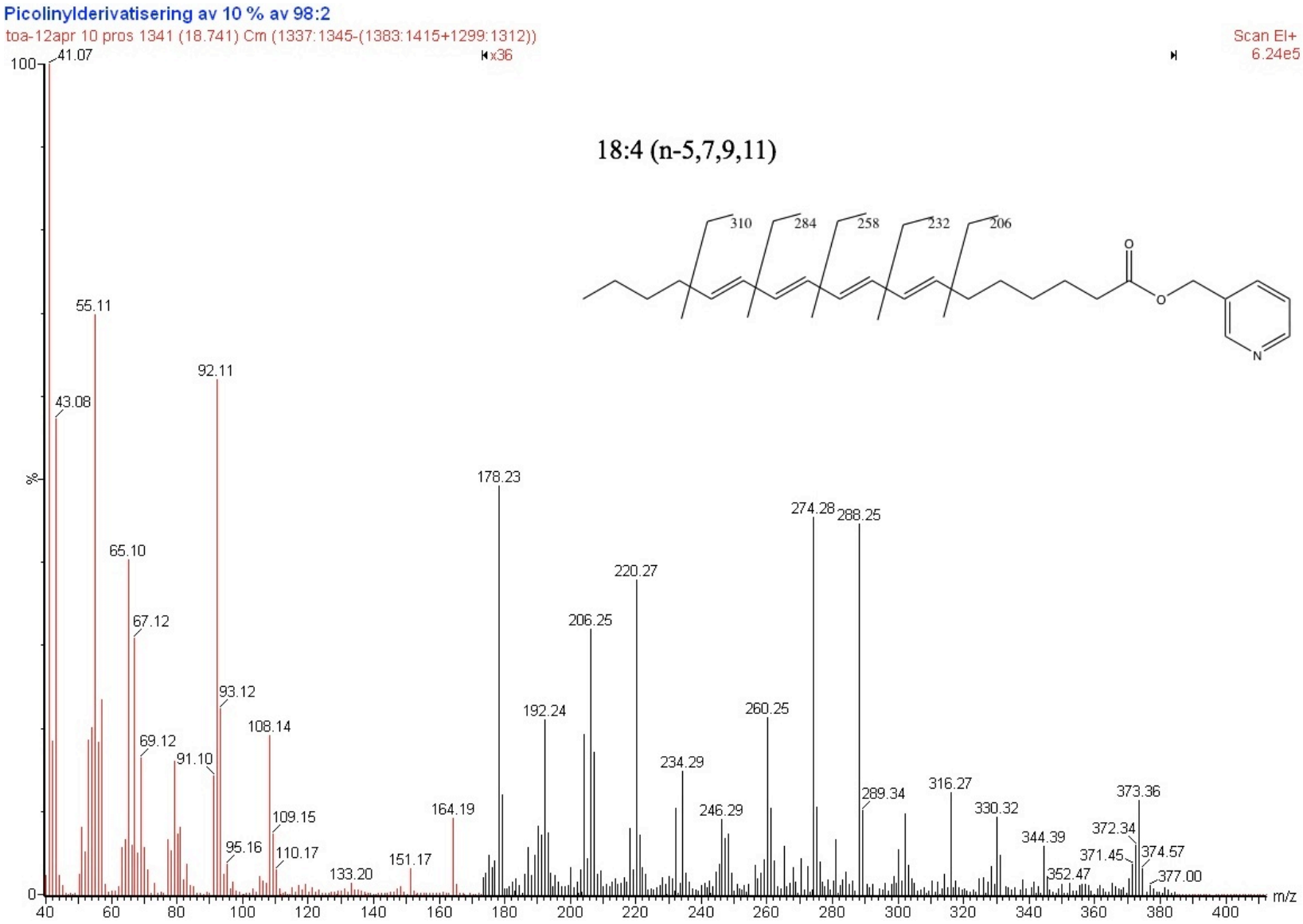
6.2 *Picolinyl spectra*

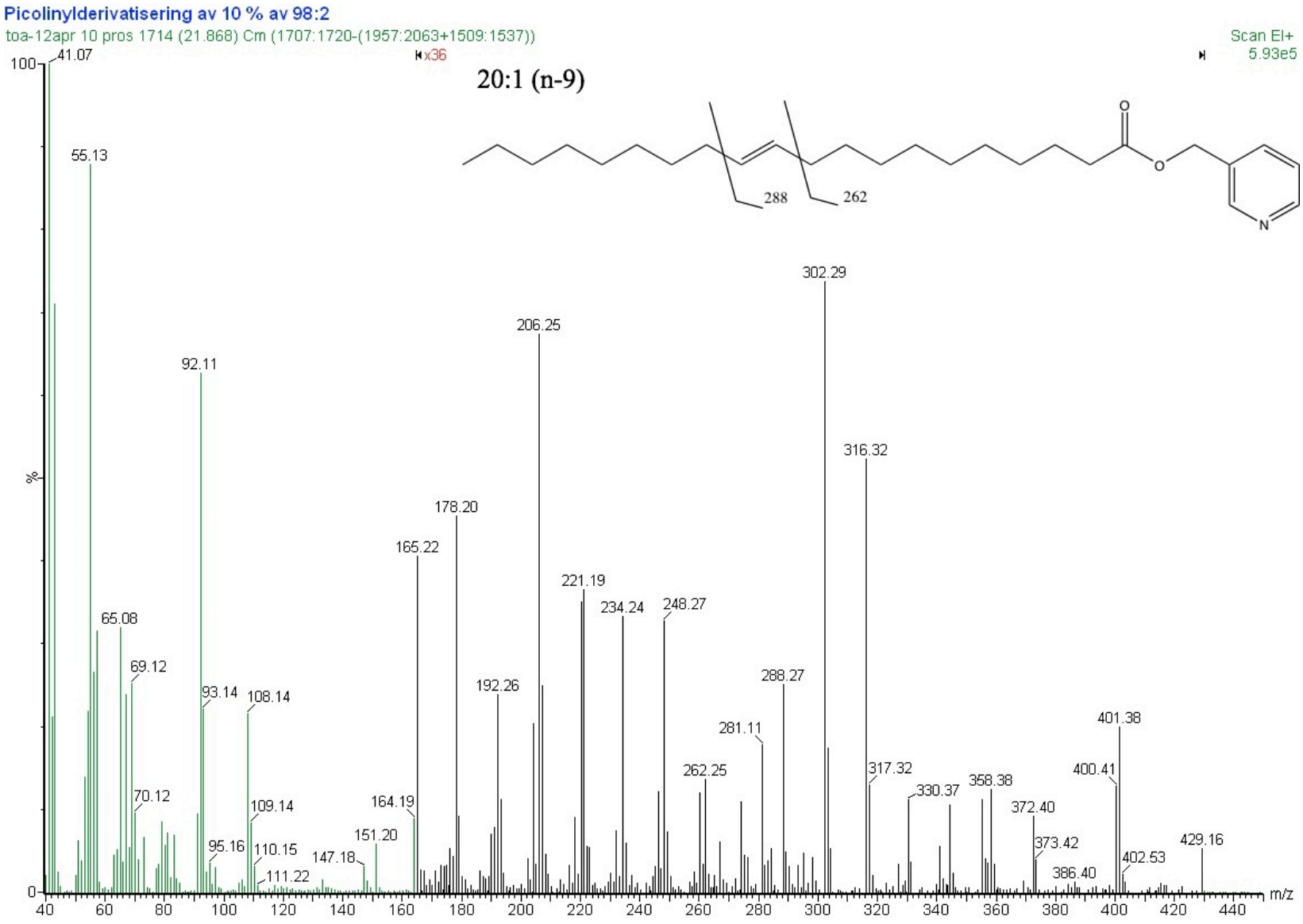


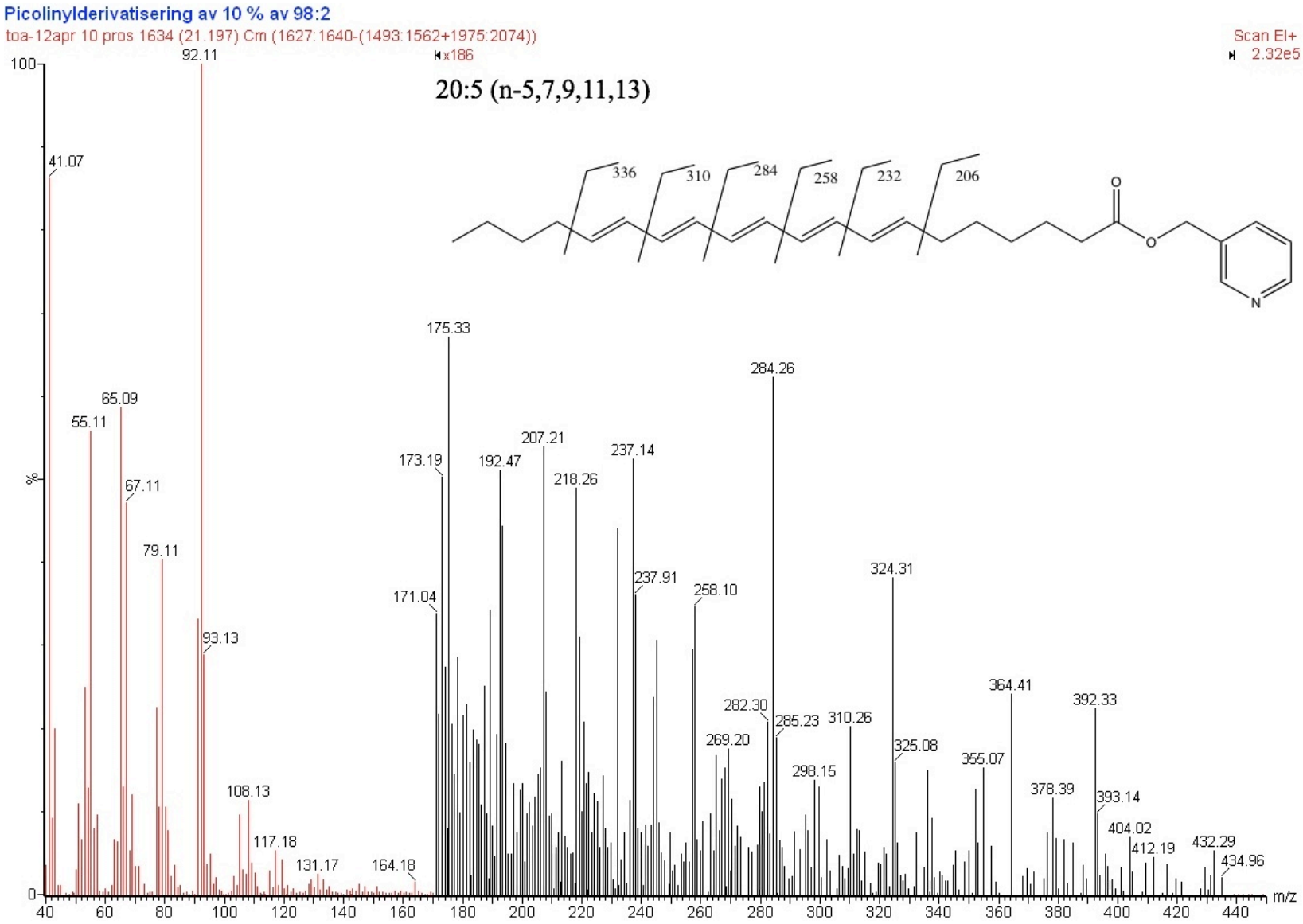

Picolinylderivatisering av 10 % av 98:2
toa-12apr 10 pros 1104 (16.754) Cm (1102:1105)
x16
Scan EI+
9.80e6
16:0
O
O
N
100
%
0
m/z
41.07
55.13
43.07
92.10
108.14
93.11
65.12
69.12
81.11
95.16
109.14
110.14
111.17
133.17
151.18
164.18
178.22
192.20
206.23
220.25
234.24
246.24
260.26
274.24
288.27
289.26
302.28
316.32
330.32
344.35
345.35
346.36
355.22
367.58
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400


