UiT
NORGES
ARKTISKE
UNIVERSITET

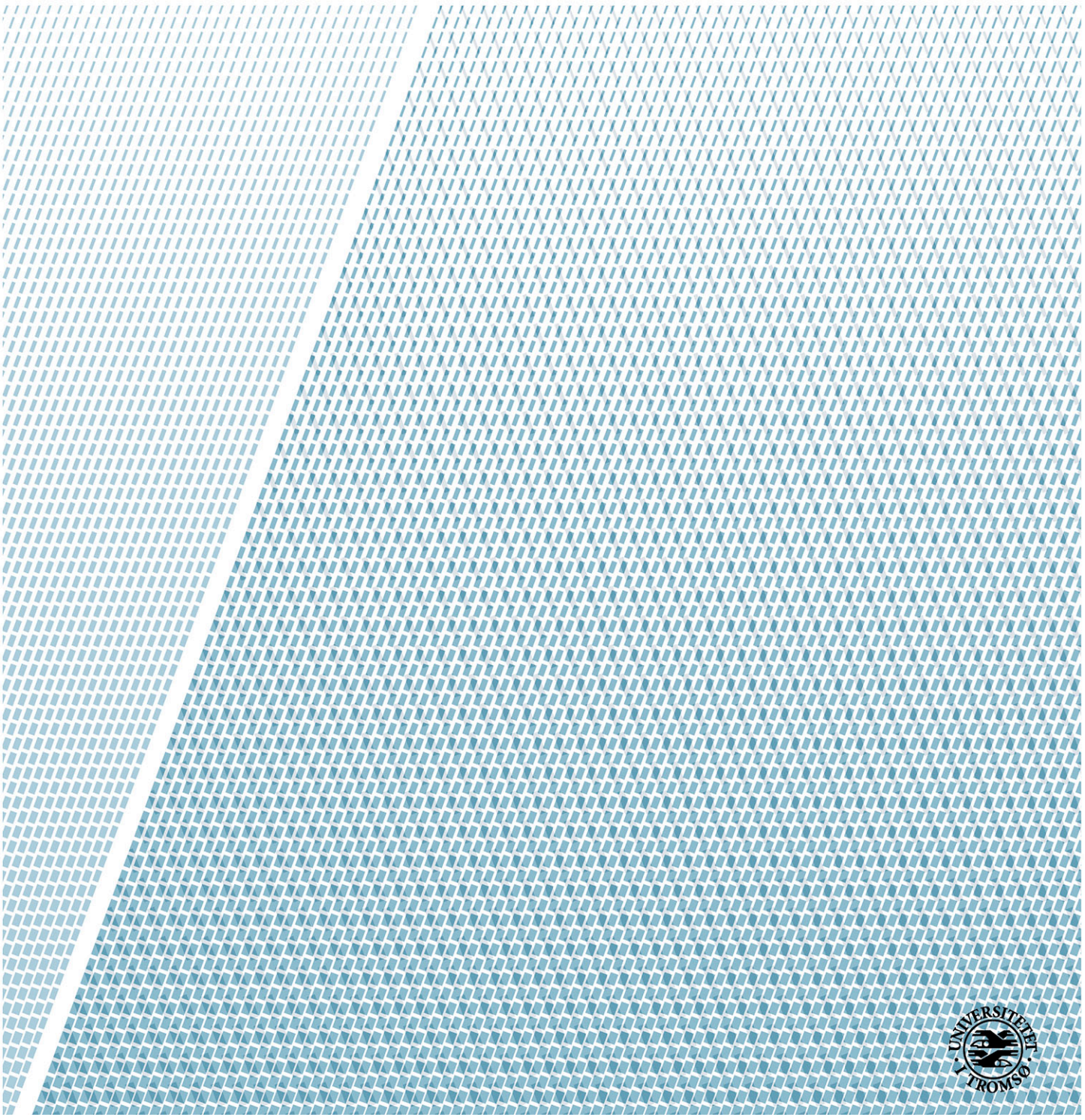
Department of Pharmacy

Structural elucidation of phospholipids in omega-3 food supplements

—

Charlotte Nikolaisen Brogård

Thesis for the degree Master of Pharmacy,
University of Tromsø, Faculty of Medicine,
Department of Pharmacy June 2014



Acknowledgements

The work presented in this thesis was carried out at the Section of Medicinal Chemistry, Department of Pharmacy (IFA), University of Tromsø in the period from August 2013 until June 2014.

First of all I would like to express my special appreciation and thanks to my main supervisor, Dr. Terje Vasskog, who has supported me throughout my thesis with his patience and knowledge. Your guidance and encouragement regarding both the experimental work and the writing of this thesis has been priceless. Thank for always being available for the bigger problems (the instrument breakdown) and for answering all my silly little questions with a smile. I would also like to thank Dr. Gøril Flaten Eide, my co-supervisor, for your help with during the start up of the project and for supplying analysing material. Thank you to Morten Kaare Moe for sharing you insight and knowledge about analysis of phospholipids. Your contribution greatly improved the experiments and interpretation of the results.

Thank you to the manufacturer, AN, for supplying relevant raw material for analysis, and to Dr. Terkel Hansen at the University of Tromsø for supplying me with chemicals at a critical point in the experiments.

A very special thanks to my fellow students, Kine Smellror, Merete Moen and Børge Renmælmo, who have continuously been there for me during this period. Thank you for all your support and advice and for sharing all the ups and downs throughout these last few months. The waffle making in the lab, the sunbaking on the roof, running around the halls on scooters when everyone else had gone home and laughing until out stomachs hurt are some of the memories I highly appreciate.

I would like to thank my friends and loved ones, who have supported me throughout entire process, thank you for your encouragement and for believing in me.

Tromsø, May 2014
Charlotte Nikolaisen Brogård

Summary

Before pharmaceuticals are released onto the marked they have to go through substantial testing to ensure their content and safety. The rules and regulations governing the dietary supplements industry on the other hand are far less strict, and as of 2013 there are no quality demands regarding dietary supplements based on marine oils, such as omega-3 and krill oil supplements. Because there is no routine testing of dietary supplements it is interesting to investigate weather the content of the supplements match the labelled ingredients and the focus of this thesis have been evaluation of the contents of marine oils based products.
The main constituents of marine oil are fatty acids, phospholipids and triglycerides. Krill oil mainly contains essential fatty acids bound to phospholipids, where phosphatidylcholine is the most dominant phospholipid.
A novel method was developed for UPLC-MS analysis of phospholipids in complex lipid samples. The methods developed allowed for identification and structural elucidation of different PL species and by utilizing MS^E technology, rapid qualitative analyses of complex lipid samples were made possible.
By using UPLC-MS it was confirmed that a high proportion of the total content of commercial marine oils contained the essential fatty acids EPA and DHA. The study also showed that the two different brands of krill oil capsules had very similar content. A raw extract intended for commercial use, where the goal was to enrich phospholipids with the essential fatty acids EPA and DHA, was also analysed. The study revealed that a higher proportion of the total content of the raw extract contained essential fatty acids. As the raw extract was obtained from other sources than krill oil this showed that, in addition to krill, there are also other marine sources that have a high content of the essential fatty acids EPA and DHA.
Few studies have been done on commercial phospholipid samples such as krill oil and raw extract intended for commercial use, and the present study is a contribution to the evaluation of marine oils used as dietary supplements and weather the content of the supplements match the labelled ingredients.

Table of contents

Acknowledgements III
Summary IV
Abbreviations VII
1. Introduction 1
2. Dietary supplements and marine oils 2
3. Lipids 4
3.1. Fatty Acyls 5
3.1.1. Structure and nomenclature of fatty acids 6
3.1.2. Function and use of fatty acids 8
3.2. Glycerolipids 12
3.2.1. Structure and nomenclature of triglycerides 12
3.2.2 Function and use of triglycerides 13
3.3. Phospholipids 13
3.3.1. Structure and nomenclature of phospholipids 13
3.3.2. Function and use of phospholipids 15
3.4. Lipids from krill oil 16
3.5. Phospholipids vs. triglycerides used as dietary supplements 17
4. Analysis of lipids 18
4.1. Ultra performance liquid chromatography 18
4.2. Mass spectrometry 19
4.3. ESI-MS of lipids 20
5. Aim of thesis 24
6. Materials and methods 25
6.1. Chemicals 25
6.2. Materials 27
6.2.1. UPLC-MS 27
7. Method development 28
7.1. Phospholipid standards analysed with UPLC-MS 28
7.1.1. Preliminary experiments 29
7.1.2. Analysis of phosphatidylcholine standards 32
7.1.3. Analysis of phosphatidylethanolamine standards 41
7.2. Krill oil capsules analysed with UPLC-MS 46
7.3. AN raw extract analysed with UPLC-MS 50
8. Results and discussion 52
8.1 Choice of methods 52
8.2. Structural determination of Rimfrost krill oil capsules 54
8.2.1. Peak 1 56
8.2.2. Peak 2 58
8.2.3. Peak 3 59
8.2.4. Peak 4 60
8.2.5. Peak 5 61
8.2.6. Peak 6 63
8.2.7. Peak 7 66
8.2.8. Peak 8 67
8.2.9. Peak 9 68
8.2.10. Peak 10 69
8.2.11. Peak 11 70
8.3. Structural determination of Life krill oil capsules 71

8.4. Structure determination of phospholipids in raw extract AN1 72
8.4.1 Peak 1 74
8.4.2. Peak 2 75
8.4.3. Peak 3 77
8.4.4. Peak 4 80
8.4.5. Peak 5 82
8.4.6. Peak 6 84
8.4.7. Peak 7 85
8.4.8. Peak 8 86
8.4.9. Peak 9 87
8.5. Evaluation of DHA and EPA content 88
9. Conclusion and future prospects 91
References 92
Appendix 1 95
Appendix 2 96
Appendix 3 97
Appendix 4 98
Appendix 5 99
Appendix 6 100
Appendix 7 101
Appendix 8 102
Appendix 9 103
Appendix 10 104
Appendix 11 105
Appendix 12 106
Appendix 13 108
Appendix 14 109
Appendix 15 110
Appendix 16 111
Appendix 17 112
Appendix 18 113
Appendix 19 114
Appendix 20 115
Appendix 21 117
Appendix 22 118
Appendix 23 119

Abbreviations

AA	Arachidonic Acid
AHA	American Heart Association
ALA	Alpha-linolenic acid
CE	Collision Energy
CHD	Chronic Heart Disease
CID	Collision Induced Dissociation
DHA	Docosahexaenoic Acid
EPA	Eicosapentaenoic Acid
ESI	Electrospray Ionisation
FA(s)	Fatty acids(s)
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LysoPC(s)	Lyso Phosphatidylcholine(s)
LysoPL(s)	Lyso Phospholipid(s)
n-3 PUFAS	Omega-3 Polyunsaturated Fatty Acids
MS	Mass Spectrometry
m/z	Mass to charge ratio
NP	Normal-phase
OAc	Acetate anion
PC(s)	Phosphatidylcholine
PE	Phosphatidylethanolamine
PL(s)	Phospholipid(s)
QTOF	Quadropol-time-of-flight
RCT	Randomised Controlled Trials
RF	Radio Frequency
RP	Reversed-phase
TEA	Triethylamine
TG	Triglycerides
UPLC	Ultra Performance Liquid Chromatography

1. Introduction

Before pharmaceuticals are released onto the market they need to go through substantial testing and the pharmaceutical industry is very well regulated when it comes to development of drugs and approval processes. The rules and regulations governing the dietary supplements industry on the other hand are far less strict. Although they vary from country to country much of the responsibility to ensure the products safety falls on the manufacturer, and generally there is no approval needed to produce or sell dietary supplements. There are rarely animal and human studies conducted to prove the effect of supplements, which leaves the consumers to evaluate the health effects for themselves. A survey was conducted among Nordic consumers, on the behalf of the Nordic Council and the Norwegian Food Authorities, to explore the consumer's knowledge, attitude and experience of dietary supplements. The survey revealed that more than 50 % of the respondents have concerns about the content of dietary supplements and more than 75 % feel they have little or no knowledge about dietary supplements (1). However studies have shown that dietary supplements have become an established part of people's daily intake (2) and that supplements based on marine oils are one of the most popular and commercially important supplements especially in the Nordic countries (1-3). Krill is emerging as one of the newcomers on the market with an ability to deliver omega-3 fatty acids. The research done on this type of marine oil is not as extensive as the work that has been done on fish oil, but preliminary studies show that this can be a new and effective delivery system for essential fatty acids (4).

2. Dietary supplements and marine oils

Dietary supplements are products intended to add nutritional value to the diet or have a physiological effect on normal body functions (5). Dietary supplements are concentrated sources of vitamins, minerals or other ingredients, such as supplements based on marine oils and plant extracts. They are sold prepacked and come in a variety of dosage forms. Most commonly are tablets, but depending on the physical and chemical properties of the substance and compliance of the product, supplements are also found as granules, hard or soft gelatine capsules, effervescent tablets or as a liquid.

The laws and regulations governing the dietary supplement industry are different from the ones that relate to pharmaceutical drugs. Dietary supplements can contain some of the same substances as pharmaceutical drugs, but then usually in a much lower concentration. Pharmaceutical drugs are defined in the Norwegian Drug regulation as "any substance, plant drug or product that is claimed to prevent, heal or alleviate disease, disease symptoms or pain, or affect human or animal physiological functions" (6). This means that a product is considered a drug if it falls under the mentioned definition or if it is presented as a product that has the mentioned effects. The laws and regulations regarding pharmaceutical drugs leave little room for interpretation, and the safety and quality of the products are strictly maintained. Dietary supplements on the other hand falls under the Norwegian law "*Lov om matproduksjon og mattrygghet mv. (matloven*)" that governs food safety. There is a regulation under this law that deals specifically with dietary supplements where it is given which vitamins and minerals the manufacturers are allowed to include in dietary supplements. This is in compliance with the European Union legislation regarding dietary supplements that Norway, as a member of the EEA, has agreed to. The maximum limits for the ingredients are also given, in addition to requirements concerning production, purity, labelling and marketing. It is however the manufacturers own responsibility that they comply with the legislation. The Norwegian Food Safety Authority has the overall responsibility, but supplements do not need to be approved before they are made available to the consumer (2) and the quality of the products mainly depend on the internal procedures and quality systems of the manufacturers.

As of 2013 there are no quality demands regarding dietary supplements based on marine oils, such as omega-3 and krill supplements. They are not included in the legislation for vitamins and minerals and therefor the only real demands the manufacturers need to comply with are the requirements for hygiene, production, storage and transport (7). Over the years there has been a substantial and steady increase in sales of marine oils and omega-3 products.

Bransjerådet for naturlegemidler, an independent Norwegian organisation representing the dietary supplement industry, reported that in 2010 marine oils and omega-3 products accounted for 22 % of the entire dietary supplements market, and from 2010 to 2011 the sales of omega-3 products increased by 30% (8). Even though this category of supplements now clearly are an important part of people's everyday diet there were per 2010 no instances that supervised the production or control the omega-3 products that are available to consumers. As mentioned the manufacturers of omega-3 and marine oils only need to comply with the regulations of the law that governs food safety. As a part of this they also have to ensure that the labelling and health claims are correct. In 2010 new regulations concerning health and nutrition benefits of dietary supplements was implemented (9), and this legislation is harmonised throughout the EEA and EU countries as the *"EU Register of nutrition and health claims made on foods"*. The main ingredients of marine oils, that the manufacturers make health claims about, are the essential nutrients eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). According to the EU legislation, if a manufacturer is to claim health benefits regarding maintenance of blood pressure and triglyceride levels, the products needs to provide DHA and EPA of 2-3 grams when consumed on a daily basis. If claims concerning maintenance of normal heart and brain function, brain development of the foetus and maintenance of normal vision are made the product needs to provide 250 mg DHA and EPA on a daily basis. Because there is no routine testing of dietary supplements it is interesting to investigate weather the content of the supplements match the labelled ingredients. The focus of this thesis have been marine oils, in particular krill oil supplements, and evaluation of the contents.

3. Lipids

Dietary supplements such as fish oil and krill oil, are lipid-based supplements. Because this category of supplements is a growing trend and an increasing part of people's everyday diet this area represents an interesting research field.

Even though the research done on lipids is substantial there is still no universally accepted definition of the term. Lipids are in general described as a very diverse group of compounds and one definition proposed by Christie states that lipids are "naturally occurring compounds, which have in common a readily solubility in organic solvents such as chloroform, benzene, ethers and alcohols." (10). This can be somewhat misleading, as some of the compounds that are considered lipids are also soluble in water, e.g. short-chain fatty acids and a definition based on more than just solubility would be useful. The same author along with others (10, 11) have later proposed that the very varied class of lipids would be best defined by structure, which is also very useful for chromatographic analysis.

Christie (10) and O'Keefe (11) divided lipids into two and three major classes; derived, simple and complex. Derived lipids are the basic building blocks of simple and complex lipids and include fatty acids and alcohols. Simple lipids are lipids that "can be hydrolysed to two different compounds". Complex lipids are structures that "yield more than three different compounds on hydrolysis" (11). Included here are the glycerolipids, glycerophospholipids and sphingolipids.

In 2005 the International Lipid Classification and Nomenclature Committee (ILCNC) saw the need for a common international language and classification to ease the communication and create a joint platform for all the research presented on the field of lipids (12). The committee developed a new classification scheme based on structure and function and has organized the lipids into eight categories (Table 1), which is now being used internationally by lipid researchers in a variety of fields.

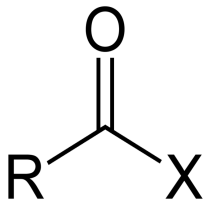
Table 1: Revised table of lipid categories of the comprehensive classification system developed by the ILCNC (13).

Category	Abbreviation
Fatty acyls	FA
Glycerolipids	GL
Glycerophospholipids	GP
Sphingolipids	SP
Sterol Lipids	ST
Prenol Lipids	PR
Saccharolipids	SL
Polyketides	PK

Lipids from a range of different sources are included in the classification system. In addition to mammalian lipids, non-mammalian sources such as plants, bacteria, fungi, algae and marine organisms are represented (13). Only three of the lipid classes are relevant for this thesis and will be explained in more detail.

3.1. Fatty Acyls

The Fatty Acyls are one of the most important lipid categories because the fatty acyl structure represents the main building block for more complex lipid structures. The general fatty acyl structure is presented in Figur 1, where R represents an alkyl chain and the X will vary for each of the 14 subcategories of the fatty acyls. This is a very broad class of compounds that all have in common the basic fatty acyl structure and the main synthesis pathway (12). The fatty acids and conjugates, eicosanoids and fatty esters are found in this category. Most commonly known and used in dietary supplements are the fatty acids.



Figur 1: The general fatty acyl structure that is a building block for many complex lipids. R = alkyl chain. The X moiety depends on the fatty acyl class

3.1.1. Structure and nomenclature of fatty acids

The fatty acids are the structurally simplest lipids and some of the most important building blocks of more complex lipids. They are "aliphatic, usually straight chain, monocarboxylic acids." (14). Generally the fatty acids contain an even number of carbon atoms in a straight chain attached to a carboxyl group in one end and a methyl group in the other end. The most common chain-length found in animal tissue is between 14 and 22 carbons (10). Fatty acids can also have one or more double bonds of either *cis* or *trans* configuration. The naturally predominant is the *cis* configuration (10). There are significant differences in chain-length, number of double bonds and their position in naturally occurring long chain fatty acids. This means that there is a great variety (15) and more than 1000 fatty acids are known that are all structurally different (14).

Fatty acids containing no double bonds are termed saturated fatty acids. They are usually straight-chained compounds, and in animal and plant tissue there are usually 14, 16 or 18 carbon atoms (10). According to the standard nomenclature developed by the International Union of Pure and Applied Chemistry (IUPAC) saturated fatty acids are named systematically after the number of carbons atoms in the chain. The carboxyl carbon is marked as number 1, and the ending is changed from "e" to "oic" (10, 11). Over the years trivial names, also called common names, have been introduced and for some fatty acids they are more frequently used. The molecular and structural formula of a saturated fatty acid with 16 carbon atoms is shown below in Figure 2.

$CH_3(CH_2)_{14}COOH$

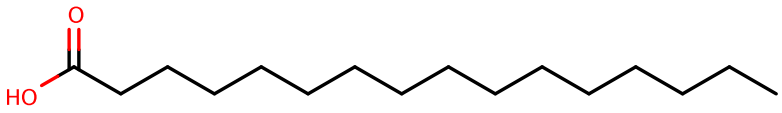


Figure 2: Molecular structure of hexadecanoic acid, also called palmitic acid (16:0)

The recommended systematic name is hexadecanoic acid. This fatty acid is the most abundant of the saturated fatty acids and is often referred to by its trivial name, palmitic acid. In shorthand nomenclature palmitic acid may be termed 'C_{16}', or more precisely '16:0', where the zero indicated that there are no double bonds present.

Unsaturated fatty acids contain one or more double bonds. The double bonds can be found in different positions and is specified in the nomenclature along with the double bond geometry, which is assigned using cis/trans, or E/Z nomenclature. The double bond position is allocated

in a number of ways. According to the recommended IUPAC rules the double bond position is indicated with a number before the fatty acid name the numbering beginning from the carboxyl end of the chain (14). The notation Δ is often used in shorthand nomenclature. The shorthand abbreviation for unsaturated fatty acids used throughout this thesis is "Number of carbon atoms:number of double bonds $\Delta^{\text{position(s) of double bond(s)}\textit{double bond configuration}}$".
The molecular and structural formula of an unsaturated fatty acid with 18 carbon atoms is shown below in Figure 3.

$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$

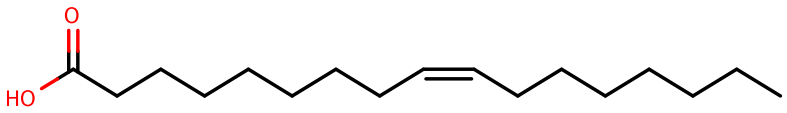


Figure 3: Molecular structure of 9-octadecenoic acid, also called oleic acid ($18{:}1\Delta^{9c}$)

The recommended systematic name is 9-octadecenoic acid, the trivial name oleic acid and the shorthand nomenclature is $18{:}1\Delta^{9c}$. For simplicity the stereochemistry is not indicated in the figures or throughout the thesis. The ω -and n-systems are sometimes used in nomenclature and this indicates the double bond positions from the methyl side of the carbon chain. It is written ω-x or n-x, where x is the double bond carbon numbered from the methyl side of the carbon chain, e.g. (ω-9)-18:1 or (n-9)-18:1 for oleic acid (14). A list of relevant fatty acids with their trivial names and shorthand nomenclature is given in Table 2.
The ω -and n-systems are sometimes used in nomenclature and this indicates the double bond positions from the methyl side of the carbon chain. It is written ω-x or n-x, where x is the double bond carbon numbered from the methyl side of the carbon chain, e.g. (ω-9)-18:1 or (n-9)-18:1 for oleic acid (14). A list of relevant fatty acids with their trivial names and shorthand nomenclature is given in table 2. .

Table 2: Relevant fatty acids with their trivial names and shorthand nomenclature. The most commonly known double bond position is indicated in these fatty acids (12).

Chain length: Double bonds	Double bond position	Trivial name (- Acid)	Systematic name	Shorthand name	Monoisotopic mass (Da)	$[M-H]^-$ (Da)
14:0		Myristic	Tetradecanoic acid	14:0	228.2089	227.2017
16:0		Palmitic	Hexadecanoic acid	16:0	256.2402	255.2330
16:1	9	Palmitoleic	9-hexadecenoic acid	$16:1\Delta^{9}$	254.2246	253.2173
18:0		Stearic	Octadecanoic acid	18:0	284.2715	283.2643
18:1	9	Oleic	9-octadecenoic acid	$18:1\Delta^{9}$	282.2559	281.2486
18:3	9, 12, 18	Apha-linolenic	9,12,15- octadecatrienoic acid	$18:3\Delta^{9,12,18}$	278.2245	277.2168
18:4	6, 9, 12, 15	Stearidonic	6,9,12,15-octadecatetraenoic acid	$18:5\Delta^{6,9,12,15}$	276.2089	275.2017
22:1	13	Eruic acid	13-docosenoic acid	$22:1\Delta^{13}$	338.3185	337.3112
20:5	5, 8, 11, 14, 17	EPA	5,8,11,14,17-eicosapentaenoic acid	$20:5\Delta^{5,8,11,14,17}$	302.2246	301.2173
22:6	4, 7, 10, 13, 16, 19	DHA	4,7,10,13,16,19-docosahexaenoic acid	$22:6\Delta^{4,7,10,13,16,19}$	328.2402	327.2324

3.1.2. Function and use of fatty acids

The body produces most of the fatty acids that are needed for optimal health, but some fatty acids need to be supplied through the diet. These fatty acids are called essential fatty acids and can broadly be divided into two different categories, omega-3 and omega-6 fatty acids. This is illustrated in Figure 4.

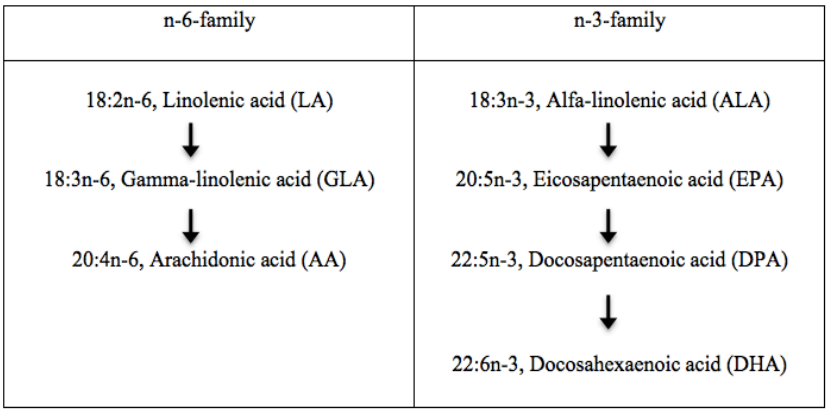


Figure 4: The two main categories of essential fatty acids. The body has the ability through several metabolic processes to convert the precursor fatty acids to longer chained ones.