Picolinylderivatisering av 10 % av 98:2
toa-12apr 10 pros 1119 (16.880) Cm (1116:1121)
x16
Scan EI+
1.30e7
16:1 (n-7)
260
234
O
O
N
%
m/z
43.09
92.10
108.14
55.13
206.23
262.26
178.17
220.22
234.24
248.28
276.27
57.13
93.14
290.28
304.27
65.12
109.14
164.18
318.31
192.19
151.16
69.12
71.12
73.12
94.14
110.14
111.15
147.15
332.31
346.37
355.14
356.20
371.09


Picolinylderivatisering av 10 % av 98:2
toa-12apr 10 pros 1327 (18.624) Cm (1322:1330)
x82
Scan EI+
1.51e6
18:1 (n-9)
260
234
O
O
N
%
m/z
41.07
92.10
65.10
67.12
55.13
79.11
91.10
93.14
53.07
178.21
298.26
173.25
192.21
206.23
218.26
232.28
258.22
272.22
284.24
246.26
171.20
108.14
299.29
310.24
324.28
338.33
366.32
352.32
367.42
368.50
382.30
119.19
133.21
151.18
164.19


Picolinylderivatisering av 10 % av 98:2
toa-12apr 10 pros 1341 (18.741) Cm (1337:1345-(1383:1415+1299:1312))
Scan EI+
6.24e5
x36
18:4 (n-5,7,9,11)
310
284
258
232
206
O
O
N
%
m/z
41.07
55.11
43.08
92.11
65.10
67.12
93.12
108.14
69.12
91.10
109.15
95.16
110.17
133.20
151.17
164.19
178.23
192.24
206.25
220.27
234.29
246.29
260.25
274.28
288.25
289.34
316.27
330.32
344.39
352.47
371.45
372.34
373.36
374.57
377.00


Picolinylderivatisering av 10 % av 98:2
toa-12apr 10 pros 1714 (21.868) Cm (1707:1720-(1957:2063+1509:1537))
Scan EI+
5.93e5
x36
20:1 (n-9)
288
262
O
O
N
100
%
0
m/z
41.07
55.13
65.08
69.12
70.12
92.11
93.14
95.16
108.14
109.14
110.15
111.22
147.18
151.20
164.19
165.22
178.20
192.26
206.25
221.19
234.24
248.27
262.25
281.11
288.27
302.29
316.32
317.32
330.37
358.38
372.40
373.42
386.40
400.41
401.38
402.53
429.16
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440


Picolinylderivatisering av 10 % av 98:2
toa-12apr 10 pros 1634 (21.197) Cm (1627:1640-(1493:1562+1975:2074))
x186
Scan EI+
2.32e5
20:5 (n-5,7,9,11,13)
336
310
284
258
232
206
O
O
N
100
%
0
m/z
92.11
41.07
65.09
55.11
67.11
79.11
93.13
108.13
117.18
131.17
164.18
175.33
173.19
171.04
192.47
207.21
218.26
237.14
237.91
284.26
258.10
324.31
282.30
285.23
269.20
310.26
325.08
298.15
355.07
364.41
392.33
378.39
393.14
404.02
412.19
432.29
434.96
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440

Picolinylderivatisering av 10 % av 98:2

toa-12apr 10 pros 2270 (26.528) Cm (2256:2280-(2144:2176+2396:2476))

Scan EI+

x16

3.02e5

22:1 (n-11)