The n-system has been used as nomenclature as this is the most frequently used when talking about essential fatty acids. Omega-3 polyunsaturated fatty acids (n-3 PUFAS) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are to a small extent converted through a series of metabolic steps from alpha- linolenic acid (ALA) together with their eicosanoidic derivates (16). Arachidonic acid (AA) is the precursor fatty acid in the Omega-6 family and are metabolically converted to pro-inflammatory eicosanoids, such as prostaglandins (17). The metabolic pathways of these two fatty acids families are similar and thus they can compete for enzymes and have an inhibitory effect on each other (16). There has been suggested that the ratio between these two families of fatty acids might influence the pathogenesis of many chronic diseases and a low omega-6/omega-3 ratio is favourable (18). EPA and DHA are the most commonly known essential fatty acids, much because they are involved in many processes in the body and play a vital role in health (17). Among others they are an important part of the phospholipids in the structure of all cell membranes. They also affect the cholesterol and the brain- and eye function. These essential fatty acids are precursors for different eicosanoids that influence inflammation, blood pressure and platelet function in the body (19, 20). EPA has a total of 20 carbon atoms and five double bonds, where the first double bond is located at the third carbon from the methyl end and the

shorthand name used throughout this thesis is 20:5$\Delta^{5,8,11,14,17}$, illustrated in Figure 5. DHA has a total of 22 carbon atoms and 6 double bonds, where the first double bond is also located at the third carbon from the methyl end thus making it an omega-3 fatty acid, illustrated in Figure 6. The shorthand nomenclature for DHA is 22:6$\Delta^{4,7,10,13,16,19}$.

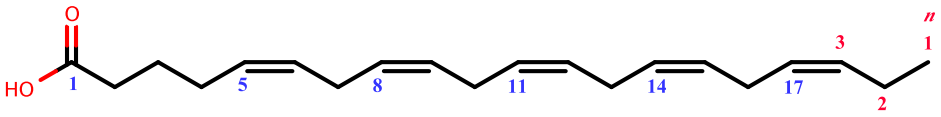


Figure 5: Molecular structure of EPA (20:5$\Delta^{5,8,11,14,17}$)

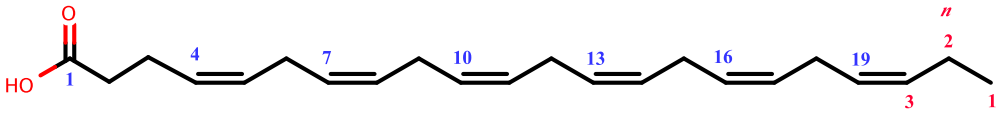


Figure 6: Molecular structure of DHA (22:6$\Delta^{4,7,10,13,16,19}$)

In recent years the fatty acids EPA and DHA have had several health benefits attributed to them and for the last three decades much research has been done in this area. Randomised controlled trials (RCT), observational- and experimental studies along with meta-analysis and systematic reviews have been conducted. There has been suggested that EPA and DHA may have a positive effect on total mortality, cardiovascular health and cancer (21-23). A meta analysis by Bucher et.al concluded that "Intake of n-3 polyunsaturated fatty acids reduces overall mortality, mortality due to myocardial infarction, and sudden death in patients with coronary heart disease." Although more recent evaluations (24, 25) of available information and a RCT study by Burr et. al (26) have not ruled out important health effects of omega 3 fats, there is some doubt with regard to the established health benefits. There is a need to investigate for example the mechanism behind and dose needed for possible beneficial outcomes and bigger and more robust studies are required to draw definite conclusions. There seems to be more benefits related to patients with diabetes, hyperlipidaemia or patients having had a myocardial infarction (24) compared to generally healthy persons.

Guidelines regarding public health both in the UK, the Nordic countries and in America include recommendations about oily fish and omega-3 fatty acids (27-29). The American Heart Association (AHA) has different recommendations for different patient categories as shown in Table 3.

Table 3: Summary of recommendations for Omega-3 fatty acids from the American Heart Association. CHD = Chronic Heart Disease

Population	Recommendation
Patients without documented CHD	Eat a variety of (preferably oily) fish at least twice a week. Include oils and foods rich in α-linolenic acid (flaxseed, canola, and soybean oils; flaxseed and walnuts).
Patients with documented CHD	Consume ≈1 g of EPA+DHA per day, preferably from oily fish. EPA+DHA supplements could be considered in consultation with the physician.
Patients needing triglyceride lowering	Two to four grams of EPA+DHA per day provided as capsules under a physician's care.

3.2. Glycerolipids

Glycerolipids are the largest of the eight lipid categories and include all glycerol-containing lipids. The basic structure of glycerol is shown in Figure 7.

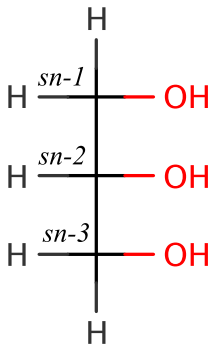


Figure 7: Basic structure of glycerol.

This category predominately consist of mono-, di-, and tri-substituted glycerols. In these compounds the hydroxygroup of the glycerol is esterified, typically by fatty acids. The glycerol backbone will then have one, two or three fatty acids attached. There is a stereospecific numbering system to describe these compounds, which is shown in Figure 7 as well. This group of lipids are the ones most commonly found in mammalian storage tissue, and the tri-substituted glycerols, also known as triglycerides, are also an important ingredient in dietary supplements (12).

3.2.1. Structure and nomenclature of triglycerides

Triglycerides, also known as triacylglycerols or TAG, are one of the simplest lipid classes and are the major building blocks of most natural fats and oils. They are esters of glycerol and consist of a glycerol moiety and fatty acids. The fatty acids can all be the same, two can be the same or they can all be different, as shown in Figure 8 (14). The recommended systematic name for the structure in Figure 8, which has palmitic acid (16:0) in *sn-1* position, EPA (20:5$\Delta^{5,8,11,14,17)}$ in *sn-2* position and oleic acid (18:0) in the *sn-3* position, is 1-octadecanoyl-2-(5,8,11,14,17-eicosapentaenoyl)-3-hexadecanolyl-*sn*-glycerol. The –ic ending id replaced with –oyl and the positions of the fatty acids are designated by using numbers.

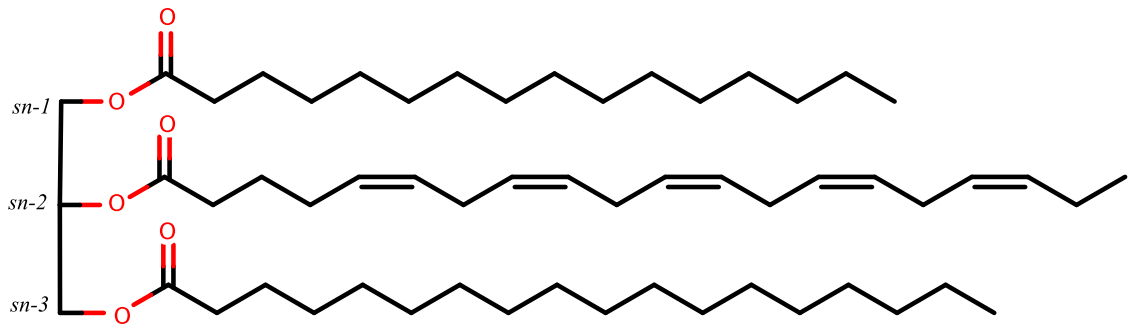


Figure 8: Molecular structure of the triglyceride 1-octadecanoyl-2-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl)-3-hexadecanolyl-*sn*-glycerol

3.2.2 Function and use of triglycerides

All that is mentioned about fatty acids in the previous section applies to the fatty acids in these compounds, and triglycerides are undoubtedly a great source of essential fatty acids. In animals and most plants triglycerides act as storage lipids and are therefore of great importance in physiology as well as in the food industry. Triglycerides are important dietary components and often the essential fatty acids in dietary supplements are bound to glycerol backbones.

3.3. Phospholipids

Glycerophospholipids, also called phospholipids, have many similarities with the previous category, glycerolipids, but have been made a separate group "because of their abundance and importance as membrane constituents, metabolic fuels, and signalling molecules" (12). They have a similar skeleton but differ, usually at the *sn*-3 position, where phospholipids (PLs) have what is called a polar head group. The polar head group consists of a phosphate group and usually an organic base or polyhydroxy compound (11). The basic structure of phospholipids is shown in Figure 9. There are several classes of phospholipids and they are most commonly divided into groups based on the characteristics of the polar head group. Attached to the *sn-1* and *sn-2* positions on the glycerol backbone are often two ester-linked fatty acids.

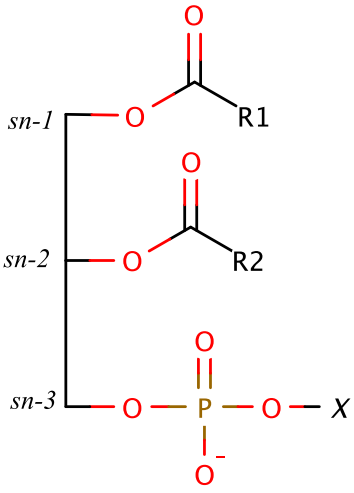


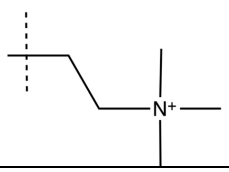
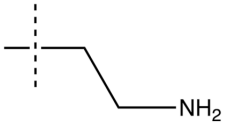
Figure 9: The general structure of a phospholipid where X denotes the part of the polar head groups that differs between the individual PL species.

3.3.1. Structure and nomenclature of phospholipids

All phospholipids (PLs) contain a glycerol backbone, a phosphate group and a polar head group that is attached to the phosphate group. This makes them di-glycerides and in most phospholipids the phosphate group is linked to the *sn-3* position (11). The glycerol backbone becomes chiral when the two substituents at *sn-1* and *sn-3* position are different, but again for

simplicity this is not indicated throughout the thesis. PLs can have fatty acids attached in both *sn-1* and *sn-2* position or only in one position. When there is only one fatty acid present, usually in *sn-1* position, they are termed lysophospholipids (lysoPLs) (10). PLs are termed complex lipids and have an amphipathic nature having both hydrophobic and hydrophilic groups. The relevant and most common phospholipids for this thesis are shown in Table 4.

Table 4: Structures of phospholipids relevant for the present thesis.

Class	Phosphatidylcholine	Phosphateidylethanolamine
Polar head group (X)		
Abbreviation	PC	PE

Phospholipids are systematically named and often the abbreviations for the head group classes are used (PC = phosphatidylcholine etc.). Figure 10 shows a phospholipid with PC as a polar head group and the fatty acid 16:0 (palmitic acid) in *sn-1* position and 22:6$\Delta^{4,7,10,13,16,19}$ (DHA) in the *sn-2* position.

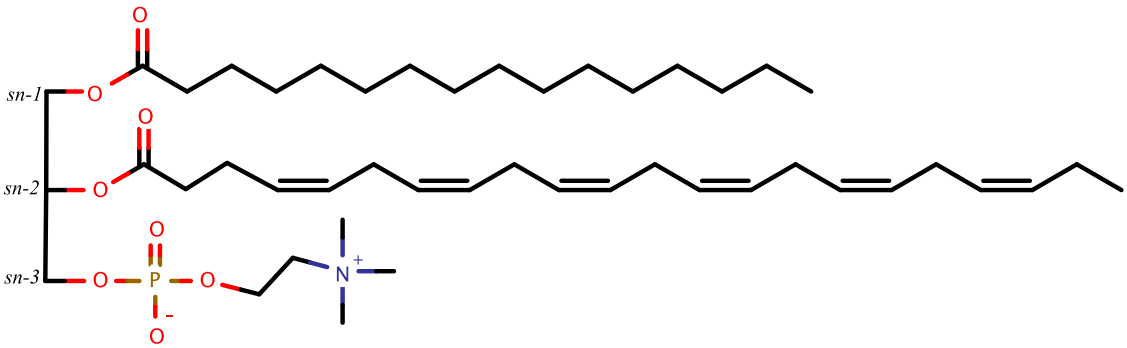


Figure 10: 1-hexadecanoyl-2-(4,7,10,13,16,19)-docosahexaenoyl-sn-glycero-3-phosphatidylcholine

The fully systematic name is 1-hexadecanoyl-2-(4,7,10,13,16,19)-docosahexaenoyl-sn-glycero-3-phosphatidylcholine. The more convenient abbreviation 16:0-22:6$\Delta^{4,7,10,13,16,19}$ PC will be used throughout the text.

3.3.2. Function and use of phospholipids

Phospholipids are amphipathic in nature and are a significant part of animal cell membranes where they form what is called the lipid bilayer. This lipid bilayer is the structural basis for all animal cells and is illustrated in Figure 11. The polar moiety of the lipid will be facing out and the neutral fatty acid "tails" will be facing inwards. PC is in abundance in the cell membranes with PE as the second most frequent phospholipid class in animal tissue (10).

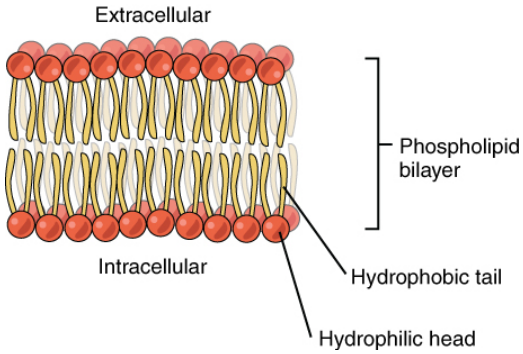


Figure 11: Phospholipids in the lipid bilayer of celle membranes

PEs are also important messengers in the endocannabinoid signalling system (14).

The main digestion of phospholipids occurs in the small intestine, and dietary phosphatidylcholine is rapidly and almost completely absorbed (30). Phospholipases, mainly Phospholipase A2, hydrolyses the PLs in stereospecific positions. Phospholipase A2 interacts with PLs at the *sn-2* position to yield free fatty acid and lysoPC.

These are both absorbed into the mucosal cells and incorporated into the chylomicron surface layer. In the bloodstream the PLs and free fatty acids are released from the chylomicrons and can be taken up by various tissue. There has been suggested that the chemical form of the phospholipid can influence the distribution of FAs to different tissues (31). PLs do not need bile salts to solubilize and form micelles, which aids the absorption and bioavailability (32). Because they are building blocks of all cell membranes they are also easily incorporated into different tissues.

PLs are used in a variety of areas and are often referred to as lechitins in the industrial field. They are commercially available for use in food industry, cosmetic and pharmaceutical fields (4). In the pharmaceutical industry it is utilized in drug delivery systems as nanocarriers and in cosmetics the conditioning and softening characteristics are exploited. Being surface-active compounds they are often used in nutritional fields as emulsifiers, lubricants and also for wetting and dispersion purposes (4). Phospholipids are naturally occurring in a range of foods but are also used as a food additive, for example in infant formulas. As a dietary supplement PLs are used to deliver essential fatty acids and one of the most frequently used phospholipids is phosphatidylcholine. It has been suggested that a high content of essential fatty acids such as EPA and especially DHA in the *sn-2* position of dietary phospholipids is beneficial (33). Choline (Figure 12), which is the polar head group of phosphatidylcholine

and phosphatidylcholine itself, are also essential nutrients and has a variety of functions in the human body. Choline affects cell structure, neurotransmitter synthesis, inflammation and cognitive function (34). It is also thought to influence diseases such as liver and heart disease (34).

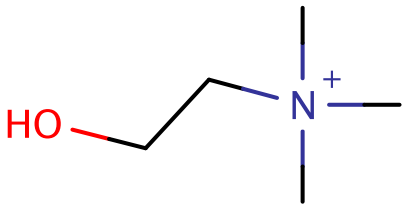


Figure 12: General structure of choline

3.4. Lipids from krill oil

Krill oil represents a new branch of marine oils that for the last few years have generated a lot of interest because of the unique form of lipids in these omega-3 products. The term krill is used to describe more than eighty species of crustaceans, which have a shrimp-like appearance. The commonly known "Arctic krill" or *Euphausia Superba* is the species of krill that is mostly harvested for human consumption (35) and one of the most commercially interesting species because of its abundance. Even with their small size they form huge surface swarms, and the density of krill can be as high as 1 million animals per cubic metre of water and stretch for several kilometres (35). They feed on marine algae (phytoplankton) that synthesize omega-3 fatty acids, DHA and EPA among others, and accumulate these in their eggs and body (35, 36). Antarctic krill is an important part of the Southern Ocean eco system and is a valuable food source for whales, seals, fish, squid and birds (35, 37).
The nutrition composition of krill has been investigated and it has been found that this is a great source of lipids and essential fatty acids. Almost all studies of krill found that the main lipid classes were phospholipids, triglycerides and free fatty acids (38). *Euphausia Superba* contains more than 40% phospholipids were phosphatidylcholine is most abundant. The dominating fatty acids have been reported to be 16:0 among saturated fatty acids and 18:1, 20:5 and 22:6 among the unsaturated. The positional analysis of fatty acids in PC and PE showed that the saturated fatty acids are mainly attached in *sn-1* position while the unsaturated fatty acids are commonly linked to the *sn-2* position (38).

3.5. Phospholipids vs. triglycerides used as dietary supplements

Krill oil represents a valuable source of omega-3 fatty acids and studies have shown that 30 - 65 % of the fatty acids in krill are attached to a phospholipid moiety while in commercial fish oil most of the fatty acids are bound as triglycerides (35). More and more studies are emerging where the bioavailability of different omega-3 formulations are compared. Studies have suggested that the chemical form of the lipid influences absorption and bioavailability. Fatty acids may be better absorbed from dietary PL than from TG as indicated in studies done on new-born infants (39), and also when infants receive formula where essential fatty acids were bound to PL this has shown a higher absorption. (40). Three clinical trials have included krill in bioavailability studies. A relatively small study by Schuchardt et al. indicated that DHA and EPA were better absorbed from krill than re-esterified triglycerides (41). Maki et al. found that DHA and EPA from krill oil was absorbed at least as well as essential fatty acids from menhaden oil (TG) and showed a trend towards greater increase in plasma DHA and EPA with krill oil (42). A third study done by Ulven et al. found no statistically significant evidence that essential fatty acids from PLs are absorbed or have a better health effect than essential fatty acids in TG form (39). All though the research done on humans is limited, there is more evidence from animal data to support that the bioavailability of krill oil is superior (43, 44) and because PLs don't require digestive enzymes to be absorbed this would be expected to increase the bioavailability.

The emerging studies indicate that the bioavailability of essential fatty acids in PL form and from krill oil are comparable to TG and are a promising way of delivering fatty acids. In addition phosphatidylcholine is an essential nutrient in itself. More randomised studies should be executed on a study population with for example elevated blood TG levels and lowered HDL-cholesterol and in addition other biomarkers such as inflammation, oxidative stress and haemostasis should be investigated to be able to predict a better health effect of PLs.

4. Analysis of lipids

Mass spectrometry (MS) has proven to be an effective tool for analysis of both simple lipids and more complex lipid structures. Coupled with the power of ultra performance liquid chromatography (UPLC) it is possible to gain information on the polar head group and the fatty acid substituents on the glycerol backbone. The identification of the phospholipid profiles is important to be able to evaluate the nutritional properties of food ingredients and products including dietary supplements (45). Dietary supplements, recommended as part of a healthy diet, often consist of very complex mixtures of different lipid structures. Krill supplements delivers essential fatty acids bound to phospholipids and often these components represent close to half of the declared content.

4.1. Ultra performance liquid chromatography

UPLC is a further development of high performance liquid chromatography (HPLC). HPLC is a separation technique where analytes are separated based on partition between a liquid mobile phase and a solid stationary phase. The distribution between the stationary phase and mobile phase depends on the nature of the analyte, the composition of the mobile phase and the properties of the stationary phase. The stronger the affinity for the stationary phase the longer the retention time will be (46). If a sample contains more than one analyte the difference in their chemical properties and affinity for the stationary phase are exploited to separate them. A liquid sample is driven through a column packed with a stationary phase by liquid flow at high pressure provided by mechanical pumps. The advancement of UPLC, using columns with smaller particle sizes and instrumentation that can withstand higher backpressures, increases the resolution, speed and sensitivity of liquid chromatography (47). UPLC is a useful tool for analysing lipids. Compared to older methods of separation, UPLC minimises oxidation of phospholipids and also reduces contamination (48). Both normal-phase UPLC (NP-UPLC) and reversed-phase UPLC (RP-UPLC) can be used when analysing lipids. NP-UPLC separates lipid classes based on polarity. The lipids elute in order of increasing polarity according to their respective classes. For the present thesis the relevant lipid classes would elute in the following order TG<PL<FA. The phospholipids also elute according to their individual classes, resulting in PEs eluting first and PCs eluting last (45).

RP-UPLC separates the lipids based on lipophilicity. The more lipophilic a compound is the more it is retained on the column. Thus in addition to polar functional groups, the fatty acids chain length and number of double bonds determines the elution sequence (48). The elution order for the relevant lipid classes is FA<PL<TG, however there is potential for overlapping of the different lipid classes. Lysophospholipids, containing only one fatty acid, also elute early in the chromatogram before the PLs containing two fatty acids. Depending on the fatty acyl groups attached overlapping of different PL groups is possible. As opposed to NP-UPLC, RP-UPLC allows for separation of each lipid class into individual species and is a suitable system for complex mixtures of lipids and has been the technique of choice for industrial and bio analytical analysis (49).

4.2. Mass spectrometry

The technique of mass spectrometry is based on separation of ions in gas phase according to their mass-to-charge ratio, *m/z*. The basic principle of MS is to generate charged ions and separate them by exposing them to dynamic or static electric fields (50). A mass spectrometer consists of an ion source, a mass analyser and a detector.

All analyses performed in the present thesis have been performed using electrospray ionisation (ESI). ESI takes place under atmospheric pressure outside the mass spectrometer. The analytes are usually in a solution that is transferred through a stainless steel capillary with high voltage applied so that an aerosol is formed. This aerosol formation is often improved by using a nebulising gas, usually nitrogen. The aerosol consists of a large number of small charged droplets. Between the capillary tip and mass analyser there is a cylindrical electrode with a counter electrode that draws the droplets towards the sampling cone. As the droplets cross the space between the needle tip and the cone the mobile phase solvent evaporates. This leads to the shrinking of the droplets and with the aid of a heated drying gas the charge density of the droplets reach a critical point and a so called columbic explosion occurs. This happens repeatedly until only the molecular species are left. Negatively charged species are formed if the potential applied to the steel capillary is negative. Usually the loss of a proton leads to the formation of $[M-H]^-$. Likewise when applying positive charge commonly a protonated molecular specie, $[M+H]^+$ is formed.

In the mass analyser the charged species are separated because of the different deflection in the magnetic or electric field. A time-of-flight (QTOF) mass analyser was used in the analysis of lipids in the present thesis. In the QTOF a linear quadropol was placed in front of a reflectron time-of-flight (TOF). A collision cell was located between the quadropole and the TOF analyser to induce fragmentation in MS/MS experiments. In a linear quadropole a continuous ion beam is sent though four parallel metallic rods where the opposing pair are connected electrically (50). The two pairs are each held at the same constant voltage and a fluctuating radio frequency (RF) voltage (46). This leads to periodic attraction and repulsion, and the ions are deflected in complex trajectories allowing only ions with particular *m/z* ratio to pass through at specific values of the applied voltages.
In a TOF an ions *m/z* ratio is determined via time measurement. Ions of different *m/z* are accelerated in pulses by an electric field and dispersed in time during their flight along a path of known length. Ions with the same charge are accelerated to the same kinetic energy, and assuming that all ions start their flight within a sufficiently short time interval the ions that have a low *m/z* value arrive earlier at the detector than the ions with a high *m/z* (50). In an appropriate detector the quantities (intensity) of the ions are plotted as a function of the *m/z* ratio.

4.3. ESI-MS of lipids

With the introduction of electrospray ionization mass spectrometry (ESI-MS) the earlier more time-demanding and nonspecific technics are outdone and makes it easier to analyse intact phospholipids (48). Being a soft ionisation technique ESI can ionise lipids with little to no fragmentation, which generates molecular ions of high intensity and makes structure determination easier because most likely the ions observed are molecular ion species (51). Although not strictly molecular ions, because neither has the correct molecular mass of the lipid, they are still referred to as molecular ions because most often they have the correct molecular mass, plus or minus a proton. PC is the exception and will be explained in more detail later. In positive ESI mode mainly information about the phospholipid head group is obtained. In negative mode more information on structure and the position of the fatty acids on the glycerol backbone can be determined when utilizing MS/MS. Class specific fragments are also more easily obtained in negative mode (52).

Mass spectrometry is frequently used because of it's high sensitivity and specificity (45). To gain more structural information, collision-induced dissociation (CID) is often used as a fragmentation technique and provides information on the polar head group and the fatty acid substituents on the glycerol backbone.

Many class specific fragments can be identified and can be used in the characterisation process (52). The literature on negative ionization of PLs is more extensive than for positive ionisation. For all PL classes there will be produced a high amount of deprotonated molecules $[M-H]^-$, except for PC, which has a permanent positively charged nitrogen. The formation of negative ions requires either removal of the quaternary nitrogen or the addition of another anionic site in the PC structure. In negative mode PC have been known to yield $[M-CH_3]^-$ ions as a result of the demethylation of choline, but $[M-H]^-$ have also been detected. Addition of another anionic site can be achieved by the formation of adducts with other anions and both $[M+Cl]^-$ and $[M+acetate]^-$ have been detected depending on the buffer agent used in the mobile phases (48, 53, 54). When an adduct formation is observed, $[M-CH_3]^-$ is also often observed. This is likely to be because of collisional decomposition of the anionic acetate, where the anion abstracts the methyl group from the quaternary nitrogen. This can be used as a diagnostic for the presence of the choline moiety in PC (54). A summary of the most important class specific product ions predicative of the polar head groups formed upon CID is presented in Table 5.

Table 5: Characteristic product ions obtained from negative ESI-MS/MS and ESI-MS/QTOF of PLs. (55)

PL class	PC	PE
Molecular ion	$[M-H]^-$	$[M-H]^-$
	$[M-CH_3]^-$	
	$[M+Cl]^-$	
	$[M+OAc]^-$	
Class specific ions	*m/z* 224	*m/z 196*
	m/z 168	*m/z* 140
$[M-(R_2COOH + R_1COOH)]^-$	*m/z* 206	*m/z* 178

Other common negative ion decomposition ions from PLs include the loss of the fatty acids in *sn-1* and *sn-2* positions (Reactions 1a and 1b).

$[M\text{-}H]^- \rightarrow R_1COO^-$ (1a)

$[M\text{-}H]^- \rightarrow R_2COO^-$ (1b)

Either one of the fatty acids might also leave the negative charge on the PC backbone to yield a lysophospholipid (Reactions 2a and 2b).

$[M\text{-}H]^- \rightarrow [M - H - R_1COOH]^-$ (2a)

$[M\text{-}H]^- \rightarrow [M - H - R_2COOH]^-$ (2b)

Either of the acyl chains may also be lost as a ketene, which leaves a –OH group on the charged lysophospholipid that is left (Reactions 3a and 3B).

$[M\text{-}H]^- \rightarrow [M - H - R'_1C{=}C{=}O]^-$ (3a)

$[M\text{-}H]^- \rightarrow [M - H - R'_2C{=}C{=}O]^-$ (3b)

All the fragmentation mechanisms mentioned are valid for $[M\text{-}CH_3]^-$ as a precursor ion and also for fragmentation after adduct formation, for example with acetate anions.
The relative yield of the difference species have by some investigators been used to assign the fatty acids to either the *sn-1* or *sn-2* position. Previous studies have revealed a potential for structural characterisation based on the ratio of the carboxylate anions, where the loss f the fatty acid in *sn-2* position would be more likely to occur and thus yield a higher signal (48). However it has been debated weather this can be used without caution as other studies have seen that the relative ratios of the carboxylate anions are dependent on the degree of saturation, the total chain length and also the collision energy applied to the experiment (48, 56). The results indicating that the collision energy may influence the fragmentation pattern, where at low collision energies there is a preferential loss of the *sn-2* carboxylate anions and at higher CEs there is a preferential loss of the *sn-1* anion. This difference observed however may also be explained by further decomposition of the *sn-2* carboxylate anion (48, 56). Because different R_2COO^-/ R_1COO^- ratios also have shown to vary between the different PL classes this might suggest that there are interactions between the acyl chains and the polar

head groups as well. Because of many observations of the preferred loss of the fatty acid in *sn-2* position (reaction 1b) and also the dominant lysoPL after loss of a neutral fatty acid in *sn-2* position (Reaction 3b) this indicates a trend. It is worth noticing that many of these studies have analysed PLs that contain mono- and di-unsaturated fatty acids. Analysis of PLs with polyunsaturated fatty acids could result in a much more difficult and complex interpretation of spectra upon CID (57). Thus care must be taken when interpreting the R_2COO^-/ R_1COO^- ratio. As the ratio seems to be dependant on both the characteristics of the PL itself and the experimental conditions it is difficult to make general conclusions for PLs as a whole and for the individual PLs of the different PL classes.

5. Aim of thesis

The general aim of the thesis was to develop UPLC and MS methods that enabled structure characterisation of phospholipids in complex lipid samples. Dietary supplements that had a high concentration of phospholipids were selected for the study.

Specific sub-goals:

- ✓ Develop a chromatographic method that can separate lipids in complex krill oil samples
- ✓ Develop and optimise a MS/MS method to be used for structural characterisation of phospholipids

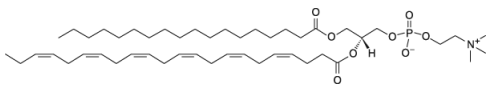
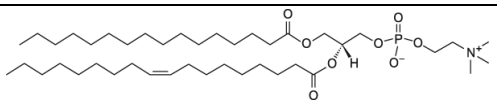
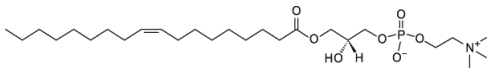
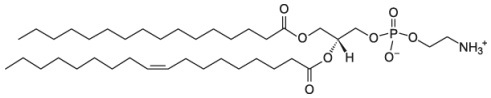
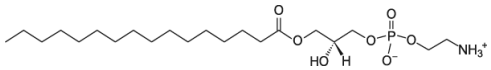
6. Materials and methods

Molecular structures are derived from http://www.chemspider.com/ (14.05.2014), provided by the Royal Society of Chemistry or drawn with molecular editing software ChemDraw version 2.0.1. developed by PerkinElmer. The molecular structures for the standards from Avanti Polar Lipids are taken from the product information sheets for the specific compounds, found at http://avantilipids.com/index.php?option=com_content&view=article&id=100&Itemid=108 (12.04.2014). Molecular and monoisotopic masses were calculated using a free online molecular mass calculator, http://www.lfd.uci.edu/~gohlke/molmass/?q=C29H58NO8P (12.05.2014), developed by Christoph Gohlke at The Laboratory for Fluorescence Dynamics, University of California Irvine.

6.1. Chemicals

The phospholipid standards 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC (1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholin), 16:0-18:1Δ^{9} PC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), 18:1Δ^{9}-0:0 PC (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine), 16:0-18:1Δ^{9} PE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine) all dissolved in chloroform and 16:0-0:0 PE (1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine) dissolved in chloroform, methanol and water were all purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Lipoid E80 standard (Mixture of lipids extracted from egg with approximately 80 per cent phosphatidylcholine. (See Appendix 1 for details) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Structures, molecular formula, exact masses and CAS registry numbers for the most relevant compounds are shown in Table 6.
Solvents for UPLC were Methanol hypergrade for LC-MS, Triethylamine for synthesis, Ammonia solution 28-30% for analysis, 2-propanole LiChrosolv gradient grade for liquid chromatography and Ammonium Acetate for analysis all purchased from Merck (Darmstadt, Germany). Acetonitrile HiPerSolv Chromanorm for HPLC was purchased from VWR BDH Prolabo (Leuven, Belgium). Water was obtained from a MilliQ purification unit from EMD Millipore Corporation (Billerica, MA, USA). Nitrogen was obtained from a Nitrogen Generator NM 32 LA (Peak Scientific) and Argon gas (99.99%) from AGA (Oslo, Norway).

Table 6: Molecular structure, formula, mass and CAS registry number of the chemical standards.

Name	Molecular structure	Molecular formula	Exact mass (Da)	CAS reg.no.
18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC		$C_{48}H_{84}NO_8P$	833,5935	59403-52-0
16:0-18:1Δ^{9} PC		$C_{42}H_{82}NO_8P$	759.5778	26853-31-6
18:1Δ^{9}-0:0 PC		$C_{26}H_{52}NO_7P$	521.3553	19420-56-5
16:0-18:1Δ^{9} PE		$C_{39}H_{76}NO_8P$	717.5309	26662-94-2
16:0-0:0 PE		$C_{21}H_{44}NO_7P$	453.2860	53862-35-4

Krill oil capsules were purchased from different health food shops. The product content declared on the packages is shown in Table 7.

Table 7: Content of krill oil capsules declared on the packages

Product name	Rimfrost Krill Oil (Euphausia Superba) 1 capsule	Life Krill Oil (Euphausia Superba) 1 capsule
Marine phospholipids (mg)	210	200
Omega-3 fatty acids (mg)	120	110
EPA (mg)	60	60
DHA (mg)	32.5	27.5

Raw extract material was donated by a Norwegian based company, who's name remains confidential and throughout the thesis will be known as AN. The raw extracts donated were given the anonymous names AN1 and AN2. The manufacturers goal was to develop a high quality marine oil product, and differences between the raw extract materials and the information about extraction methods were confidential and not provided. The aim of the extraction however was to enrich phospholipids with the essential fatty acids EPA and DHA.

6.2. Materials

6.2.1. UPLC-MS

Ultra performance liquid chromatography was performed using a Waters Acquity Ultra Performance LC I-Class instrument. Five different columns, all purchased from Waters corp. (Milford, MA, USA) were used to find optimal separation for different phospholipids.

Table 8: Properties of columns used in method development

UPLC-column	**Properties**	
Acquity UPLC® BEH C18 column	Dimensions:	2,1 x 100 mm
	Particle size:	1,7 µm
Acquity UPLC® BEH Amide column	Dimensions:	2,1 x 100 mm
	Particle size:	1,7 µm
Acquity UPLC® BEH C18 VanGuard	Dimensions:	2,1 x 5mm
Pre-column	Particle size:	1,7 µm
Acquity UPLC® BEH C18 column	Dimensions:	2,1 x 50 mm
	Particle size:	1,7 µm
Acquity UPLC® BEH C18 column	Dimensions:	1 x 150 mm
	Particle size:	1,7 µm

Mass spectrometric detection was conducted on a Waters Xevo G2 Q-TOF with an electrospray ionization source. The software used for analysis was MassLynx version 4.1.

7. Method development

The phospholipid standards were used to find the optimal conditions for separation and mass spectrometric analysis of phospholipids. These methods were then applied to unknown krill oil samples and raw extracts to identify the different phospholipid classes and try to determine their structures based on literature and information from chromatograms and mass spectra.

7.1. Phospholipid standards analysed with UPLC-MS

All standards were stored under nitrogen at -18°C in the dark to keep oxidation to a minimum. The phospholipid standards were made fresh approximately every third day.
Operating conditions for all full scan TOF-MS analysis are shown in Table 9.

Table 9: Parameters for full scan TOF-MS mode

Conditions	Parameters
Capillary (kV)	0.6
Sampling cone (V)	55
Extraction cone	4
Source temperature (C)	130
Desolvation temperature (C)	350
Cone gas (Lh^{-1})	20
Desolvation gas (Lh^{-1})	800
Low Mass (*m/z*)	100
High mass (*m/z*)	1200
Scan time (Sec.)	0.25
Data Format	Centroid

Nitrogen was used as the desolvation and cone gas. Direct infusion of available phospholipid standards was used to find the optimal cone voltage. The ESI was operated in negative mode for all samples. During MS/MS experiments Argon was used as collision gas, and the collision energy was varied for the different standards and analytes. Leucine enkephaline (*m/z* 554.2651 in negative ionization) was used as lock spray. Full scan MS, MS/MS and MS^E Continuum was utilized. MS^E is a technique were both precursor and fragment mass spectra

are simultaneously acquired by alternating between high and low collision energy during a single chromatographic run. When using MS^E mode it was not necessary to pre-select precursor ions. The parameters for MS^E mode were the same as for MS and MS/MS apart from the data format, which was set to continuum. The low collision energy was off and the high collision energy set to 28 (V).

7.1.1. Preliminary experiments

During the initial experiments the standard E80 from lipoid was analysed. This standard was tested with different mobile phase compositions and used to develop gradient profiles for further lipid analysis. The lipid standards were dissolved in methanol or ethanol and as much 0.1% ammonia solution as needed for complete dissolution.
E80 was first analysed with the **Acquity UPLC BEH C18 100 mm column** using two different mobile phase compositions. Mobile phase composition 1 consisted of water and methanol with 0.1% ammonia and is displayed in Table 10.

Table 10: Mobile phase composition 1

Mobile phase A	Water with 0.1 % ammonia
Mobile phase B	Methanol with 0.1 % ammonia
Column temperature	50°C.

Different gradient profiles were tested where the initial conditions were varied. These are illustrated below in Table 11.

Table 11: Gradient profiles used for analysis of E80 (10 µg/ml) on Acquity UPLC BEH C18 100mm column using mobile phase composition 1

Time (min.)	Flow (ml/min)	Mobile phase A Water with 0.1% ammonia	Mobile phase B Methanol with 0.1% ammonia
Initial	0.6	95 %	5 %
10	0.6	5 %	95 %

Time (min.)	Flow (ml/min)	Mobile phase A Water with 0.1% ammonia	Mobile phase B Methanol with 0.1% ammonia
Initial	0.6	70 %	30 %
10	0.6	5 %	95 %

Time (min.)	Flow (ml/min)	Mobile phase A Water with 0.1% ammonia	Mobile phase B Methanol with 0.1% ammonia
Initial	0.6	95 %	5 %
30	0.6	5 %	95 %

Time (min.)	Flow (ml/min)	Mobile phase A Water with 0.1% ammonia	Mobile phase B Methanol with 0.1% ammonia
Initial	0.6	70 %	30 %
30	0.6	5 %	95 %

Neither of these gradients produced chromatograms that were possible to analyse or gain relevant information from. There was little separation of different lipid compounds and few, not distinct peaks of low intensity. The few peaks detected were broad with long tails, showing poor selectivity. This is displayed in Appendix 2.

In mobile phase composition 2 the buffer component was changed to Triethylamine (TEA) and tested on the same Acquity UPLC BEH C18 100 mm column.

Table 12: Mobile phase composition 2

Mobile phase A	Water with 2% TEA
Mobile phase B	Methanol with 2% TEA
Column temperature	50°C.

The E80 standard was tested using gradient displayed below in Table 13.

Table 13: Gradient used for analysis of E80 (10 µg/ml) with mobile phase composition 2.

Time (min.)	Flow (ml/min)	Mobile phase A Water with TEA	Mobile phase B Methanol with
Initial	0.6	30 %	70 %
10	0.6	10 %	90 %

However none of the experiments gave results that could be used for further analysis and as a result of this a shorter column was tested. The **Acquity UPLC® BEH C18 VanGuard Pre-column** was employed as it was suspected that the lipid standards did not elute from the longer 100mm column. Because TEA gives a lasting signal in the LC-MS instrument when run in positive mode, it was attempted to keep the concentration of TEA as low as possible to avoid contamination in other analyses. Six different concentrations of TEA were tested, 0.02 %, 0.05%, 0.10%, 0.50%, 1% and 2%, where 2% proved the best. Two different gradient profiles, shown in Table 14, were developed where the latter proved the best.

Table 14: Gradient profiles for E80 (100 µg/ml) tested on Acquity UPLC BEH C18 VanGuard Pre-column

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	30 %	70 %
10	0.6	5 %	95 %

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	50 %	50 %
10	0.6	0 %	100 %

The first sign of separation of lipids was achieved using this method and the chromatogram is shown in Appendix 3. The efficiency of this column for analysing lipids was not optimal as there were overlapping peaks that clearly contained more than one compound.
On the basis of this a longer column was tested to gain better separation of the compounds in the lipid mixture. The **Acquity UPLC BEH C18 50 mm column** was tried using mobile phase composition 2 with 2% TEA. The gradient profiles were varied and the optimal gradient is displayed below in Table 15.

Table 15: The gradient profile that provided the best separation of lipid compounds in the E80 standard using Acquity UPLC BEH C18 50 mm column and mobile phase composition 2.

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	30 %	70 %
14	0.6	0 %	100 %

The chromatogram is shown in Appendix 4. Even though the peaks shapes are far from optimal and visibly contain several compounds it was possible to locate different lipid compounds. This proved that a longer column was more efficient for analysing a sample with different lipid components. Other gradient profiles that were tested but did not provide sufficient separation are also displayed in Appendix 4.

7.1.2. Analysis of phosphatidylcholine standards

The three phosphatidylcholine standards 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC, 16:0-18:1Δ^{9} PC and 18:1Δ^{9}-0:0 PC from Avanti was used as a basis for development of optimal chromatography and MS methods for more complex phospholipid samples. To be able to identify the structures in complex lipid samples good chromatography is necessary, and a method was developed using two different mobile phase compositions and three different columns. All PC standards were dissolved in ethanol and 0.1% ammonia solution (50:50).

Development of UPLC method for analysing phosphatidylcholine standards

Mobile phase composition 2

The short **Acquity UPLC® BEH C18 VanGuard Pre-column** was tried first and the gradient profile that provided the best chromatography for the E80 standards (Table 15) was optimized further. It was shortened and a wash out period of 7 minutes with 100% methanol was added. The gradient is displayed in Table 16.

Table 16: Optimal gradient for analysing PC standards on Acquity UPLC BEH C18 VanGuard Pre-column with mobile phase composition 2

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	30 %	70 %
8	0.6	0 %	100 %
15	0.6	0 %	100%

This gradient was used in all further analysis of PC standards. All PC standards provided chromatograms with one distinct peak with little to no tailing. Even though the intensity of the peak was high there was still a lot of background noise recorded. The chromatograms are displayed in Appendix 5.

When the longer **Acquity UPLC BEH C18 50 mm column** was employed this resulted in longer retention times, from approximately 4 minutes to 7.08 minutes for 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC, from approximately 3.6 to 7.02 minutes for 16:0-18:1Δ^{9} PC and from approximately 0.29 to 2.38 minutes for 18:1Δ^{9}-0:0 PC. The retention times are only approximate because with this method the peaks were broad. There was also less background noise recorded. The chromatogram for 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC is illustrated in Appendix 6. When all three standards where mixed and analysed in one sample, it was difficult to tell 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC and 16:0-18:1Δ^{9} PC apart just by looking at the chromatogram as they elute almost at the same time. This is also displayed in Appendix 6.

Mobile phase composition 3

A third mobile phase composition was tested with the PC standards. This contained water, acetonitrile, isopropanol and ammonium acetate as a buffer and is displayed in Table 17. The flow was set to 0.5 ml/min because this mobile phase composition generated more backpressure.

Table 17: Mobile phase composition 3

Mobile phase A	Acetonitrile/Water (40/60, v/v) with 10 mM Ammonium Acetate
Mobile phase B	Acetonitrile/Isopropanol (10:90, v/v) with 10 mM Ammonium Acetate
Column temperature	50°C.

This mobile phase composition was tried because, as seen with the E80 standards, the lipids were difficult to separate and elute from the columns. Isopropanol is a stronger eluent than methanol when using C18 columns. Two different gradient profiles, illustrated in Table 18 and Table 19 were tested, also using the Acquity UPLC BEH C18 50 mm column.

Table 18: 10 minute gradient profile tested with mobile phase composition 3.

Time (min.)	Flow (ml/min)	Mobile phase A *Acetonitrile/Water (40/60, v/v) with 10 mM Ammonium Acetate*	Mobile phase B *Acetonitrile/Water (40/60, v/v) with 10 mM Ammonium Acetate*
Initial	0.5	60 %	40 %
10	0.5	0 %	100 %

The 10-minute gradient was tested with all three PC standards (10 µg/ml), resulting in improved chromatography. The retention time for all standards changed and the peaks had higher intensity. For 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC and 16:0-18:1Δ^{9} PC there were some background noise but the peak from the precursor ion was easy to locate. The chromatograms are displayed Appendix 7. All three standards were mixed together and analysed with a longer 30-minute gradient, which is displayed in Table 19.

Table 19: 30 minute gradient profile tested with mobile phase composition 3.

Time (min.)	Flow (ml/min)	Mobile phase A *Acetonitrile/Water (40/60, v/v) with 10 mM Ammonium Acetate*	Mobile phase B *Acetonitrile/Water (40/60, v/v) with 10 mM Ammonium Acetate*
Initial	0.5	80 %	20 %
30	0.5	0 %	100 %

Also with this mobile phase composition the 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC and 16:0-18:1Δ^{9} PC standards co-eluted. As seen from the individual spectra in Appendix 7 the retention times are quite similar, 5.19 and 5.26 minutes respectively, and in a mixture they yielded one unsymmetrical peak. Compared to mobile phase composition 2 the chromatographic separation was better as it was more apparent that the peak contained two compounds. The intensity of the signal was higher as well. This is displayed in Appendix 8.
To be able to differentiate between various PC compounds in a mixture of phospholipids with fatty acids in both *sn-1* and *sn-2* position better chromatographic separation was desirable.
The **Acquity UPLC® BEH C18 150 mm column** in addition to being longer had a smaller

inner diameter. To cope with the smaller inner diameter the column temperature was changed to 60°C. The gradient profile used was an optimised version of the 30-minute gradient used with the previous column.

Table 20: Gradient used for Acquity UPLC® BEH C18 150 mm column with mobile phase composition 3.

Time (min.)	Flow (ml/min)	Mobile phase A Acetonitrile/Water (40/60, v/v) with 10 mM Ammonium Acetate	Mobile phase B Acetonitrile/Isopropanol (10:90, v/v) with 10 mM Ammonium Acetate
Initial	0.5	60 %	40 %
20	0.5	0 %	100 %
30	0.5	0 %	100 %

The chromatography was far better using this longer column and the chromatogram for 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC, displayed in Appendix 9, showed a more symmetrical peak for the phospholipid standard. The peak was narrower proving this column was more efficient for analysing PC standards. The optimal UPLC conditions for analysing PC standards are summarised below.

Table 21: Column and mobile phase composition for optimal analysis of PC standards

Column	Acquity UPLC BEH C18 150 mm column
Column temperature	60 °C

Table 22: Gradient profile for optimal analysis of PC standards

Time (min.)	Flow (ml/min)	Mobile phase A Acetonitrile/Water (40/60, v/v) with 10 mM Ammonium Acetate	Mobile phase B Acetonitrile/Isopropanol (10:90, v/v) with 10 mM Ammonium Acetate
Initial	0.5	60 %	40 %
20	0.5	0 %	100 %
30	0.5	0 %	100 %

MS method development for phosphatidylcholine standards

To develop MS methods for identifying different structures in phospholipid samples both MS and MS/MS experiments were set up. Full scan MS was used to find the precursor ions and MS/MS was used to gain information on the fatty acid components and if possible their position on the glycerol backbone. Whenever MS^E was utilized this information was gained during a single chromatographic run.

Precursor ion detection

The conditions for all full scan TOF-MS data were the same as in the preliminary experiments and shown in Table 9. The initial experiments were performed using mobile phase composition 2 (Table 12) and from the full scan MS spectra the precursor ions for the three different PC standards were detected. These are summarised in Table 23. For all full scan MS experiments both $[M-H]^-$ and $[M-CH_3]^-$ were visible. When MS^E mode was used for analysis of PC standards both $[M-H]^-$ and $[M-CH_3]^-$ were visible, but the dominant precursor ion was $[M-H]^-$ (Appendix 10).

Table 23: Precursor ions detected for PC standards (10 µg/ml) using mobile phase composition 2.

PC standards	$[M-H]^-$	$[M-CH_3]^-$
18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC	832	818
16:0-18:1Δ^9 PC	758	744
18:1Δ^9-0:0 PC	520	506

Changing to mobile phase composition 3 altered the detected precursor ion for all PC standards. When using ammonia acetate as a buffer, the acetate anion (OAc) formed an adduct with phosphatidylcholine because of it's permanently charged nitrogen (Figure 13). In full scan MS mode there were two precursor ions observed for all three PC standards, $[M+OAc]^-$ and $[M-CH_3]^-$. A summary of the precursor ions observed using this mobile phase composition is shown below in Table 24 and spectra are displayed in Appendix 11. These precursor ions had a higher intensity than the ones observed when using mobile phase composition 2.

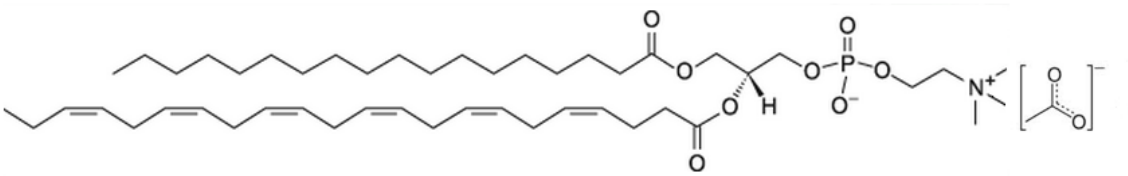


Figure 13: The acetate adduct formation with 22:6Δ^{4,7,10,13,16,19} PC when using mobile phase composition 3.

Table 24: Precursor ions detected for PC standards (10 µg/ml) using mobile phase composition 3.

PC standards	$[M-CH_3]^-$	$[M+OAc]^-$
18:0-22:6Δ^{4,7,10,13,16,19} PC	818	892
16:0-18:1Δ^{9} PC	744	818
18:1Δ^{9}-0:0 PC	506	580

MS/MS experiments

The conditions for all MS/MS experiments were the same as for full scan experiments (Table 9). Initially the collision energy (CE) was varied for all standards, and through several experiments a CE of 28V proved best for 18:0-22:6Δ^{4,7,10,13,16,19} PC and 16:0-18:1Δ^{9} PC. For the lysophospholipid, 18:1Δ^{9}-0:0 PC, a CE of 25V provided fragment ions of high intensity where also the precursor ion was visible. Mass spectra of all standards are displayed in Appendix 12. A series of 10 runs of each of the PCs were run to establish that this method was reliable. Expected fragmentations of all PC standards are shown in the figures below. As seen from the spectra in Appendix 12 expected fragments described in 4.3. ESI-MS of lipids, were visible, some only upon magnification.

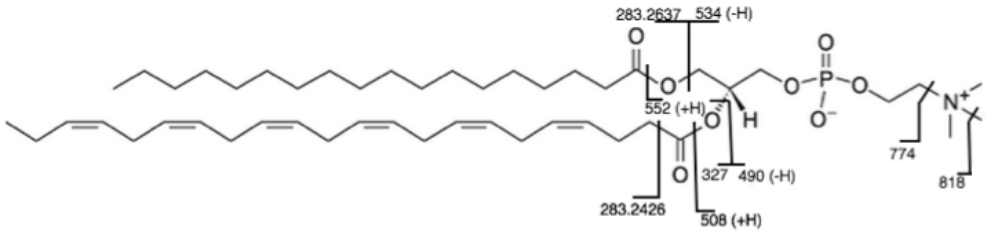


Figure 14: Expected fragmentation 18:0-22:6Δ^{4,7,10,13,16,19} PC upon CID

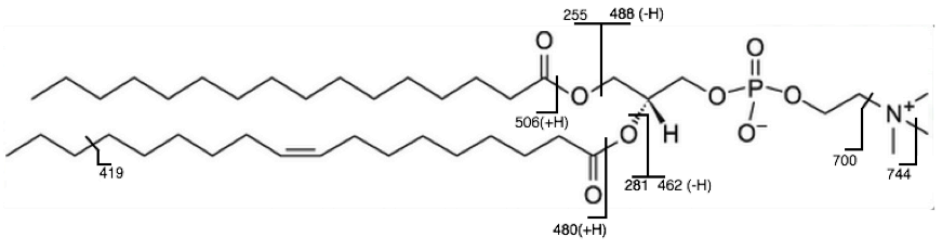


Figure 15: Expected fragmentation 16:0-18:1Δ^{9} PC upon CID

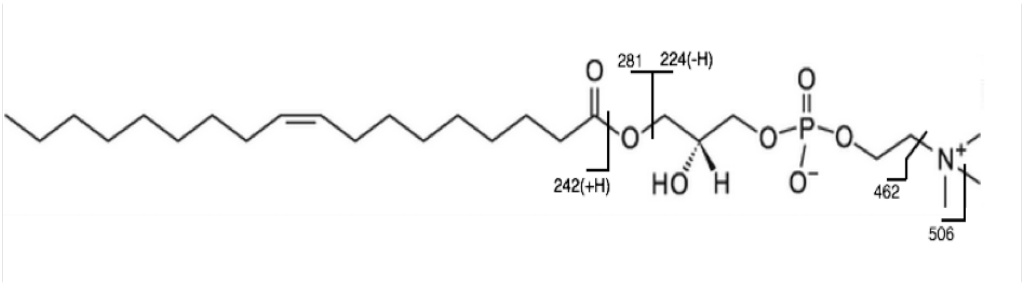


Figure 16: Expected fragmentation 18:1-0:0 PC upon CID

In 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC both fatty acid 18:0 and 22:6$\Delta^{4,7,10,13,16,19}$ was present. The 18:0 fatty acid has a monoisotopic mass of 283.2637 Da, while 22:6$\Delta^{4,7,10,13,16,19}$ has a monoisotopic mass of 327.2324 Da. The fatty acid in *sn-2* position, 22:6$\Delta^{4,7,10,13,16,19}$, will loose CO_2 during fragmentation witch yields a fragment of 283. 2426 Da. To be able to establish which fatty acid signal that has the highest intensity it is essential to differentiate between the two. When the data format was set to centroid mode the signals 283.2426 (22:6) and 283.2637 (18:0) was displayed as one signal and thus information was lost. It is not possible to change the way the instrument generates centroid data during an acquisition and the data format was therefore changed to continuum where both signals were visible. This is shown in Appendix 12 and was also transferred to the MS^E continuum experiments as well. The relative intensity of the two different fatty acids may be used to assign the position of the fatty acids on the glycerol backbone, as the fatty acid in *sn-2* position have been thought to more likely undergo fragmentation and produce a higher intensity signal in the mass spectrometer. Only the data acquired in MS/MS continuum mode and MS^E mode could be used to investigate this. For 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC only data from MS^E experiments were available for analysis. In a series of 6 runs the relative ratio between R_2COO^-/ R_1COO^- fragment ions were studied, and the results are displayed in Table 25. The ratio was stable in all six consecutive runs. In these experiments mobile phase composition 2 was used.

Table 25: MS^E experiments of 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC (CE 28V) and the ratio between R_2COO^-/ R_1COO^- fragment ions

Experiment	Intensity of FA 327 (R_2COO^-)	Intensity of FA 327 ÷ CO_2	Intensity of FA 283 (R_1COO^-)	Ratio R_2COO^-/ R_1COO^-
1	100	22	82	1,49
2	100	25	76	1,64
3	100	26	76	1,66
4	100	24	79	1,57
5	100	23	78	1,58
6	100	24	82	1,51

Also when mobile phase composition 3 was tested the R_2COO^-/ R_1COO^- ratio proved stable for 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC. The results of these experiments are displayed in Table 26.

Table 26: MS^E experiments of 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC (CE 28V) and the ratio between R_2COO^-/ R_1COO^- fragment ions

Experiment	Intensity of FA 327 (R_2COO^-)	Intensity of FA 327 ÷ CO_2	Intensity of FA 283 (R_1COO^-)	Ratio R_2COO^-/ R_1COO^-
1	100	24	38	3,26
2	100	24	36	3,44
3	100	22	34	3,59
4	100	26	36	3,50
5	100	25	34	3,68
6	100	24	38	3,26

For 16:0-18:1Δ^9 PC a series of 10 consecutive runs were performed and also in these experiments the ratio between the carboxylate anions proved stable. The results can be viewed in Appendix 13.

These experiments showed a stable ratio between the fragments of the two fatty acids in *sn-1* and *sn-2* position of PC compounds. Because recent studies had suggested that CID spectrum alone was not enough to reveal the regioisomerism and that the relative intensity of the carboxylate ions also could be influenced by the collision energy, experiments with different collision energies were set up. The experiments only had one parallel each, which is a limitation, but the results could be an indication of the effect the collision energy has on the

R_2COO^-/ R_1COO^- fragment ratio. As these experiments were performed when operating in centroid mode the data for 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC is not valid because of the inability to distinguish between the two different *m/z* 283 fragments. For 16:0-18:1Δ^9 PC however the MS/MS centroid data revealed that there was a greater variation between R_2COO^-/ R_1COO^- ratio for the different collision energies than when the same collision energy was applied. This indicated that care are must be taken when using the carboxylate anion ratios of PCs to assign the position of the fatty acids on the glycerol backbone. The ratios for the different CEs are displayed in Table 27.

Table 27: MS/MS experiments of 16:0-18:1Δ^9 PC where the collision energy was varied to study the ratio between R_2COO^-/ R_1COO^- fragment ions

	$[M-H]^-$ = 758			**$[M-CH_3]^-$ = 744**		
Collision energy (V)	**Intensity of FA 281 (R_2COO^-)**	**Intensity of FA 255 (R_1COO^-)**	**Ratio R_2COO^-/ R_1COO^-**	**Intensity of FA 281 (R_1COO^-)**	**Intensity of FA 255 (R_1COO^-)**	**Ratio R_2COO^-/ R_1COO^-**
15	N/A	N/A	N/A	N/A	N/A	N/A
25	100	49	2,04	100	48	2,08
28	100	46	2,17	100	61	1,64
30	100	53	1,89	100	56	1,79
32	100	45	2,22	100	69	1,45
35	100	53	1,89	100	82	1,22

7.1.3. Analysis of phosphatidylethanolamine standards

The phosphatidylethanolamine (PE) standards 16:0-18:1Δ^9 PE and 16:0-0:0 PE from Avanti have also been used as a basis for development of optimal chromatography and MS methods. All PE standards were dissolved in ethanol and 0.1% ammonia solution (50:50). The research done one PE standards is less extensive than for the PC standards. The PE standards have been tested throughout the project with some of the more successful methods developed for PC standards. Full scan MS was used to find the precursor ions and MS/MS analysis to gain information on the fatty acid composition.

Development of UPLC method for analysing phosphatidylethanolamine standards

Mobile phase composition 2

The gradient profile that was optimised for PC standards for analysis on the **Acquity UPLC® BEH C18 VanGuard Pre-column** was transferred to the PE standards. The gradient is displayed in Table 28.

Table 28: Gradient profile used in analysis of PE standards (10 µg/ml) on Acquity UPLC BEH C18 VanGuard Pre-column with mobile phase composition 2

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	30 %	70 %
8	0.6	0 %	100 %
15	0.6	0 %	100%

PE standards analysed on this column also resulted in chromatographic separation where the intensity of the precursor ion peaks were high and the peaks had little to no tailing. The PE standards did not display as much background noise as the PC standards analysed, and therefore produced better chromatograms that are displayed in Appendix 14.

Mobile phase composition 3

The standard 16:0-18:1Δ^9 PE was analysed using **Acquity UPLC BEH C18 50 mm column** and mobile phase composition 3. The chromatography was improved, as the peak of the precursor ion was more symmetrical and had higher intensity and a shorter gradient profile

could be used. The gradient is displayed below in Table 29 and the chromatogram in Appendix 14.

Table 29: 10 minute gradient profile used for analysis of 16:0-18:1Δ^9 PE (10 µg/ml) with mobile phase composition 3 using Acquity UPLC BEH C18 column 50 mm

Time (min.)	Flow (ml/min)	Mobile phase A *Acetonitrile/Water (40/60, v/v) with 10 mM Ammonium Acetate*	Mobile phase B *Acetonitrile/Water (40/60, v/v) with 10 mM Ammonium Acetate*
Initial	0.5	60 %	40 %
10	0.5	0 %	100 %

The PE standards were not tested with the longer **Acquity UPLC® BEH C18 150 mm column**, but it is natural to assume that this would as for PC improve the chromatography further.

MS method development for phosphatidylethanolamine standards

To develop MS methods for identifying different structures in phospholipid samples both MS and MS/MS experiments of PE standards were set up. Full scan MS was used to find the precursor ions and MS/MS was used to gain information on the fatty acid components, and if possible their position on the glycerol backbone. Whenever MS^E was utilized this information was gained during a single chromatographic run.

Precursor ion detection

The conditions for all full scan TOF-MS data were the same as in the preliminary experiments and are shown in Table 9. The initial experiments were performed using mobile phase composition 2 and from the full scan MS spectra the precursor ions for the two PE standards were detected. These are summarised in Table 30. The dominant precursor ion was the deprotonated molecular ion $[M-H]^-$.

Table 30: Precursor ions detected for PE standards (10 µg/ml) using mobile phase composition 2.

PE standards	$[M-H]^-$
16:0-18:1Δ^9 PE	716
16:0-0:0 PE	452

Changing to mobile phase composition 3 did not alter the detected precursor ion for PE as it did for PC standards. $[M-H]^-$ remained the dominant precursor ion as seen in Appendix 15.

MS/MS experiments

The conditions for all MS/MS experiments were the same as for full scan experiments (Table 9). Initially the collision energy was varied for both standards and through several experiments a CE of 28V for 16:0-18:1Δ^9 PE and a CE of 25V for 16:0-0:0 PE provided fragment ions of high intensity where also the precursor ion was visible. A series of 10 runs of each of the PE standards were run to establish that this method was reproducible. Expected fragmentation of both PE standards is shown in Figure 17 and Figure 18 below. As seen from the spectra in Appendix 16 expected fragments, as described in 4.3. ESI-MS of lipids, were visible. Some were only upon magnification.

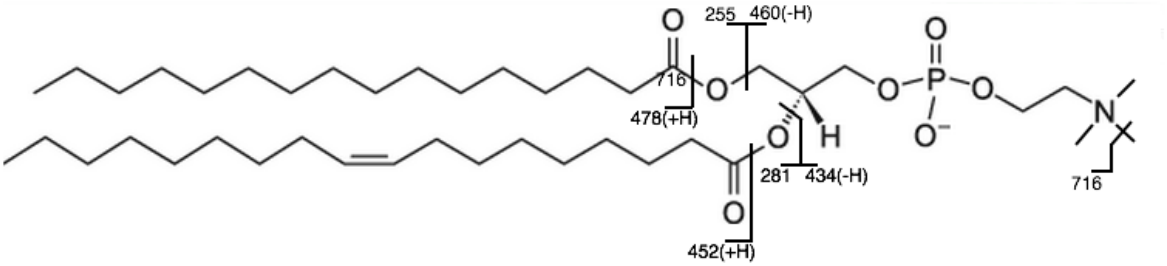


Figure 17: Expected fragmentation of 16:0-18:1Δ^9 PE upon CID.

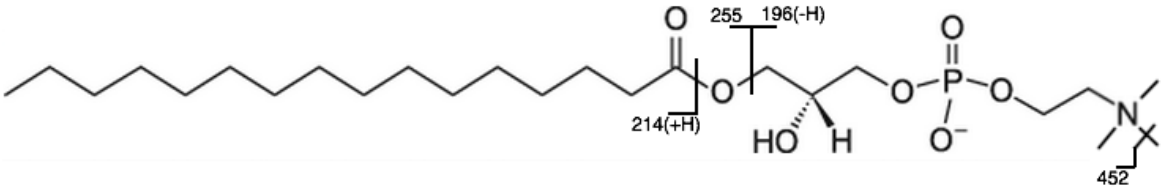


Figure 18: Expected fragmentation of 16:0-0:0 PE upon CID.

The relative intensity of the two different fatty acids may be used to assign the position of the fatty acids on the glycerol backbone. Only the data acquired in MS/MS continuum mode and MS^E mode could be used to investigate this and for the PE standards only 16:0-18:1Δ^9 PE was analysed. In a series of 10 consecutive runs the relative ratio between R_2COO^-/ R_1COO^- fragment ions were studied, and the results are displayed below in Table 31.

Table 31: MS/MS experiments of 16:0-18:1Δ^9 PE (CE 28V) and the ratio between R_2COO^-/ R_1COO^- fragment ions

$[M-H]^- = 716$			
Experiment	**Intensity of FA 281 (R_2COO^-)**	**Intensity of FA 255 (R_1COO^-)**	**Ratio R_2COO^-/ R_1COO^-**
1	100	50	2,00
2	100	51	1,96
3	100	50	2,00
4	100	48	2,08
5	100	49	2,04
6	100	50	2,00
7	100	48	2,08
8	100	50	2,00
9	100	50	2,00
10	100	51	1,96

In these experiments the ratio between the carboxylate anions proved stable, but because the relative intensity of the carboxylate ions also could be influenced by the collision energy, experiments with different CEs were set up. For 16:0-18:1Δ^9 PE these experiments revealed that even though the CE was varied the R_2COO^-/ R_1COO^- ratio proved stabile. The experiments only had one parallel each, which is a limitation and care should be taken when using the carboxylate anion ratios of PEs to assign the position of the fatty acids on the glycerol backbone. The ratios for the different CEs are displayed in Table 32.

Table 32: MS/MS experiments of 16:0-18:1Δ^9 PE where the collision energy was varied to study the the ratio between R_2COO^-/ R_1COO^- fragment ions

$[M-H]^-$ = 716			
Collision energy (V)	**Intensity of FA 281 (R_2COO^-)**	**Intensity of FA 255 (R_1COO^-)**	**Ratio R_2COO^-/ R_1COO^-**
15	N/A	N/A	N/A
25	100	51	1,96
28	100	50	2,00
30	100	51	1,96
32	100	52	1,92
35	100	51	1,96

7.2. Krill oil capsules analysed with UPLC-MS

Two different brands of krill oil were analysed. To be able to analyse commercial krill oil capsules it is necessary to dissolve it in a media that is applicable to LC-MS instrumentation. The gradient profile used for all krill oil samples during dissolution experiments are illustrated in Table 15. This was all conducted on the **Acquity UPLC VanGuard Pre-column** with mobile phase composition 2 using MS^E technology. The krill oil capsules were cut open and dissolved in ethanol. The capsules needed approximately 12 minutes to reach a homogenous orange coloured solution. Before introducing the samples into the LC-MS system they were diluted. The strength of the krill solution was gradually increased to find the best but lowest concentration needed for analysis of the phospholipids. Concentrations from 10 µg µg /ml to 1000 µg/ml were tested, and 1000 µg/ml was chosen for further analysis. The samples were diluted with a mixture of ethanol and MilliQ water or ethanol and 0.1% ammonia solution. The dissolving and dilution method that proved best for analysis is displayed below in Table 33.

Table 33: Preparation of krill oil capsules prior to analysis.

1. Cut open krill capsule.
2. Dissolve in 100 ml Ethanol, while stirring at 200rpm for approximately 12 minutes.
3. Dilute with pure MilliQ water to a concentration of 1000 µg/ml.
4. Filter with 0.25 mm filter.

Development of UPLC method for analysing krill oil capsules

The krill oil samples have been tested throughout the project with some of the more successful methods developed for PC and PE standards.

Mobile phase composition 2

These complex lipid samples were first analysed on the short **Acquity UPLC® BEH C18 VanGuard Pre-column**. The gradients tested resembled the optimal gradient for phospholipid standards, Table 16, and the best chromatography was achieved with the gradient displayed below in Table 34.

Table 34: Optimal gradient for analysing complex lipid samples on Acquity UPLC BEH C18 VanGuard Pre-column

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	60 %	40 %
10	0.6	0 %	100 %
15	0.6	0 %	100%

The chromatogram is shown in Appendix 17. All peaks were broad and are likely to contain several different phospholipid compounds. Other gradients were also explored, but did not provide as good results, and are also displayed in Appendix 17.

To achieve better separation of the lipids in the krill oil capsules the **Acquity UPLC BEH C18 50 mm column** was tested. The samples were first analysed using mobile phase composition 2 with 2% TEA and the gradient that proved to provide the best separation for krill oil on the 5mm pre-column (Table 34) was used. Because this proved to be a very complex mixture of lipids extended gradient profiles were tested and the flow decreased to 0.5 ml/min. A 40-minute gradient with a slower increase of mobile phase B at the end was the one that provided the best chromatographic separation of compounds in the krill oil capsules. The gradient is displayed below and the chromatogram in Appendix 18. Other gradient profiles that were tested are also displayed in Appendix 18.

Table 35: 40-minute gradient used to analyse Life krill oil capsules on the Acquity UPLC BEH C18 50 mm column

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.5	60 %	40 %
20	0.5	20 %	80 %
40	0.5	0 %	100 %
50	0.5	0 %	100%

Mobile phase composition 3

Using mobile phase composition 3 improved the chromatography when analysing krill oil capsules. The gradients that were developed for PC standards were transferred to these complex lipid samples. Both the 10-minute gradient (Table 18) and 30-minute gradient (Table 19) were tested with the **Acquity UPLC BEH C18 50 mm column** and provided better base

line separation and also more intense and symmetrical peaks. The 30-minute gradient separated the different lipid compounds to a greater extent and the chromatogram is displayed in Appendix 19.

The optimal UPLC conditions for analysing PC standards, using the **Acquity UPLC® BEH C18 150 mm column,** were also used for analysis of the krill oil samples. The gradient profiles developed for these experiments were based on the gradient that provided the best chromatographic results for the krill oil samples using mobile phase composition 2 (Table 35). The gradients were extended and the starting conditions were experimented with. The optimal gradient when taking both chromatographic separation and time management into consideration proved to be a 57-minute gradient, which is displayed below in Table 36 with the corresponding chromatogram in Figure 19.

Table 36: Optimal gradient for analysing Rimfrost krill oil capsules on the Acquity UPLC BEH C18 150 mm column

Time (min.)	Flow (ml/min)	Mobile phase A *Acetonitrile/Water (40/60, v/v) with 10 mM Ammonium Acetate*	Mobile phase B *Acetonitrile/Water (40/60, v/v) with 10 mM Ammonium Acetate*
Initial	0.5	95 %	5 %
55	0.5	20 %	80 %
57	0.5	0 %	100 %
60	0.5	0 %	100 %

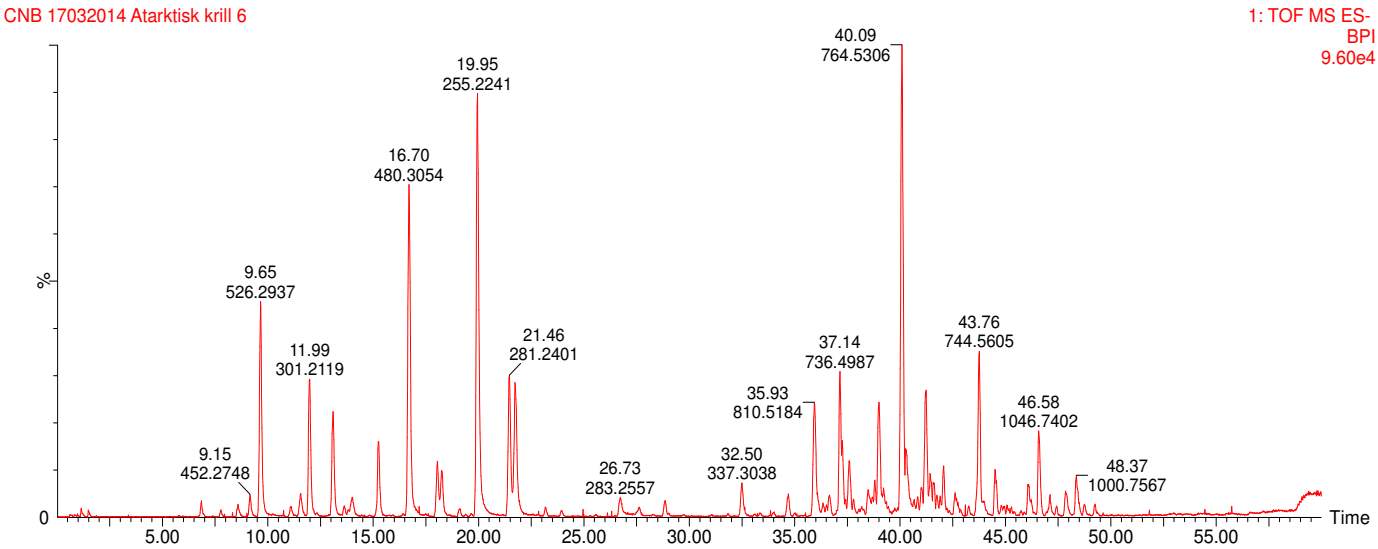


Figure 19: Rimfrost krill oil (1000 µg/ml) analysed with mobile phase composition 3 on Acquity UPLC® BEH C18 column 150 mm using a 57 minute gradient.

There was improved separation of the compounds in the krill oil sample. The peaks in the chromatograms had a higher intensity and there were more base line separation. Other gradients that were tested are shown in Appendix 20 with corresponding chromatograms.

MS method development for krill oil capsules

All full scan MS and MS/MS experiments done with krill oil samples were executed using MS^E technology taking advantage of the feature of not having to preselect precursor ions. The parameters for these MS^E experiments are displayed in Table 37.

Table 37: Parameters for MS^E experiments.

Conditions	Parameters
Capillary (kV)	0.6
Sampling cone (V)	55
Extraction cone	4
Source temperature (C)	130
Desolvation temperature (C)	350
Cone gas (Lh^{-1})	20
Desolvation gas (Lh^{-1})	800
Low Mass (*m/z*)	100
High mass (*m/z*)	1200
Scan time (Sec.)	0.5
Data Format	Continuum
Collision energy (V)	28

The mass spectra and interpretation of the different compounds in the krill oil samples are displayed in the results and discussion chapter.

7.3. AN raw extract analysed with UPLC-MS

A raw extract with unknown phospholipid content was also analysed using the UPLC and MS methods that were developed for krill oil capsules. The methods that provided the best results were transferred to these experiments. There were two raw extracts where two different extraction methods were used. The raw extract was solid and was dissolved in the same way as the krill oil capsules following the steps in Table 33.

Development of UPLC method for phospholipid raw extract

Mobile phase composition 2

The AN raw extract was first analysed on the **Acquity UPLC BEH C18 50 mm column** using mobile phase composition 2 with 2% TEA. The gradient profile that proved the best for krill oil capsules (Table 35) were used for the raw extract samples as well. Concentrations of 1000 µg/ml, 2000 µg/ml and 4000 µg/ml were tested and the samples with the highest concentration provided results that could be used for analysis and interpretation. The chromatogram can be seen in Appendix 21. There are many similarities between the two different raw extracts and there were several peaks of high intensity, but they were all broad and not symmetrical. There was clearly more than one compound in each peak making it hard to identify the structures of the different lipids.

Mobile phase composition 3

Using mobile phase composition 3 improved the chromatography for the raw extracts. Both the 10-minute gradient (Table 18) and 30-minute gradient (Table 19) that were developed for PC standards and also used for analysis of the krill oil samples were tested with the **Acquity UPLC BEH C18 50 mm column.** Even with shorter gradients this provided better base line separation and also more intense peaks. The 30-minute gradient separated the different lipid compounds in the raw extracts to a greater extent and the chromatograms for both extraction methods are displayed in Appendix 22.

The optimal UPLC conditions for analysing PC standards and krill oil capsules, using the **Acquity UPLC® BEH C18 150 mm column,** were also tested with raw extracts. All the different gradients that were tried for krill oil samples were also tried with the raw extracts and, as for the krill oil samples, the 57-minute gradient proved the best when considering both separation and time management (Table 36). The chromatogram is displayed below in Figure 20.

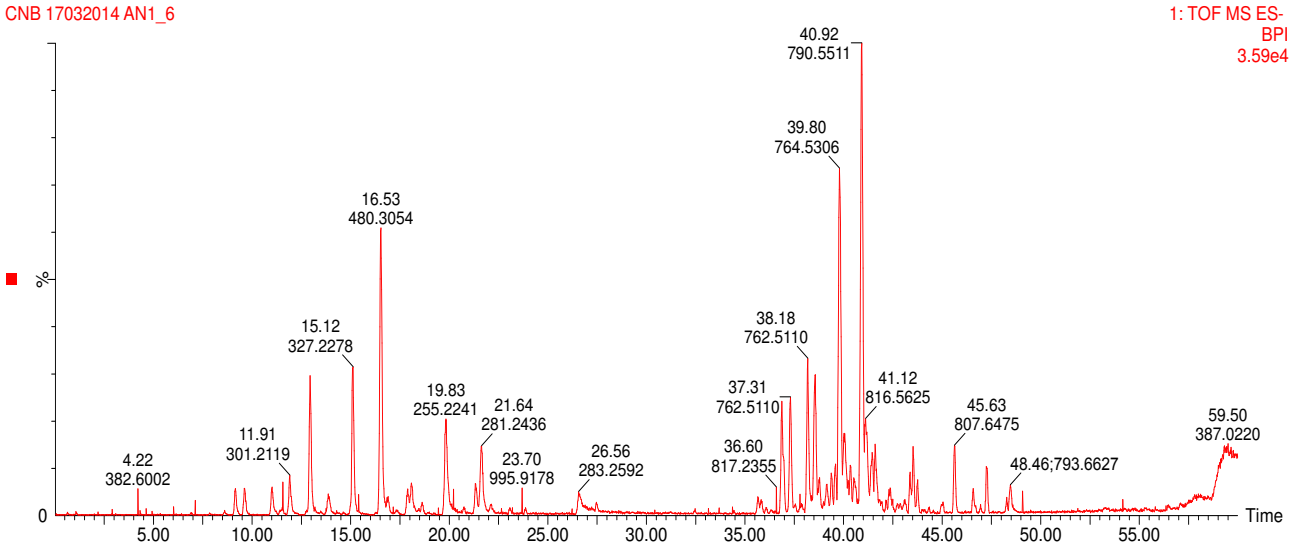


Figure 20: Phospholipid raw extract (4000 µg/ml) analysed with mobile phase composition 3 on Acquity UPLC® BEH C18 column 150 mm using a 57 minute gradient.

There was improved separation of the compounds in the raw extract sample as well. The peaks in the chromatograms had a higher intensity and there were more base line separation. Other gradients that were tested are shown in Appendix 23 with corresponding chromatograms.

MS method development for phospholipid raw extract

All full scan MS and MS/MS experiments done with raw extracts were executed using MS^E technology taking advantage of the feature of not having to preselect precursor ions. The parameters for these MS^E experiments were the same as for krill oil samples, and are displayed in Table 37.

The mass spectra and interpretation of the different compounds in the raw extract samples are displayed in the results and discussion chapter.

8. Results and discussion

8.1 Choice of methods

The methods for analysing complex lipid samples were developed by testing different columns, mobile phases and gradient profiles.

When utilizing the shorter columns separation was accomplished, but it was clear from the chromatograms that several compounds co-eluted and further separation was needed to more precisely identify the structures. The best result was achieved when the longest column was utilized, and ideally to gain even better separation of the compounds within the same time frame, an even longer column could have been employed.

The different mobile phase compositions tested provided different opportunities for analysing both the standards and the more complex lipid mixtures. Using mobile phase composition 2, with methanol and TEA (Table 12), there was poor reproducibility. The results varied from day to day, even with the same conditions applied both for samples and instrumentation. Ion suppression occurred in some of the experiments, and most of the chromatographic peaks were broad and not ideal for further analysis. Mobile phase 3, consisting of acetonitrile, isopropanol and ammonium acetate (Table 17), greatly improved the chromatography of both standards and complex samples. It also allowed shorter gradients without compromising the chromatographic separation of the compounds in the krill and raw extract samples. Because of the increased separation this provided a better opportunity for identifying different lipid structures. Identifying phospholipid structures and assigning the position of the fatty acid components was achieved by studying full scan MS and MS/MS spectra. MS^E technology was able to provide all the needed information in a single run and was therefore the optimal choice. In negative ESI mode information about the PL class and the fatty acid components were obtained. The position of the fatty acids on the glycerol backbone could be determined from studying the CID recorded spectra. Experiments with the PC and PE standards revealed that the relative ratio of the carboxylate anions could be used to assign the fatty acids to either the *sn-1* or sn-2 position. For the two PC standards that had fatty acids attached in both *sn-1* and *sn-2* position (18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC and 16:0-18:1Δ^{9} PC), the relative ratio between R_2COO^-/ R_1COO^- fragment ions were studied in a series of consecutive runs. For 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC a series of 6 consecutive runs were performed with both mobile phase composition 2 and mobile phase composition 3. In these experiments the CE was held constant at 28V and for both mobile phases the ratio proved stabile. For 16:0-18:1Δ^{9} PC a

series of 10 consecutive runs were performed using mobile phase composition 3. The R_2COO^- / R_1COO^- ratio proved stabile also in these experiments. Because it has been suggested that the relative intensity of the carboxylate ions also could be influenced by the collision energy, experiments with different collision energies were set up. The results revealed that there was a greater variation between R_2COO^-/ R_1COO^- ratio for the different collision energies than when the same collision energy was applied. Because these experiments only had one parallel more research is needed to properly establish the effect of the collision energy and also to investigate degree of saturation and the total chain length.

When the same experiments were performed with the 16:0-18:1Δ^9 PE standard, a series of 10 consecutive runs proved that the relative ratio between R_2COO^-/ R_1COO^- fragment ions was stable when a CE of 28V was applied. Experiments were the CE was varied also provided stabile results. As with the PC standard these experiments only had one parallel each, which is a limitation and care should be taken when using the carboxylate anion ratios of PEs to assign the position of the fatty acids on the glycerol backbone.

8.2. Structural determination of Rimfrost krill oil capsules

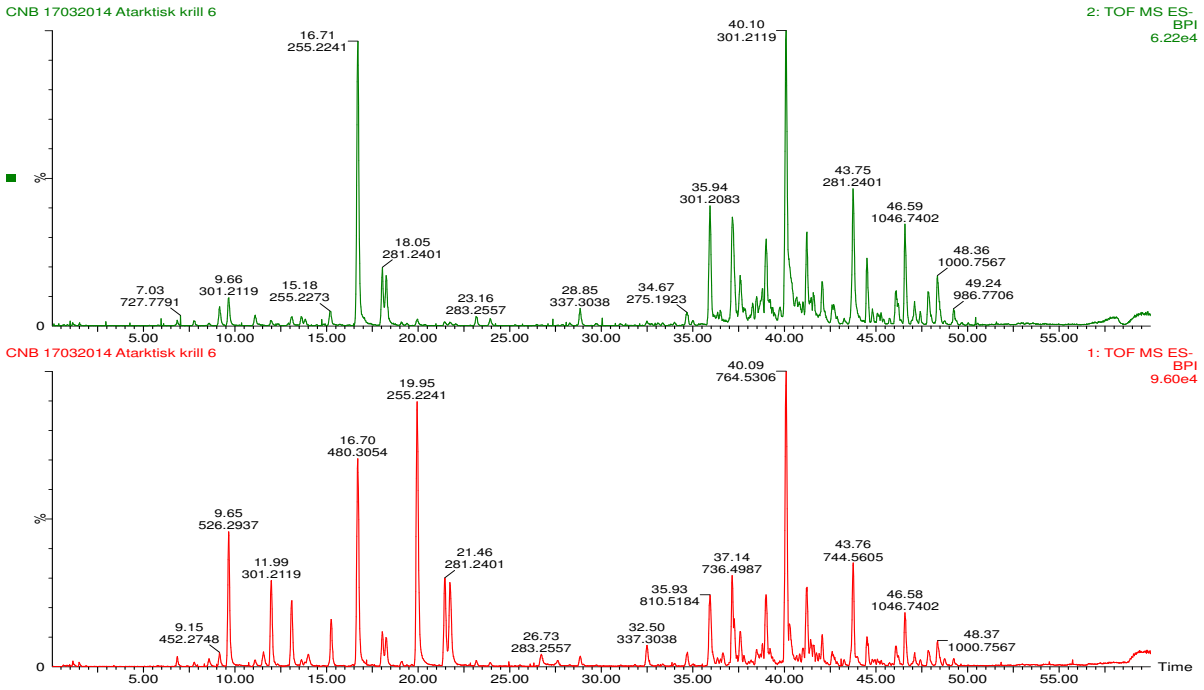


Figure 21: Full scan MS (1) and MS/MS (2) acquired using MS^E. Analysis of Rimfrost krill oil capsules (4000 µg(ml) using Acquity UPLC® BEH C18 150 mm column and mobile phase composition 3 with a 57-minute gradient profile.

The MS (1) and MS/MS (2) chromatograms of Rimfrost krill oil capsules revealed that this was a complex mixture of compounds. When taking both chromatographic separation and time management into consideration, the 57-minute gradient provided the best separation of compounds. Several peaks were of high intensity and there was adequate base line separation. There were two distinct areas of peaks in the chromatogram. The first half was the area where free fatty acids and the lysoPLs eluted and the second half where the less polar phospholipids and triglycerides eluted. Below are the magnified chromatograms of each of the two distinct areas (Figure 22 and Figure 23). The area between 28 and 32 minutes is not magnified, as this does not contain peaks of high intensity or of interest. The numbered peaks are the ones that were possible to interpret as their intensity was high enough and the chromatographic separation was sufficient, and the structural interpretation is given in the coming sections

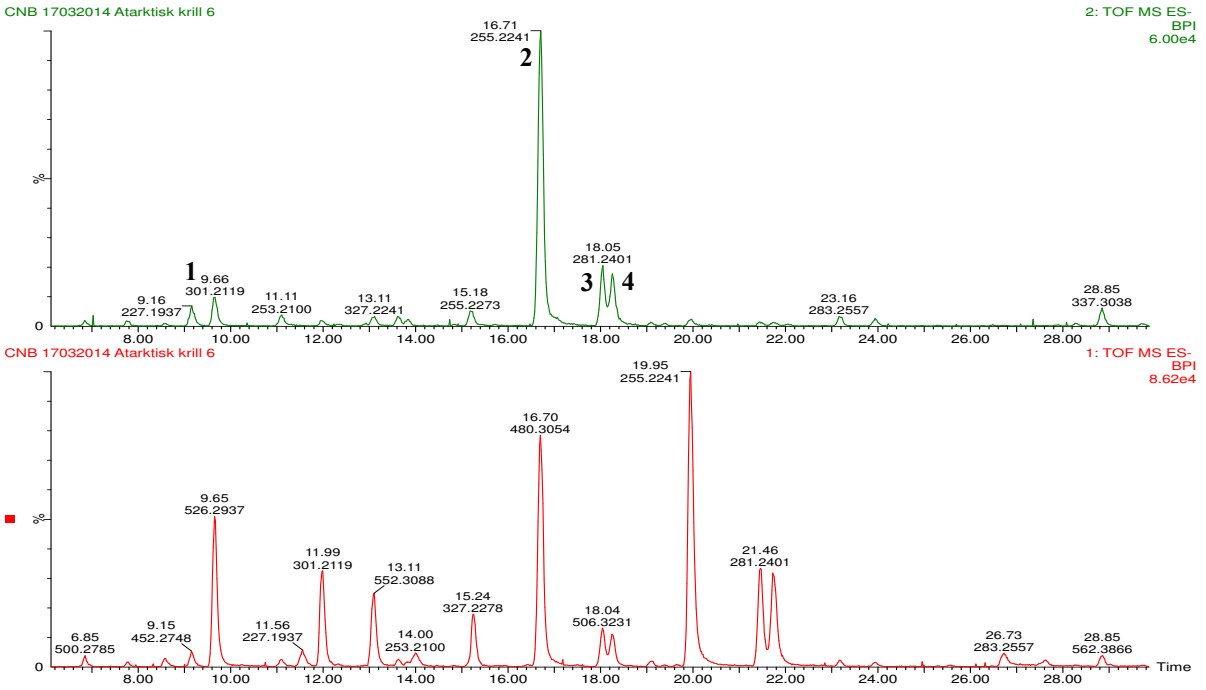


Figure 22: Magnified full scan MS (1) and MS/MS (2) of Rimfrost krill oil capsules (1000 µg/ml)

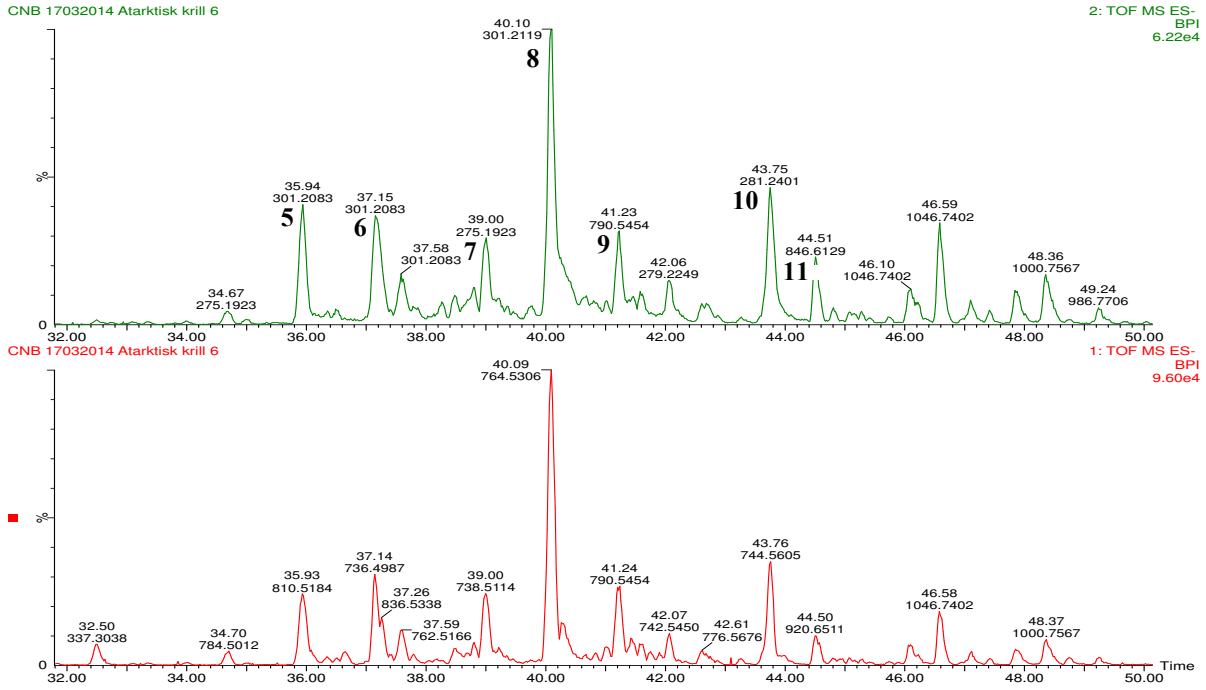


Figure 23: Magnified full scan MS (1) and MS/MS (2) of Rimfrost krill oil capsules (1000 µg/ml)

8.2.1. Peak 1

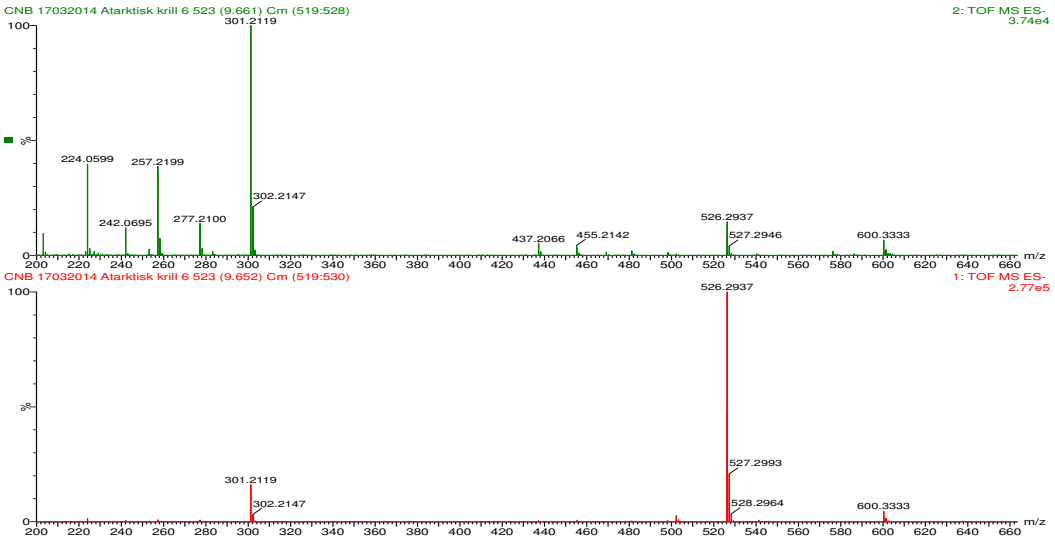


Figure 24: MS (1) and MS/MS (2) spectra for the chromatographic peak 1

From the MS and the MS/MS spectra one compound could be identified. The signal at *m/z* 526 was likely to be a PC as an acetate adduct at *m/z* 600 was also observed. The dominant fatty acid component was at *m/z* 301 (20:5$\Delta^{5,8,11,14,17}$), with the corresponding loss of CO_2 to yield a *m/z* 257 signal. The signal at *m/z* 224 also indicated that this was a PC as this is a class specific fragment. To further confirm the compound, the chromatograms for the individual peaks in the spectra were studied. Similar retention time and peak shape indicated that the elements originated from the same phospholipid. Figure 25 shows that the *m/z* 301 signal showed a similar retention time and peak shape as the *m/z* 526 and *m/z* 600, which confirmed the proposed compound.

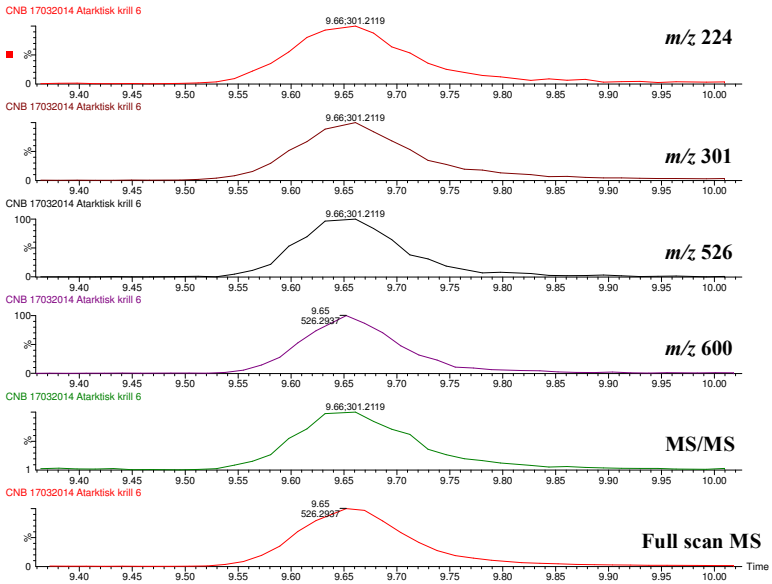
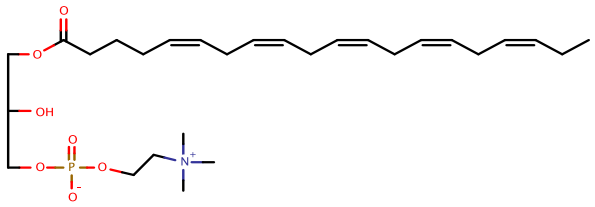


Figure 25: Full scan MS and MS/MS chromatograms along with the chromatograms for the individual *m/z* signals.

The relatively short retention time and $[M\text{-}CH_3]^-$ at *m/z* 526 indicated that this was a lysophospholipid with only one fatty acid attached. It was not possible from the data obtained to identify the position of the fatty acid. Since most lysophospholipids have the fatty acid attached in *sn-1* position the compound was drawn in accordance with this. Table 38 summarizes the elements of the compound identified in peak 1.

Table 38: Summary of the elements of the compound identified in peak 1

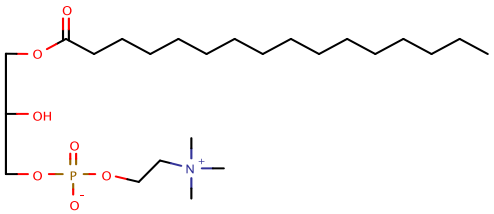
	$[M+OAc]^-$	**$[M\text{-}CH_3]^-$**	**Fatty acid components**
Compound 1	*m/z* 600	*m/z* 526	*m/z* 301 ($20{:}5\Delta^{5,8,11,14,17}$)
			

8.2.2. Peak 2

Figure 26: MS (1) and MS/MS (2) spectra for the chromatographic peak 2

From the MS and the MS/MS spectra one compound could be identified. The signal at *m/z* 480 was likely to be a PC as an acetate adduct at *m/z* 554 also was observed. The class specific fragment at *m/z* 224 also indicated that this was a PC. The dominant fatty acid component was at *m/z* 255 (16:0). The structure elucidation was performed as for the lysoPC in peak 1. The relatively short retention and $[M\text{-}CH_3]^-$ at *m/z* 480 indicated that this was a lysophospholipid with only one fatty acid attached. It was not possible from the data obtained to identify the position of the fatty acid. Since most lysophospholipids have the fatty acid attached in *sn-1* position the compound was drawn in accordance with this. Table 39 summarizes the elements of the compounds identified in peak 2.

Table 39: Summary of the elements of the compound identified in peak 2

	$[M+OAc]^-$	$[M\text{-}CH_3]^-$	Fatty acid components
Compound 1	*m/z* 554	*m/z* 480	*m/z* 255 (16:0)
			

8.2.3. Peak 3

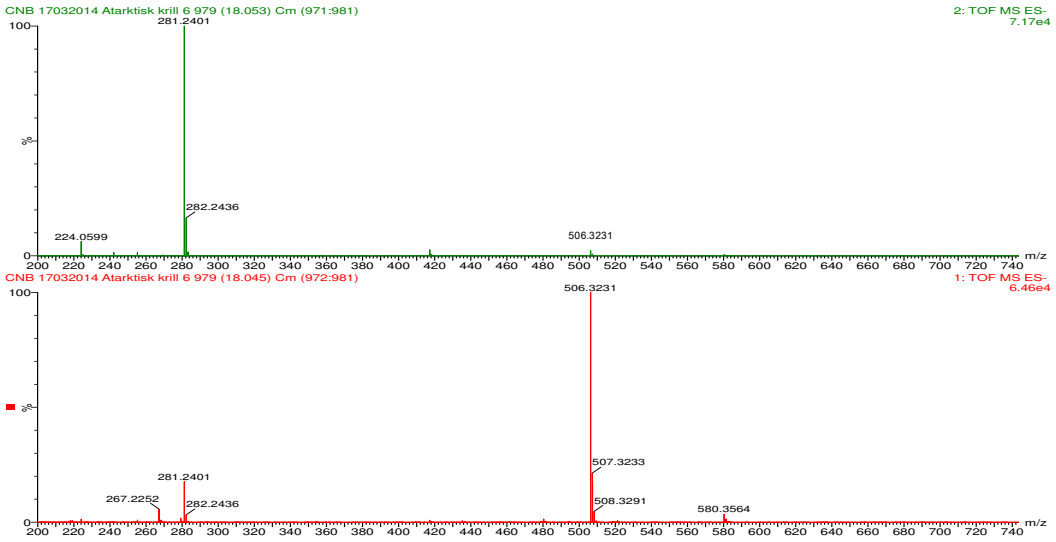
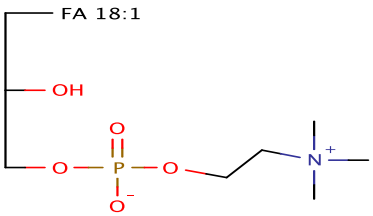


Figure 27: MS (1) and MS/MS (2) spectra for the chromatographic peak 3

From the MS and the MS/MS spectra one compound could be identified. The signal at *m/z* 506 was likely to be a PC as an acetate adduct at *m/z* 580 also was observed. The class specific fragment at *m/z* 224 also indicated that this was a PC. The dominant fatty acid component was the *m/z* 281 (18:1) signal. The structure elucidation was performed as for the lysoPC in peak 1 and for the fatty acid component 18:1 it is was not possible to identify where the double bond was situated. Table 47 summarizes the elements of the compounds identified in peak 10. Table 40 summarizes the elements of the compounds identified in peak 3.

Table 40: Summary of the elements of the compound identified in peak 3

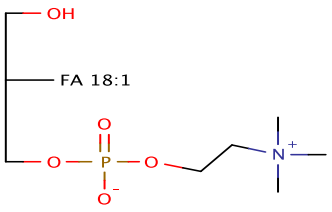
	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 580	*m/z* 506	*m/z* 281 (18:1)
			

8.2.4. Peak 4

Figure 28: MS (1) and MS/MS (2) spectra for the chromatographic peak 4

From the MS and the MS/MS spectra of peak 4 one compound could be identified. This peak contained all the same elements as peak 3, the only difference being the retention time. This peak eluted 0.12 minutes later. One possible explanation could be that the compound contains the same PC backbone and fatty, acid the only difference being the position of the fatty acid. For peak 3 and 4 it was not possible to determine if the fatty acid was situated in *sn-1* or *sn-2* position. Table 41 summarizes the elements of the compounds identified in peak 4.

Table 41: Summary of the elements of the compound identified in peak 4

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 580	*m/z* 506	*m/z* 281 (18:1)
			

8.2.5. Peak 5

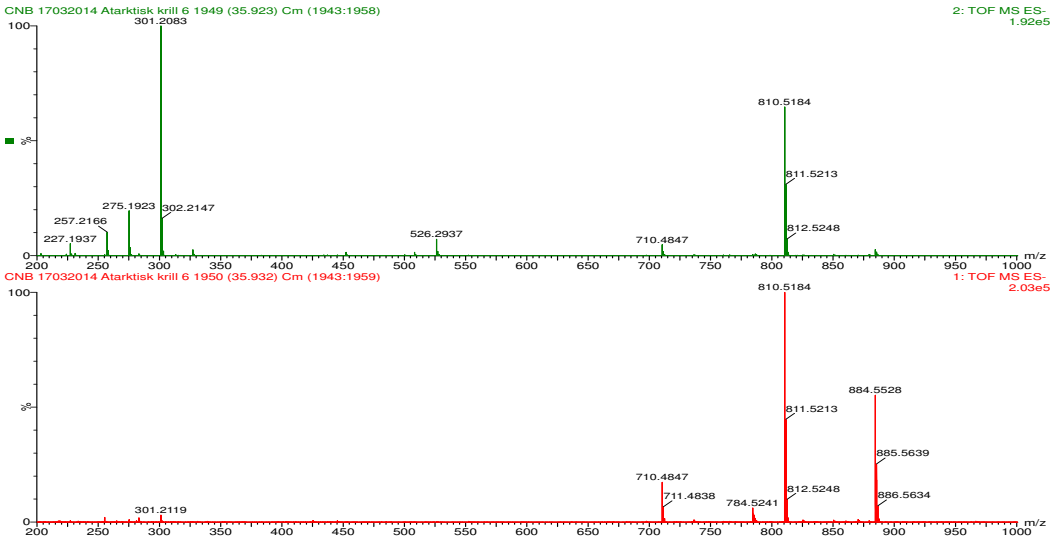
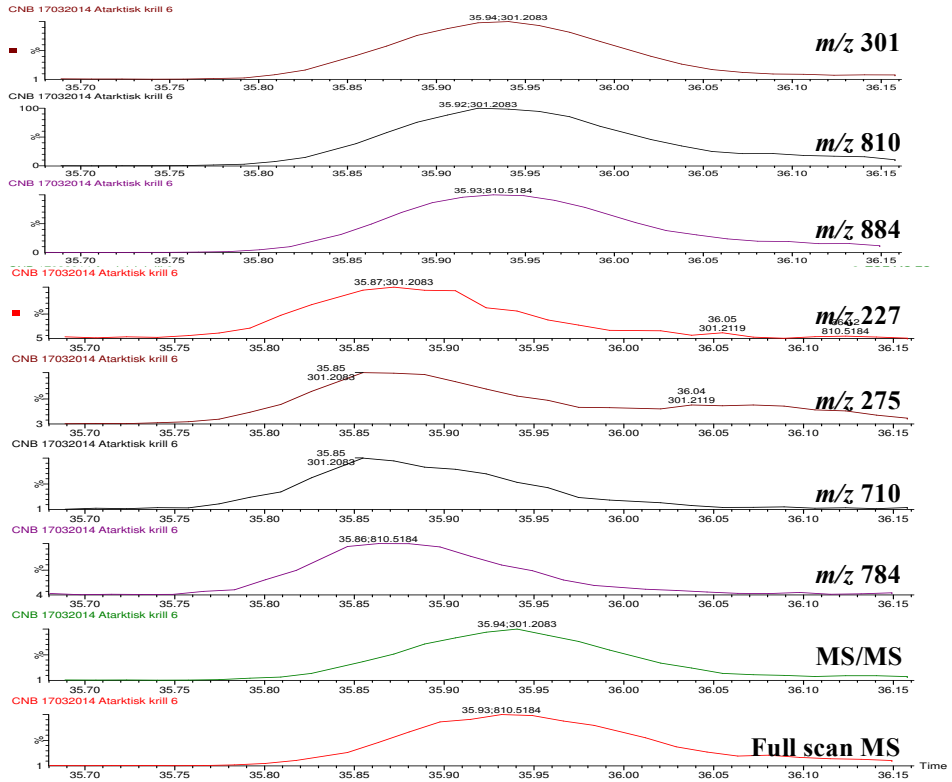


Figure 29: MS (1) and MS/MS (2) spectra for the chromatographic peak 5

From the MS and the MS/MS spectra two compounds could be identified. The signals at *m/z* 710 and *m/z* 810 are likely to be two different PCs. In the MS spectra they both form acetate-adducts $[M+OAc]^-$, at *m/z* 784 and *m/z* 884 respectively. The dominant fatty acid component was at *m/z* 301($20:5\Delta^{5,8,11,14,17}$), with the corresponding loss of CO_2 to yield a *m/z* 257 signal. To gain more information on the structure of the compounds the chromatograms of the individual peaks in the spectra were studied.

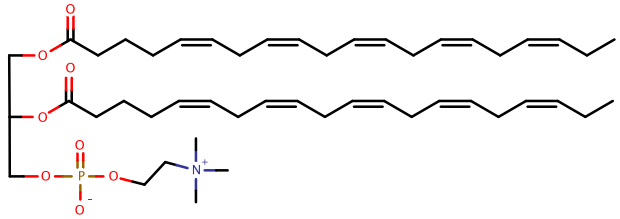
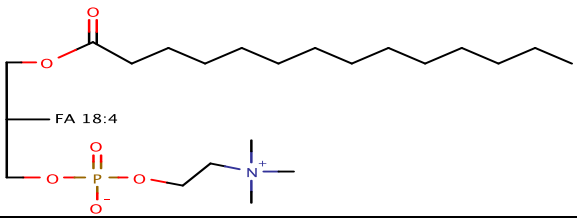


Figur 30: Full scan MS and MS/MS chromatograms along with the chromatograms for the individual *m/z* signals.

As seen from Figure 31 the $[M\text{-}CH_3]^-$ at *m/z* 884, *m/z* 810 and *m/z* 301 co-eluted. This indicated that they came from the same phospholipid where the fatty acid $20{:}5\Delta^{5,8,11,14,17}$ was attached both in *sn-1* and *sn-2* position. This also explains the high intensity of this signal. The assumed $[M\text{-}CH_3]^-$ at *m/z* 710 had a lower intensity and consequently so had the corresponding fatty acid components. This PC compound contained *m/z* 227 (14:0) and *m/z* 275 (18:4). For the fatty acid 18:4 it is was not possible to identify where the double bonds were situated. This compound is drawn with the *m/z* 275 (18:4) in *sn-2* position and *m/z* 227 (14:0) in *sn-1* position. This is based on the ratio of the carboxylate anions, where the loss of the fatty acid in *sn-2* position would be more likely to occur and thus yield a higher signal. Throughout the thesis this has been used to assign the positions of the fatty acids, as experiments performed with PC and PE standards proved that on this instrument using the optimised CE in the collision cell, the R_2COO^-/ R_1COO^- ratios were stable.

The described method for identifying compounds in this peak was utilized in structural elucidation of all chromatographic peaks that partly co-eluted. Using the MS^E feature to identify compounds is not possible if the peaks in the chromatogram completely co-elute, a small difference in elution time is required. It would be possible to identify structural elements but not the complete compound. This could be resolved by conducting MS/MS experiments of all single compounds, but this will be very time consuming compared with the MS^E method. Table 42 summarizes the elements of the compounds identified in peak 5.

Table 42: Summary of the elements of compounds identified in peak 5

	$[M+OAc]^-$	$[M\text{-}CH_3]^-$	Fatty acid components
Compound 1	*m/z* 884	*m/z* 810	*m/z* 301 ($20{:}5\Delta^{5,8,11,14,17}$)
			
Compound 2	*m/z* 784	*m/z* 710	*m/z* 227 (14:0) *m/z* 275 (18:4)
			

8.2.6. Peak 6

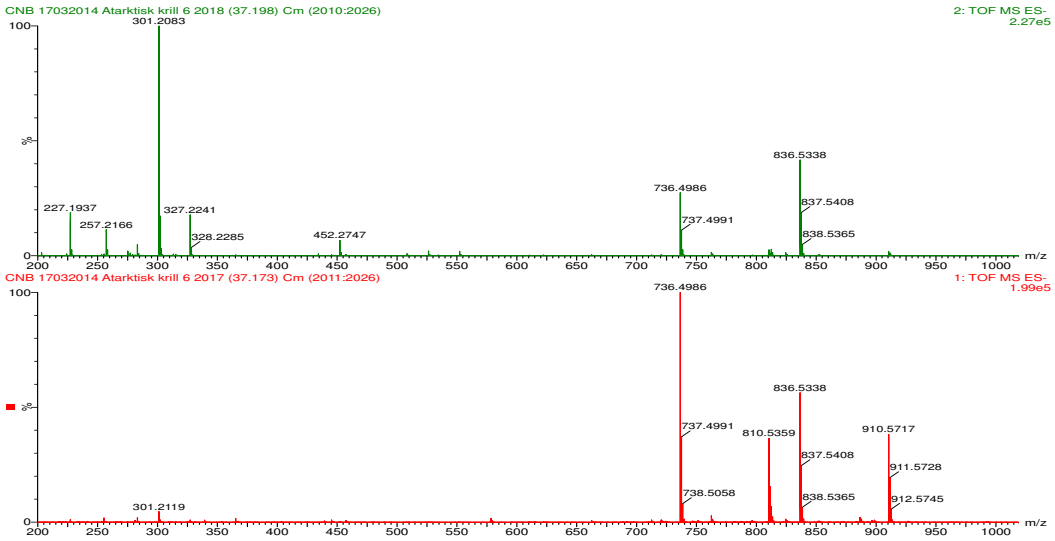


Figure 31: MS (1) and MS/MS (2) spectra for the chromatographic peak 6

From the MS spectra (1) and the MS/MS spectra (2) two compounds could be identified. The signals at *m/z* 736 and *m/z* 836 are likely to be two different PCs. In the full scan MS spectra they both form acetate-adducts $[M+OAc]^-$, at *m/z* 810 and *m/z* 910 respectively. The dominant fatty acid component was at *m/z* 301($20:5\Delta^{5,8,11,14,17}$), with the corresponding loss of CO_2 to yield a *m/z* 257 signal. Other fatty acid components observed were *m/z* 227 (14:0) and *m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$). The *m/z* at 283 corresponds to the loss of CO_2 to from *m/z* 327. The structure elucidation was performed as for the PLs in peak 5, but this peak was more complicated as the *m/z* 301 was present in both compounds. The chromatograms for the individual peaks are shown below.

Figure 32 shows that the $[M-CH_3]^-$ at *m/z* 736 had a similar retention time and peak shape as the *m/z* 227 signal. The *m/z* 810 signal also correlated with this. The chromatographic peak for the fatty acid with *m/z* 301 showed a similar retention time, but the peak shape indicated two partly overlapping peaks. This indicated that *m/z* 301 was part of both phospholipids, which also explains the high intensity of the signal. The $[M-CH_3]^-$ at *m/z* 836 had a similar retention time and peak shape as the *m/z* 910 signal, as well as the *m/z* 301 and *m/z* 327 signal. All this confirmed that there were two compounds included in this peak.

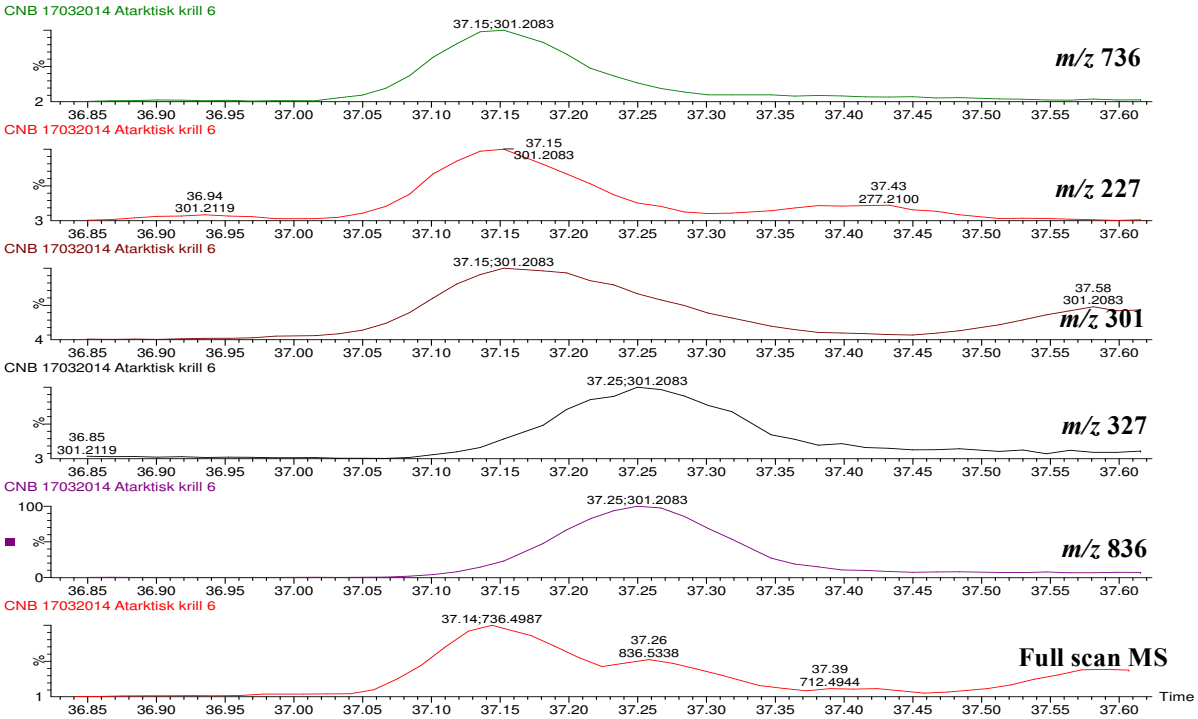
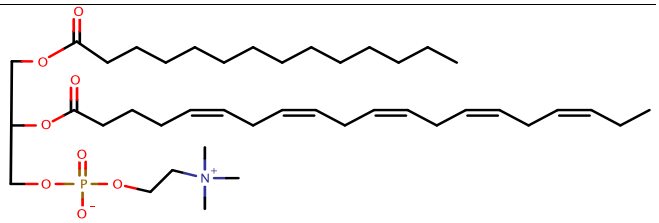
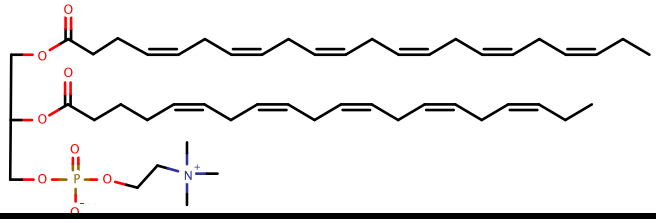


Figure 32: Chromatograms showing full scan MS and MS/MS experiment, in addition to the individual chromatograms for the different *m/z* signals.

It is assumed that the relative intensity of the *m/z* 301 fragment from *m/z* 736 and *m/z* 836 signal is similar to the integrated ratio between *m/z* 736 and *m/z* 836 themselves. When the *m/z* 736 and *m/z* 836 were integrated this showed a 64:36 ratio. For compound 1 the relative intensity of *m/z* 301 ($20{:}5\Delta^{5,8,11,14,17}$) was 68% when the signal intensity for *m/z* 257 was added. The division of the *m/z* 257 was done similarly to *m/z* 301. For *m/z* 227 (14:0) the relative intensity was calculated to be 29%, and therefor this fatty acid was determined to be in *sn-1* position.

This was used to assign the fatty acids of the PC based on the R_2COO^-/ R_1COO^- ratio. Assigning the *sn-1* and *sn-2* positions for compound 2 was more challenging. The relative intensity of *m/z* 301 ($20{:}5\Delta^{5,8,11,14,17}$) was 38% when the signal intensity of *m/z* 257 was added. The relative intensity of *m/z* 327 ($22{:}6\Delta^{4,7,10,13,16,19}$) was approximately 33% when the intensity of fragment *m/z* 283 was added. Because the ratio between the carboxylate anions was quite similar, assigning the position of the fatty acids based on this was more uncertain. To confirm the positions of the fatty acids experiments with standards should be performed. Table 43 summarizes the elements of the compounds identified in peak 6.

Table 43: Summary of the elements of compounds identified in peak 6

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 810	*m/z* 736	*m/z* 227 (14:0) *m/z* 301 (20:5$\Delta^{5,8,11,14,17}$)
			
Compound 2	*m/z* 910	*m/z* 836	*m/z* 327 (22:6$\Delta^{4,7,10,13,16,19}$) *m/z* 301 (20:5$\Delta^{5,8,11,14,17}$)
			

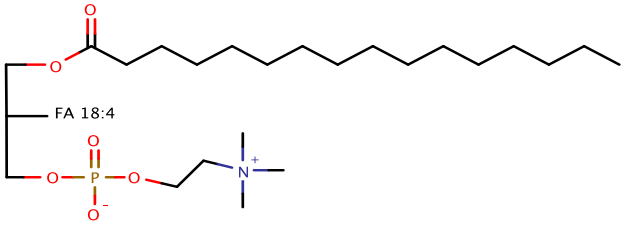
8.2.7. Peak 7

Figure 33: MS (1) and MS/MS (2) spectra for the chromatographic peak 7

From the MS and the MS/MS spectra one compound could be identified. The signal at *m/z* 738 is likely to be a PC as an acetate adduct at *m/z* 812 also could be observed. Four different fatty acid components were identified where *m/z* 275 (18:4) was the dominant one.

For the fatty acid 18:4 it is was not possible to identify where the double bonds were situated. The structure elucidation was performed as for the PLs in peak 5. Other visible elements such as the fatty acid components *m/z* 327 ($22{:}6\Delta^{4,7,10,13,16,19}$) and *m/z* 301 ($20{:}5\Delta^{5,8,11,14,17}$) did not co-elute with peak 7 and is assumed to come from another compound. From the late retention time this could originate from a triglyceride, as these compounds tend to elute at similar times as PLs and thus the chromatographic peaks may overlap. Table 44 summarizes the elements of the compounds identified in peak 7.

Table 44: Summary of the elements of the compound identified in peak 7

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 812	*m/z* 738	*m/z* 255 (16:0) *m/z* 275 (18:4)
			

8.2.8. Peak 8

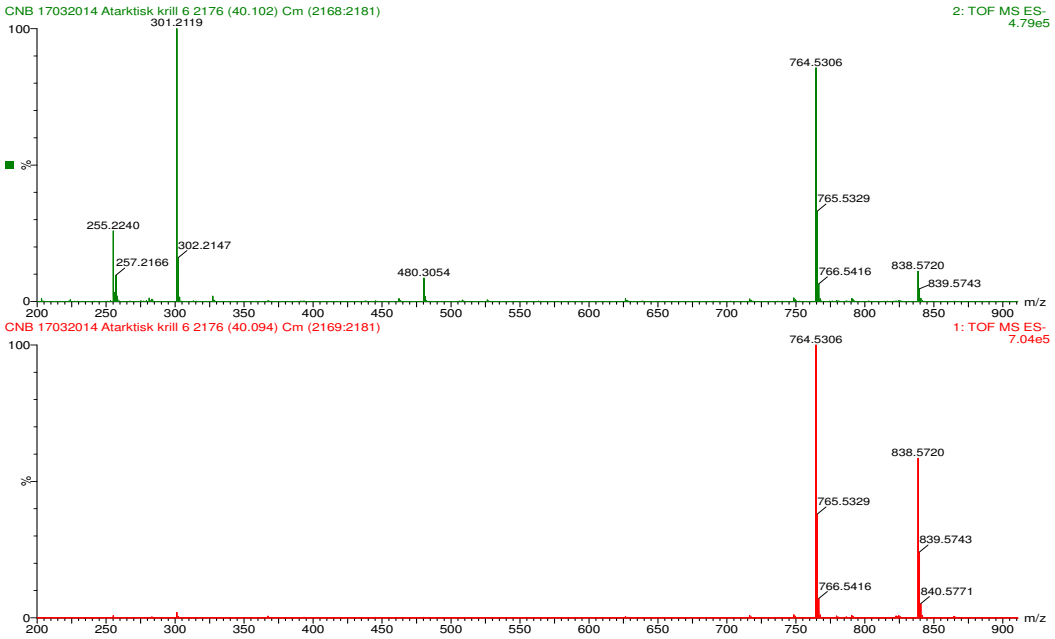
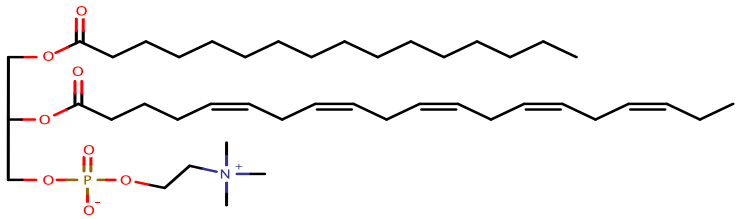


Figure 34: MS (1) and MS/MS (2) spectra for the chromatographic peak 8

From the MS and the MS/MS spectra one compound could be identified. The signal at *m/z* 764 is likely to be a PC as an acetate adduct at *m/z* 838 also could be observed. Two different fatty acid components were identified where *m/z* 301 (20:5$\Delta^{5,8,11,14,17}$) had a higher intensity than *m/z* 255 (16:0). The fragment at *m/z* 257 results from 20:5$\Delta^{5,8,11,14,17}$ loosing CO_2 during fragmentation. The structure elucidation was performed as for the PLs in peak 5. Table 45 summarizes the elements of the compounds identified in peak 8.

Table 45: Summary of the elements of the compound identified in peak 8

	$[M+OAc]^-$	**$[M-CH_3]^-$**	**Fatty acid components**
Compound 1	*m/z* 838	*m/z* 764	*m/z* 255 (16:0) *m/z* 301 (20:5$\Delta^{5,8,11,14,17}$)
			

8.2.9. Peak 9

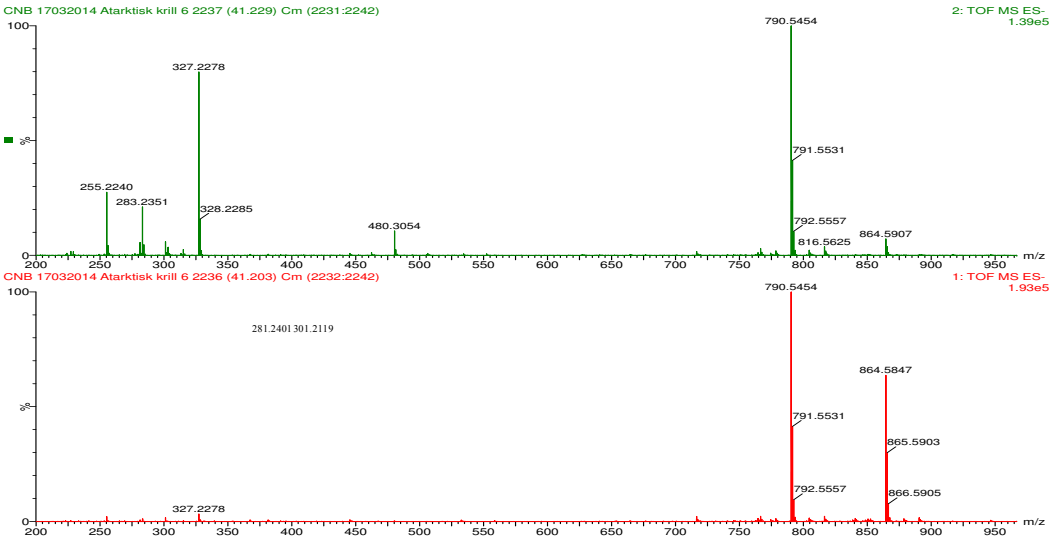
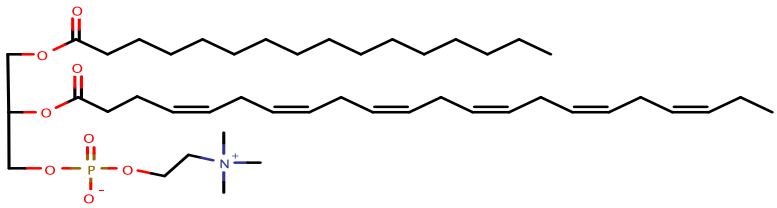


Figure 35: MS (1) and MS/MS (2) spectra for the chromatographic peak 9

From the MS spectra (1) and the MS/MS spectra (2) one compounds could be identified. The signals at *m/z* 790 was likely to be a PC as in the full scan MS spectra an acetate-adduct $[M+OAc]^-$ at *m/z* 864 was observed. There were four different fatty acid fragments observed, *m/z* 327 (22:6$\Delta^{4,7,10,13,16,19}$), *m/z* 255 (16:0), *m/z* 301 (20:5$\Delta^{5,8,11,14,17}$) and *m/z* 281 (18:1), where the two first dominated. The structure elucidation was performed as for the PLs in peak 5. The signals at *m/z* 301 and *m/z* 281 had low intensity compared to the other fatty acid components and could possibly originate from a compound where the parent ion is not visible because the signal is too low. When the chromatography for the individual peaks were compared *m/z* 301 and *m/z* 281 eluted later than the three other signals and were therefore likely to be part of partly overlapping peaks as seen from the full MS and MS/MS spectra in Figure 23. Table 46 summarizes the elements of the compound identified in peak 9.

Table 46: Summary of the elements of the compound identified in peak 9

	$[M+OAc]^-$	**$[M-CH_3]^-$**	**Fatty acid components**
Compound 1	*m/z* 864	*m/z* 790	*m/z* 255 (16:0) *m/z* 327 (22:6$\Delta^{4,7,10,13,16,19}$)
			

8.2.10. Peak 10

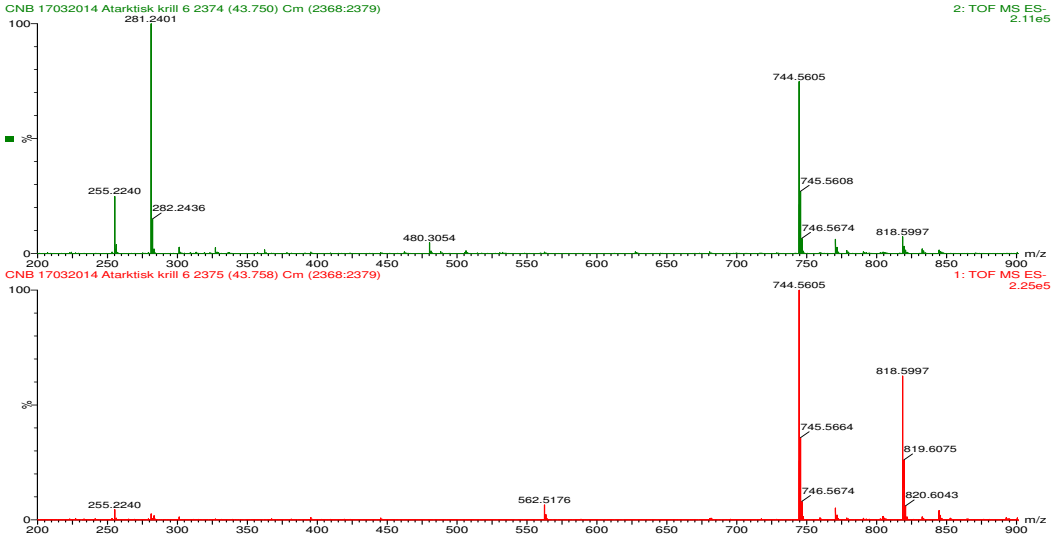
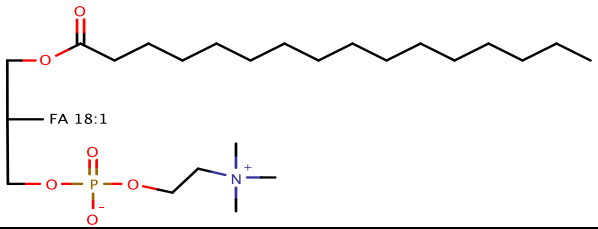


Figure 36: MS (1) and MS/MS (2) spectra for the chromatographic peak 10

From the MS and the MS/MS spectra one compound could be identified. The signal at *m/z* 744 is likely to be a PC as an acetate adduct at *m/z* 818 also could be observed. Two different fatty acid components were identified, *m/z* 255 16:0) and *m/z* 281 (18:1), where the latter had the highest intensity. The structure elucidation was performed as for the PLs in peak 5. For the fatty acid component 18:1 it is was not possible to identify where the double bond was situated. Table 47 summarizes the elements of the compounds identified in peak 10.

Table 47: Summary of the elements of the compound identified in peak 10

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 818	*m/z* 744	*m/z* 255 (16:0) *m/z* 281 (18:1)
			

8.2.11. Peak 11

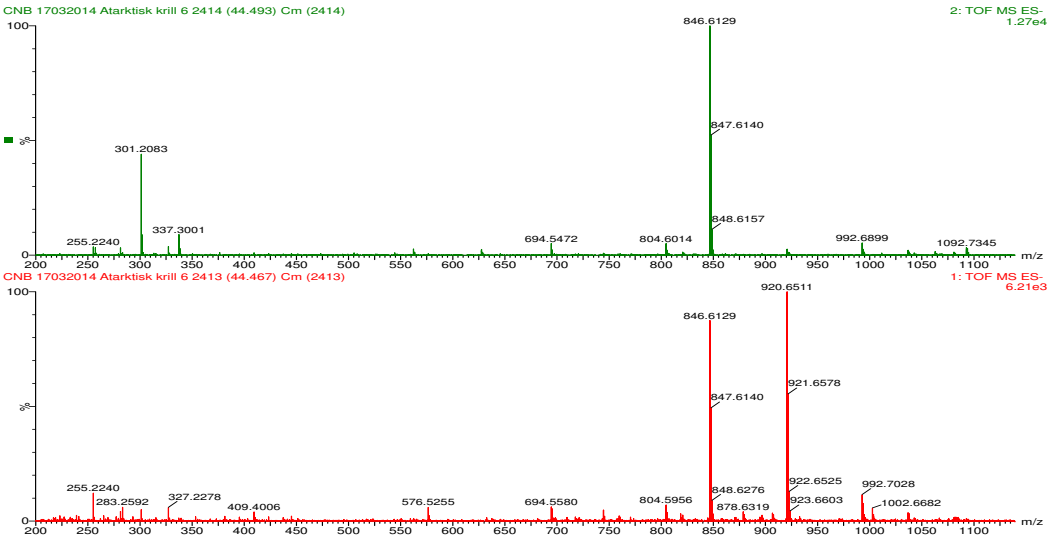
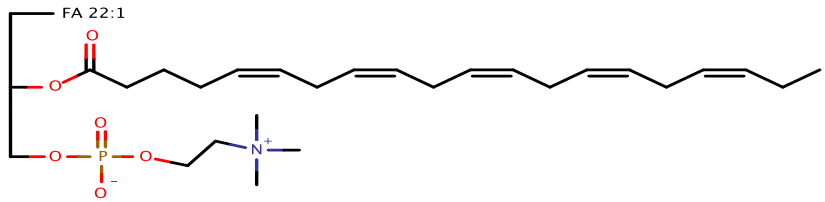


Figure 37: MS (1) and MS/MS (2) spectra for the chromatographic peak 11

From the MS and the MS/MS spectra one compound could be identified. The signal at *m/z* 846 is likely to be a PC as an acetate adduct at *m/z* 920 also could be observed. Two different fatty acid components were identified, *m/z* 337 (22:1) and *m/z* 301 (20:5$\Delta^{5,8,11,14,17}$), where the latter had the highest intensity. The structure elucidation was performed as for the PLs in peak 5. For the fatty acid component 22:1 it is was not possible to identify where the double bond was situated. Other observed ions such as *m/z* 992 and *m/z* 1092 eluted later than the other three components. Upon magnification the chromatogram showed that this peak was asymmetrical and most likely contained two overlapping peaks. The other ions were therefore likely to be part of other compound(s) that are not likely to be phospholipids. Table 48 summarizes the elements of the compounds identified in peak 11.

Table 48: Summary of the elements of the compound identified in peak 11

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 920	*m/z* 846	*m/z* 337 (22:1) *m/z* 301 (20:5$\Delta^{5,8,11,14,17}$)
			

8.3. Structural determination of Life krill oil capsules

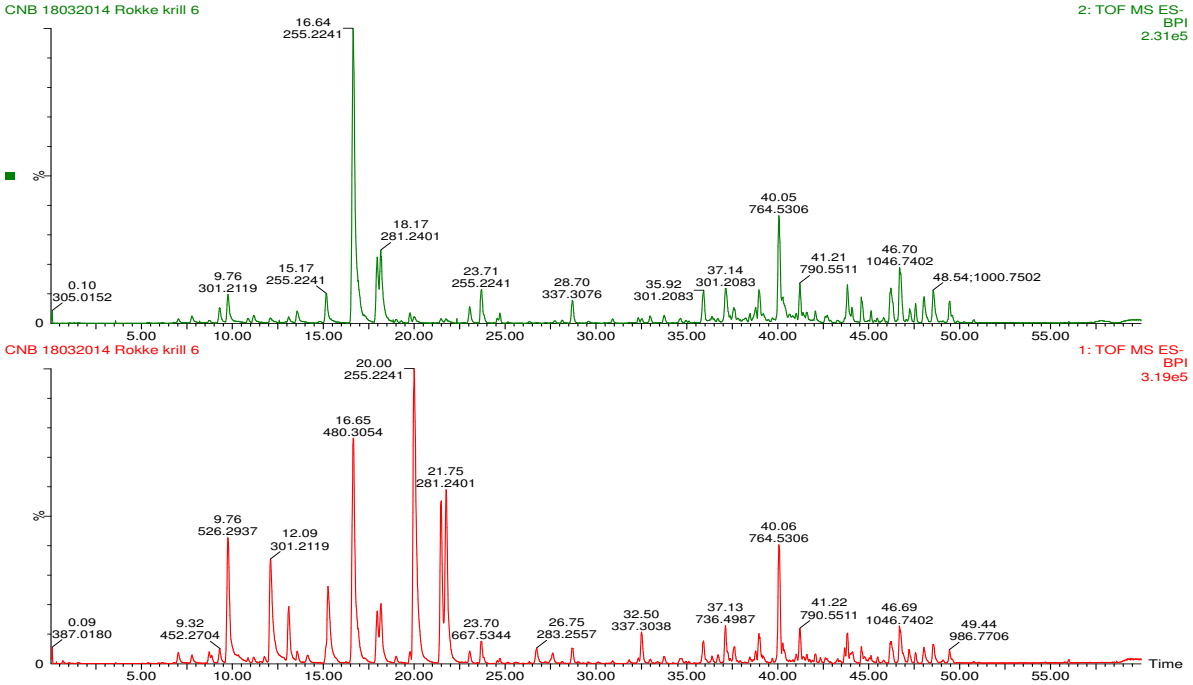


Figure 38: MS (1) and MS/MS (2) chromatogram for Life krill oil capsules.

The MS (1) and MS/MS (2) chromatograms of Life krill oil capsules also proved to be a complex mixture of compounds. Similarly to the Rimfrost krill oil capsules the 57-minute gradient provided the best separation of compounds when taking both chromatographic separation and time management into consideration. Several peaks were of high intensity and upon comparison the chromatograms proved very similar. This chromatogram also had two distinct areas of peaks where in the first half the free fatty acids and the lysoPLs were detected and in the second part the less polar phospholipids and triglycerides were detected. When studying the individual mass spectra of the most intense peaks it was found that the compounds detected were very similar and when identifying PL structures the same compounds were observed. For some of the less intense peaks small differences were noticed but the signals were not high enough to be analysed.

8.4. Structure determination of phospholipids in raw extract AN1

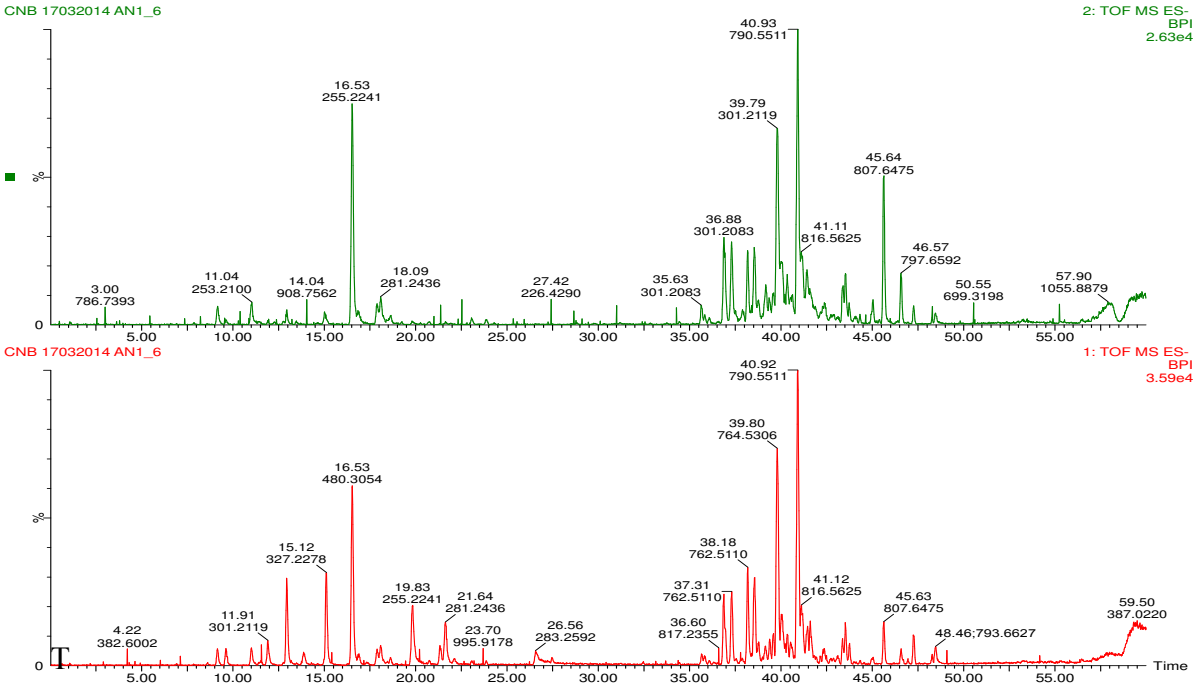


Figure 39: Full scan MS (1) and MS/MS (2) spectra acquired using MS^E. Analysis of raw extract AN1 (4000µg(ml) using Acquity UPLC® BEH C18 150 mm column and mobile phase composition 3 with a 57-minute gradient profile.

The MS (1) and MS/MS (2) chromatograms of the phospholipid raw extract proved that this was a complex mixture of compounds. As for the two other complex lipid samples the 57-minute gradient provided the best separation of compounds. Several peaks were of high intensity and suited for structure determination of compounds. The two distinct areas of peaks that were observed for the previous krill samples were also observed here. Below are the magnified chromatograms of each of the two distinct areas where the free fatty acids and lysoPLs were detected and the area where the phospholipids were detected. The area between 28 and 37 minutes are not magnified, as this does not contain peaks of high intensity or of interest.

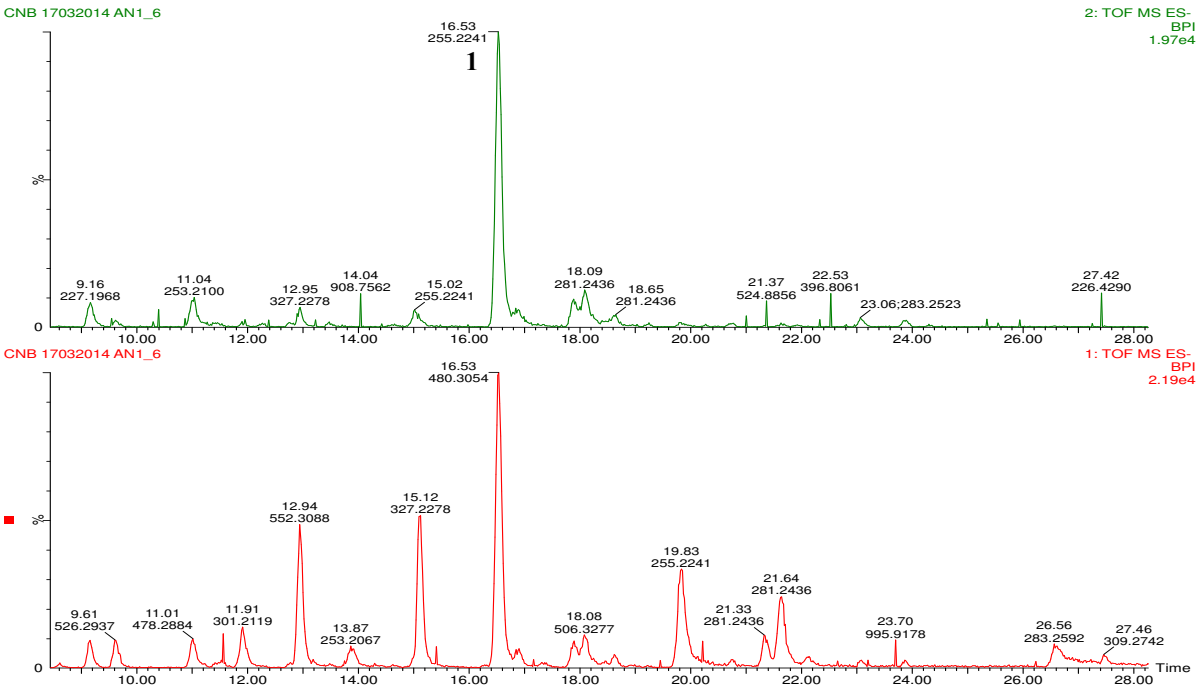


Figure 40: Magnified full scan MS (1) and MS/MS (2) raw extract AN1 (4000µg/ml).

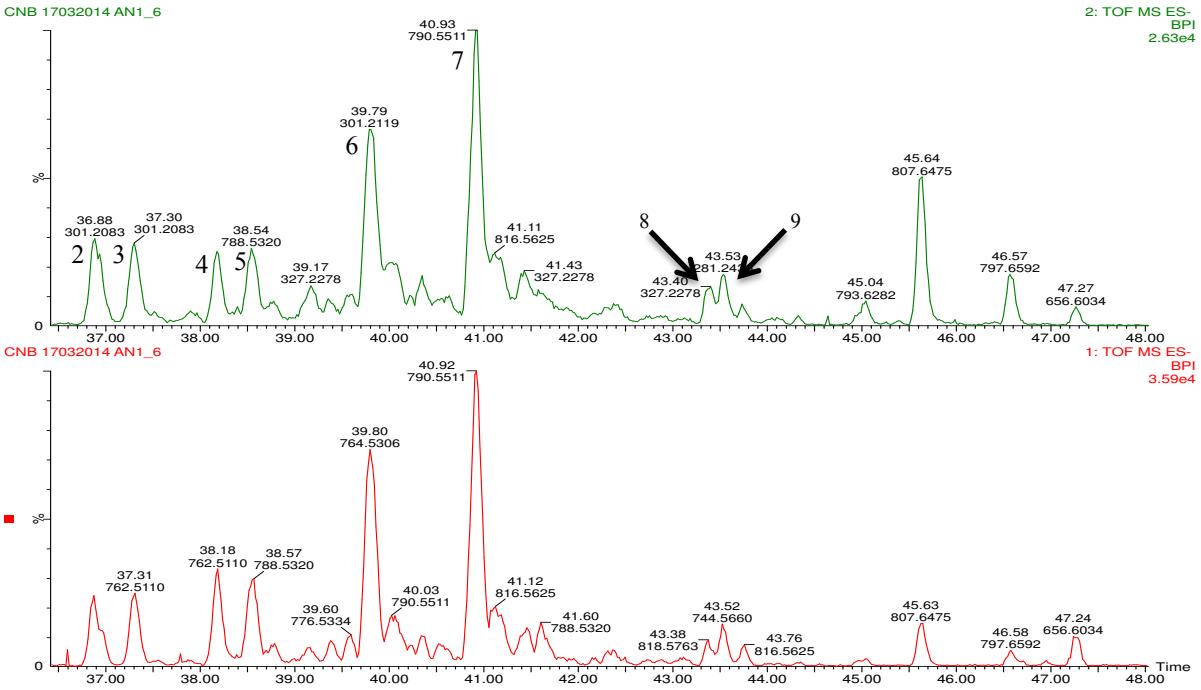


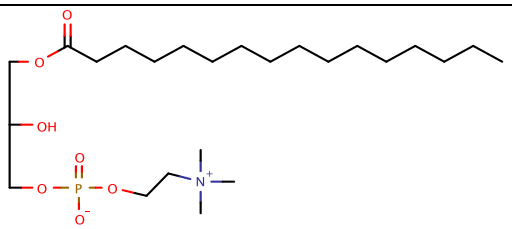
Figure 41: Magnified full scan MS (1) and MS/MS (2) of raw extract (4000µg/ml).

8.4.1 Peak 1

Figure 42: MS and MS/MS spectra for the chromatographic peak 1

From the MS (1) and the MS/MS (2) spectra one compound could be identified. The signal at *m/z* 480 was likely to be a PC as an acetate adduct at *m/z* 554 also was observed. The dominant fatty acid component was at *m/z* 255 (16:0). The structure elucidation was performed as for the lysoPC in peak 1 of the Rimfrost krill oil capsules. As seen from Figure 40, this peak eluted early in the chromatogram. This combined with a $[M\text{-}CH_3]^-$ at *m/z* 554 indicated that this was a lysophospholipid with only one fatty acid attached. It was not possible from the data obtained to identify the position of the fatty acid. As with the previous lysoPLs the compound has been drawn with the fatty acid attached in *sn-1* position. Table 49 summarizes the elements of the compounds identified in peak 1.

Table 49: Summary of the elements of the compound identified in peak 1

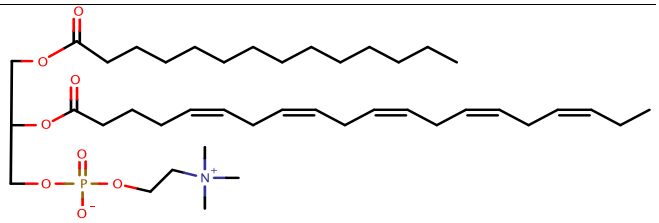
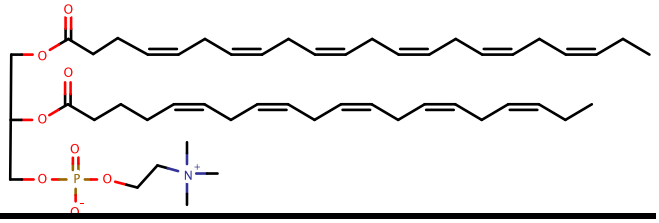
	$[M+OAc]^-$	$[M\text{-}CH_3]^-$	Fatty acid components
Compound 1	*m/z* 554	*m/z* 480	*m/z* 255 (16:0)
			

8.4.2. Peak 2

Figure 43: MS and MS/MS spectra for the chromatographic peak 2

From the MS spectra (1) and the MS/MS spectra (2) two compounds could be identified. The signals at *m/z* 736 and *m/z* 836 are likely to be two different PCs. In the full scan MS spectra they both form acetate-adducts $[M+OAc]^-$, at *m/z* 810 and *m/z* 910 respectively. The dominant fatty acid component was at *m/z* 301($20:5\Delta^{5,8,11,14,17}$), with the corresponding loss of CO_2 to yield a *m/z* 257 signal. The signal at *m/z* 227 (14:0) and *m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$) had similar intensities. The mass spectra revealed that this peak contains the same compounds as the ones observed in peak 6 for the Rimfrost krill oil capsules. The retention times for the peaks are also quite similar, at 36.90 minutes for the raw extract and 37.20 minutes for the krill oil sample. The structure elucidation was performed as for the PLs in peak 6. Also here the occurrence of *m/z* 301 ($20:5\Delta^{5,8,11,14,17}$) in both peaks made the assigning of the fatty acid position harder. When both *m/z* 736 and *m/z* 836 were integrated the relative ratio between them proved to be 41:59. For compound 1 the relative intensity of *m/z* 301 ($20:5\Delta^{5,8,11,14,17}$) was 43,9% when the intensity of *m/z* 257 was added, while it for *m/z* 227 (14:0) was 20%. This was used to assign the fatty acids of the PC based on the R_2COO^-/ R_1COO^- ratio. For compound 2 the relative intensity of *m/z* 301 ($20:5\Delta^{5,8,11,14,17}$) was 63.1%, while the relative intensity of *m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$) was approximately 35% when the intensity of fragment *m/z* 283 was added. This was used to assign the fatty acids based on the R_2COO^-/ R_1COO^- ratio. Table 50 summarizes the elements of the compounds identified in peak 2.

Table 50: Summary of the elements of compounds identified in peak 2

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 810	*m/z* 736	*m/z* 227 (14:0) *m/z* 301 ($20:5\Delta^{5,8,11,14,17}$)
			
Compound 2	*m/z* 910	*m/z* 836	*m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$) *m/z* 301 ($20:5\Delta^{5,8,11,14,17}$)
			

8.4.3. Peak 3

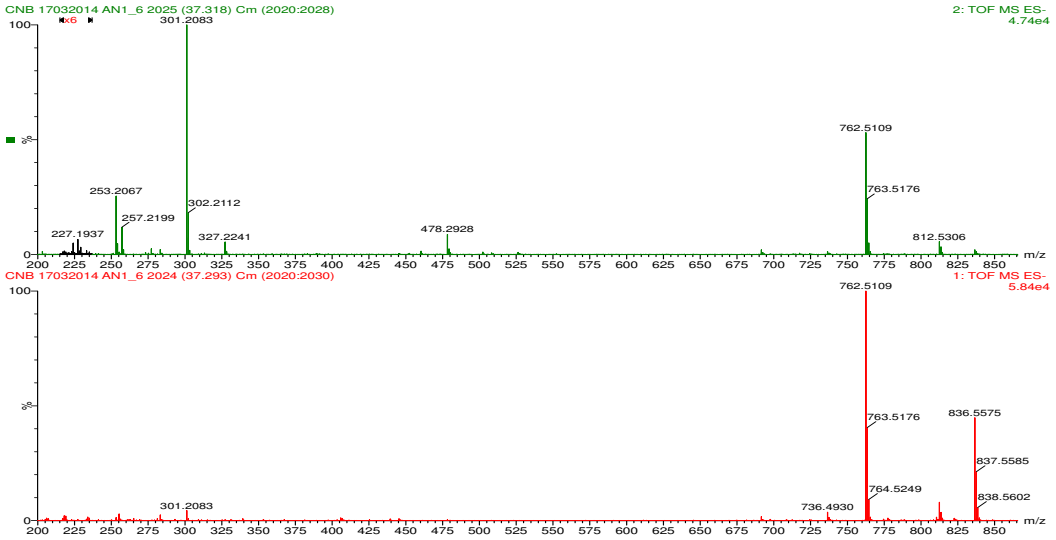


Figure 44: MS (1) and MS/MS (2) spectra for the chromatographic peak 3

From the MS and the MS/MS spectra two compounds could be identified. The signals at *m/z* 762 and *m/z* 812 are likely to be two different PCs. In the MS spectra they both form acetate-adducts $[M+OAc]^-$, at *m/z* 836 and *m/z* 886 respectively. Four different fatty acid components were identified where *m/z* 301 ($20:5\Delta^{5,8,11,14,17}$) and *m/z* 253 (16:1) were the dominating ones and the *m/z* at 257 were the fragment from *m/z* 301 loosing CO_2. For the fatty acid component 16:1 it is was not possible to identify where the double bond was situated. There were two possible combinations of fatty acids that could be present in the phospholipid that corresponded to the $[M-CH_3]^-$ at *m/z* 762. Both *m/z* 301 and 253, and *m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$) and *m/z* 227 (14:0) add up to *m/z* 762 and to confirm the compound the chromatograms for the individual peaks in the spectra were studied. These chromatograms are shown below in Figure 45.

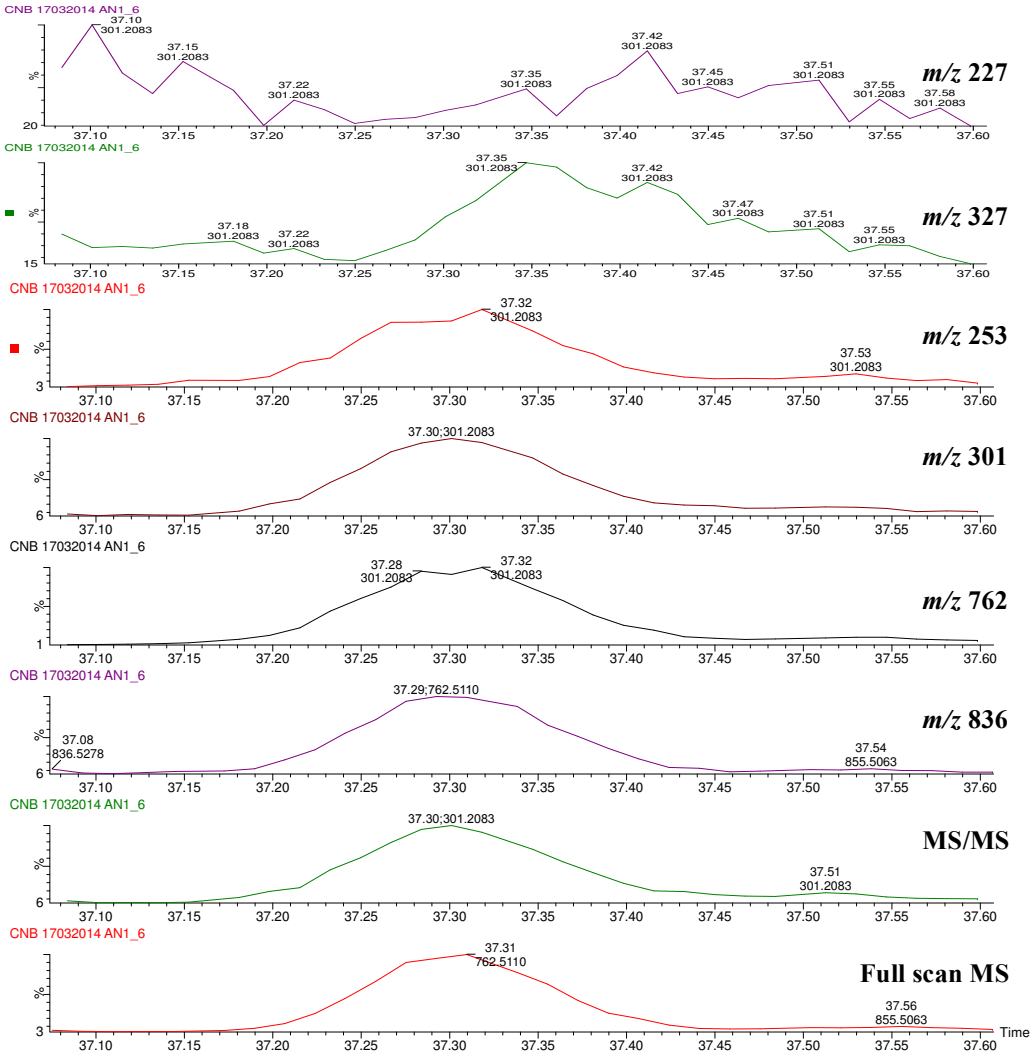
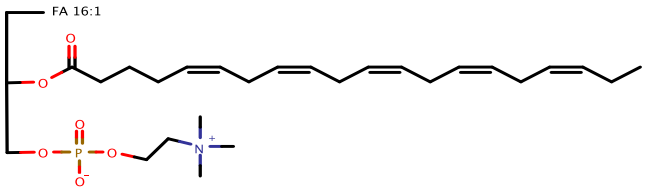
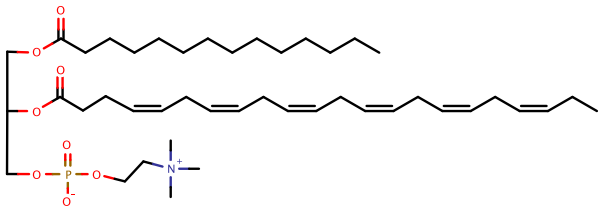


Figure 45: Chromatograms showing full scan MS and MS/MS experiment., in addition to the individual chromatograms for single signals.

From the chromatograms it was revealed that *m/z* 762 had similar retention time and peak shape as the *m/z* 301 and *m/z* 253 signals, which indicated that the elements originate from the same phospholipid. The *m/z* 327 and *m/z* 227 signal did not co-elute with the $[M\text{-}CH_3]^-$ at *m/z* 762 as seen in Figure 46 and this made it possible to identify the different compounds.
For the $[M\text{-}CH_3]^-$ at *m/z* 812 the studies of the individual chromatograms showed that *m/z* 327 and *m/z* 227 had similar retention times and peaks shapes. For these three *m/z* signals the intensity were lower and the *m/z* 227 was only visible upon magnification. This confirmed that there were two compounds included in peak 3, where $[M\text{-}CH_3]^-$ at *m/z* 762 with the fatty acid components *m/z* 301 and *m/z* 253 was the dominant phospholipid. For the fatty acid 16:1 it is was not possible to identify where the double bonds were situated. Table 51 summarizes the elements of the compounds identified in peak 3.

Table 51: Summary of the elements of compounds identified in peak 3

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 836	*m/z* 762	*m/z* 253 (16:1) *m/z* 301 ($20:5\Delta^{5,8,11,14,17}$)
			
Compound 2	*m/z* 886	*m/z* 812	*m/z* 227 (14:0) *m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$)
			

8.4.4. Peak 4

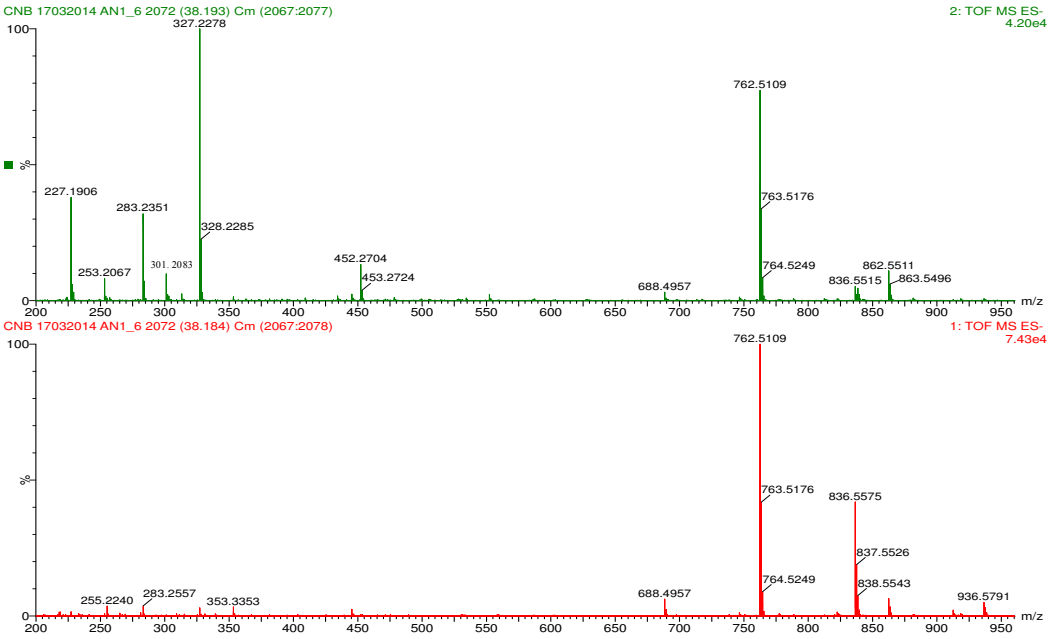
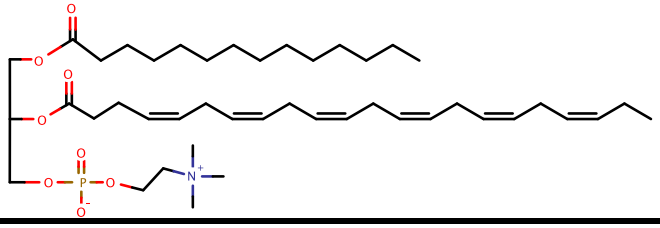


Figure 46: MS (1) and MS/MS (2) spectra for the chromatographic peak 4

From the MS and the MS/MS spectra one compound could be identified. The signals at *m/z* 762 and *m/z* 862 are likely to be two different PCs. In the MS spectra they both form acetate-adducts $[M+OAc]^-$, at *m/z* 836 and *m/z* 936 respectively. Four different fatty acid components were identified where *m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$) and *m/z* 227 (14:0) were the dominating ones, while the *m/z* 301 and *m/z* 253 (16:1) signal were of lower intensities. The *m/z* at 283 was the fragment from *m/z* 327 loosing CO_2 during fragmentation. There were two possible combinations of fatty acids that could be present in the phospholipid that corresponded to the $[M-CH_3]^-$ at *m/z* 762. Both *m/z* 327 and 227, and *m/z* 301 and 253 adds up to *m/z* 762. The structure elucidation was performed as for the PLs in peak 3 on the raw extract. The signals at *m/z* 301 and *m/z* 253 could originate from a compound where the parent ion is not visible because the signal is too low. There were signals in the spectra to indicate that there was a PC compound with a $[M-CH_3]^-$ at *m/z* 862 because it also formed an acetate adduct. None of the observed fatty acid components however corresponded with this. Table 52 summarizes the elements of the compounds identified in peak 4.

Table 52: Summary of the elements of the compound identified in peak 4

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 836	*m/z* 762	*m/z* 227 (14:0) *m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$)
			

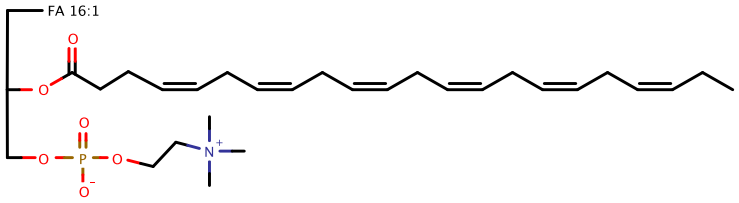
8.4.5. Peak 5

Figure 47: MS (1) and MS/MS (2) spectra for the chromatographic peak 5

From the MS and the MS/MS spectra one compounds could be identified. The signal at *m/z* 788 is likely to be a PC as an acetate adduct at *m/z* 862 also could be observed. Six different fatty acid components were identified where *m/z* 327 (22:6$\Delta^{4,7,10,13,16,19}$) and *m/z* 253 (16:1) were the dominating ones. The peak at *m/z* 283 resulted from the fatty acid at *m/z* 327 loosing CO_2 during fragmentation. Other observed elements such as the fatty acid components *m/z* 227 (14:0), *m/z* 255 (16:0), *m/z* 275 (18:4) and *m/z* 301 (20:5$\Delta^{5,8,11,14,17}$) as well as the signals *m/z* 738 and *m/z* 762. Some of these signals had low intensities and were only visible upon magnification. This made identification of the compounds difficult. The structure elucidation was performed as for the PLs in peak 3 in the raw extract.

One possible PC compound could be a $[M\text{-}CH_3]^-$ at *m/z* 738 with the fatty acids *m/z* 255 (16:0) and *m/z* 275 (18:4) attached. The retention times for these signals were similar but the peak shapes only party resembled each other, which made it hard to draw conclusions regarding the compound. As peak 5 was not entirely symmetrical it is likely that it comprises more than one compound. Table 53 summarizes the elements of the compounds identified in peak 5.

Table 53: Summary of the elements of the compound identified in peak 5

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 862	*m/z* 788	*m/z* 253 (16:1) *m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$)
			

8.4.6. Peak 6

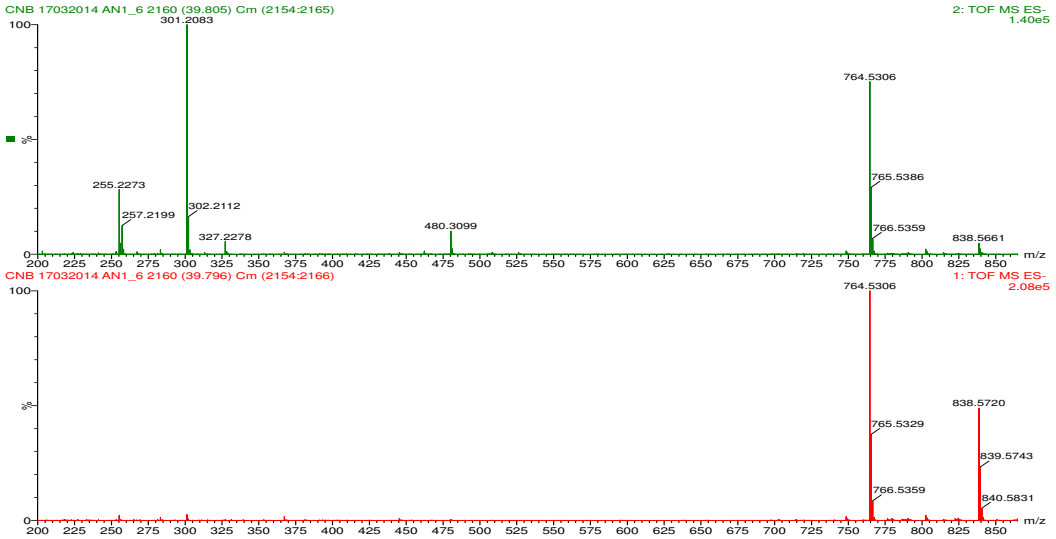
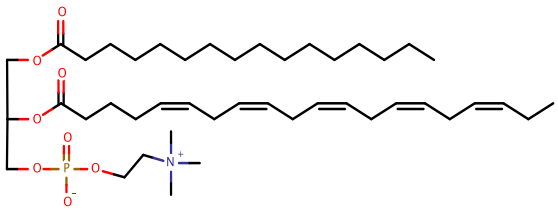


Figure 48: MS (1) and MS/MS (2) spectra for the chromatographic peak 6

From the MS and the MS/MS spectra one compound could be identified. The signal at *m/z* 764 is likely to be a PC as an acetate adduct at *m/z* 838 also could be observed. The different fatty acid components were identified where were *m/z* 301 (20:5$\Delta^{5,8,11,14,17}$) and *m/z* 255 (16:0) were the dominating ones. The peak at *m/z* 257 resulted from the fatty acid at *m/z* 327 loosing CO_2 during fragmentation. Other observed elements were the fatty acid component *m/z* 327 (22:6$\Delta^{4,7,10,13,16,19}$). The structure elucidation was performed as for the PLs in peak 3 of the raw extract. Table 54 summarizes the elements of the compounds identified in peak 6.

Table 54: Summary of the elements of the compound identified in peak 6

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 838	*m/z* 764	*m/z* 255 (16:0) *m/z* 301 (20:5$\Delta^{5,8,11,14,17}$)
			

8.4.7. Peak 7

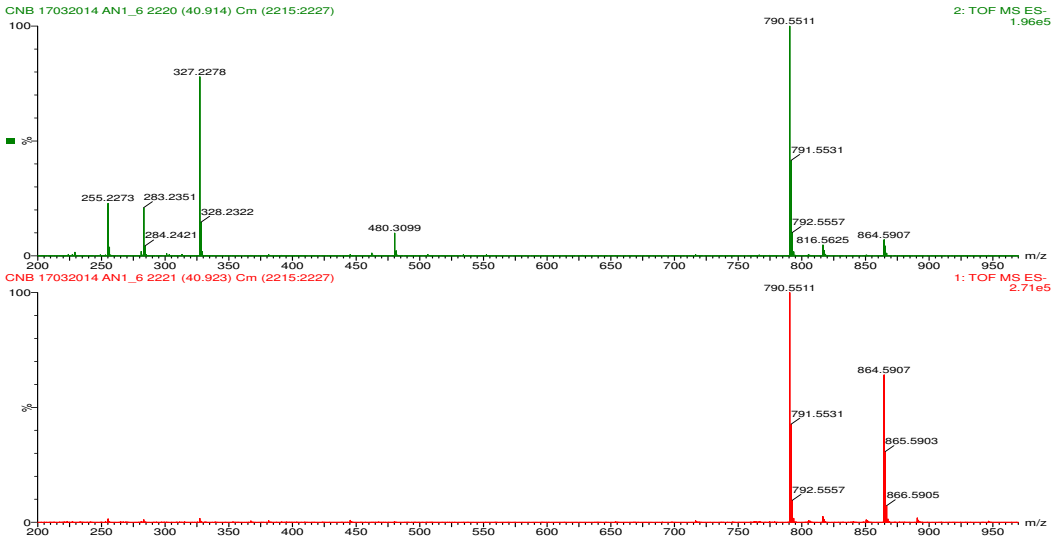
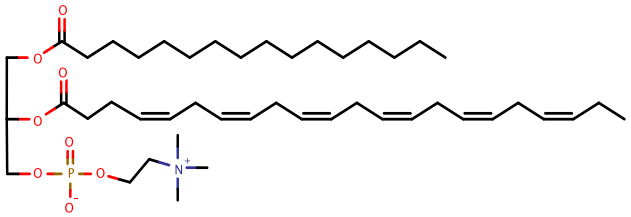


Figure 49: MS (1) and MS/MS (2) spectra for the chromatographic peak 7

From the MS and the MS/MS spectra one compound could be identified. The signal at *m/z* 790 is likely to be a PC as an acetate adduct at *m/z* 864 also could be observed. Two different fatty acid components, *m/z* 327 (22:6$\Delta^{4,7,10,13,16,19}$) and *m/z* 255 (16:0), were identified. The peak at *m/z* 283 resulted from the fatty acid at *m/z* 327 loosing CO_2 during fragmentation. The structure elucidation was performed as for the PLs in peak 3 in the raw extract and Table 55 summarizes the elements of the compounds identified in peak 7.

Table 55: Summary of the elements of the compound identified in peak 6

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 864	*m/z* 790	*m/z* 255 (16:0) *m/z* 327 (22:6$\Delta^{4,7,10,13,16,19}$)
			

8.4.8. Peak 8

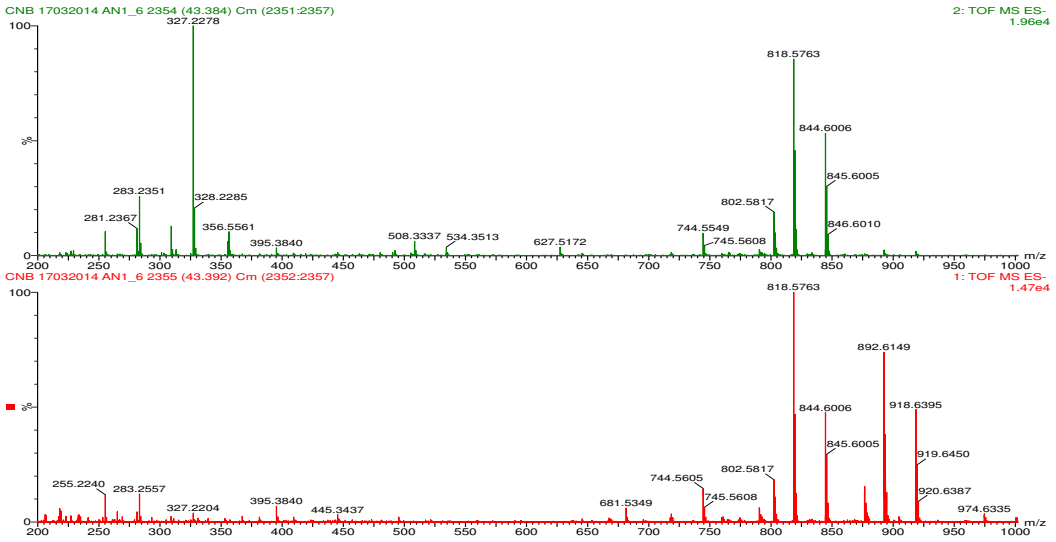
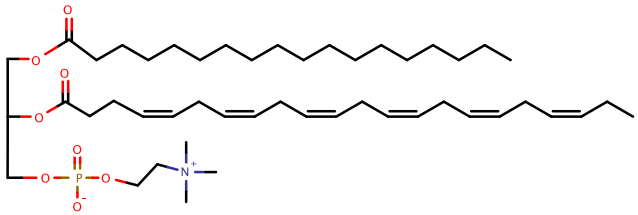


Figure 50: MS (1) and MS/MS (2) spectra for the chromatographic peak 8

From the MS and the MS/MS spectra one compounds could be identified. The signal at *m/z* 818 was likely to be a PC as an acetate adduct $[M+OAc]^-$ at and *m/z* 892 was also observed. Several fatty acids were identified where *m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$) and *m/z* 283 (18:0) dominated. Upon magnification it was visible that the *m/z* 283 signal represented two different signals, where one came from *m/z* 327 loosing CO_2 upon fragmentation to yield 283.2351 and the other was the fatty acid 18:0 with a *m/z* 283.2557. The structure elucidation was performed as for the PLs in peak 3 in the raw extract. There were a lot of other signals in these spectra that did not co-elute with the identified compound. As seen from the chromatogram (Figure 41) this peak was not well separated from other peaks and thus interfering signals will appear. Table 56 summarize the elements of the compound identified in peak 8.

Table 56: Summary of the elements of the compound identified in peak 8

	$[M+OAc]^-$	**$[M-CH_3]^-$**	**Fatty acid components**
Compound 1	*m/z* 892	*m/z* 818	*m/z* 283 (18:0) *m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$)
			

8.4.9. Peak 9

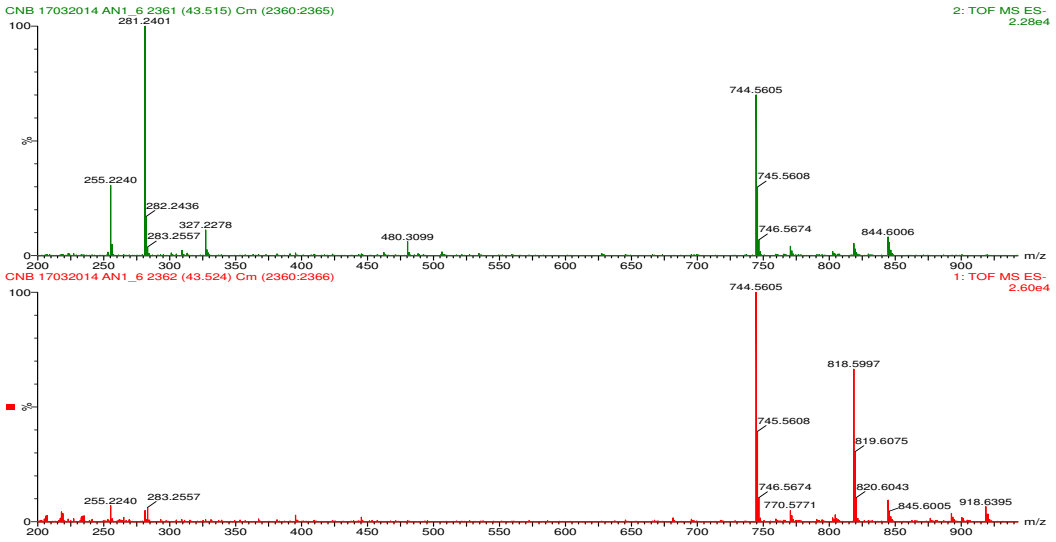
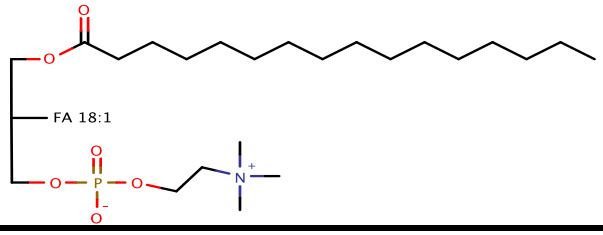


Figure 51: MS (1) and MS/MS (2) spectra for the chromatographic peak 9

From the MS and the MS/MS spectra one compound could be identified. The signal at *m/z* 744 was likely to be a PC as an acetate adduct $[M+OAc]^-$ at and *m/z* 818 was also observed. Three fatty acids were identified where *m/z* 281 (18:1) and *m/* 255 (16:0) dominated. A *m/z* 327 ($22:6\Delta^{4,7,10,13,16,19}$) signal was also seen, and the corresponding *m/z* 283 from the loss of CO_2. The structure elucidation was performed as for the PLs in peak 3 in the raw extract. As with the previous peak the chromatographic separation was not optimal and resulted in interfering signals from nearby peaks. Table 57 summarize the elements of the compound identified in peak 9.

Table 57: Summary of the elements of the compound identified in peak 9

	$[M+OAc]^-$	$[M-CH_3]^-$	Fatty acid components
Compound 1	*m/z* 818	*m/z* 744	*m/z* 255 (16:0) *m/z* 281 (18:1)
			

8.5. Evaluation of DHA and EPA content

As previously mentioned the main ingredients of marine oils, that the manufacturers make health claims about, are the essential fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). As seen from the EU legislation there are requirements to the contents of the products if the producers are to make claims about them, and therefor it was interesting to see weather the main detected ingredients in krill oil capsules and raw extract were found to be EPA and DHA. In Figure 52 the chromatographic peaks containing EPA and/or DHA are labelled for Rimfrost krill oil capsules.

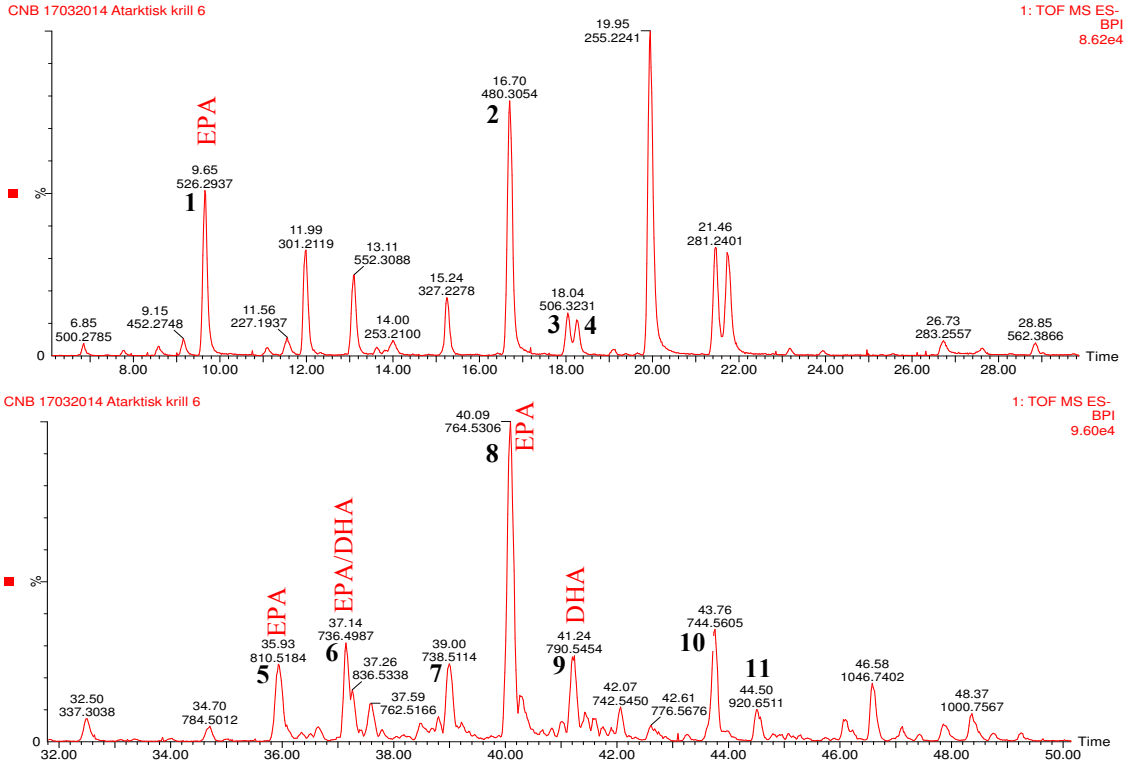


Figure 52: Peaks in the chromatogram of Rimfrost krill oil capsules (1000 µg/ml) that contained the essential fatty acids EPA and DHA

The EPA and DHA containing peaks were labelled in the full scan MS chromatogram as the intensity of the fragment peaks in the MS/MS chromatogram can vary. This showed that some of the most intense peaks contained either one or both essential fatty acids. A high proportion of the overall content contained EPA and/or DHA. The krill oil also contained many phospholipid compounds that had other fatty acids attached, in addition to several peaks that were only of high intensity in the full scan MS spectra. Because the method was optimised for PLs they were likely to be other lipid compounds. The most dominant peak in the

chromatogram was peak 8, which contained EPA. Whenever EPA was observed, the essential fatty acid was situated in *sn-2* position. One of the EPAs detected were in the form of lysoPC. When DHA was detected in the peaks it was attached in *sn-1* position for peak 6 and in *sn-2* position for peak 9. The krill oil capsules and the raw extract chromatograms were not directly comparable because of the difference in concentration, however the basic compounds of the two products were proved to be comparable. Because the samples were analysed on the same instrument and under the same conditions, a similar degree of ionisation was achieved and the results from the full scan MS can therefore be compared.

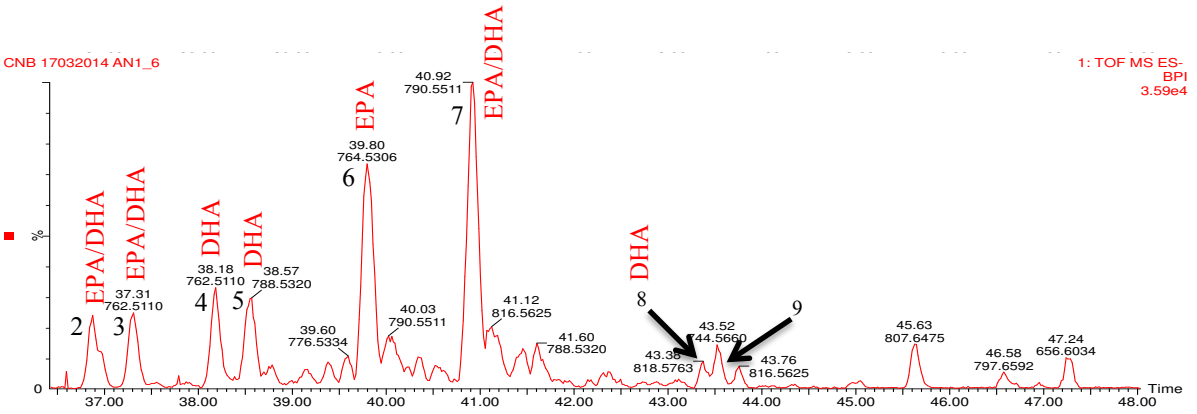


Figure 53: Peaks in the chromatogram of phospholipid raw extract (4000µg/ml) that contained the essential fatty acids EPA and DHA

The full scan MS chromatogram of the raw extract displayed in Figure 53 showed that, compared to the krill oil sample, a higher proportion of the total content contained EPA and/or DHA. None of the peaks in the lysoPL region contained either of the essential fatty acids, however in the PL region most of the dominant peaks detected contained EPA or DHA. In the two most dominant peaks, peak 6 and peak 7, both the essential fatty acids were detected. Whenever EPA was identified it was attached in the *sn-2* position, the exception being peak 3. The same trend was observed for DHA, where in all but one compound, the essential fatty acid was attached in *sn-2* position. The analyses revealed that the raw extract had a relatively higher EPA and DHA content than the krill oil capsules. This showed that the methods developed by the manufacturer AN was successful in enriching the phospholipids with EPA and DHA. As the raw extract was extracted from other sources than krill oil this shows that in addition to krill there are also other marine sources that have a high content of the essential fatty acids EPA and DHA.

In these experiments only phosphatidylcholine species were identified, and from the knowledge of krill oil and the raw extract content this was also expected.

9. Conclusion and future prospects

A novel UPLC and MS method was developed for analysing phospholipid standards and complex phospholipid samples. The methods developed allowed for identification and structural elucidation of different PL species and by utilizing MS^E technology, rapid qualitative analyses of complex lipid samples were made possible. A mobile phase consisting of acetonitrile, isopropanol and water with ammonium acetate provided the best reversed phase chromatographic separation when analysed with a narrow bore (1mm) 150mm C18 column. By using UPLC-MS it was confirmed that a high proportion of the total content of commercial marine oils contained the essential fatty acids EPA and DHA. The study also showed that the two different brands of krill oil capsules had very similar content. There was greater variation in the fatty acid species of the krill oil than of the raw extract, and the raw extract seems successfully enriched with EPA and DHA.
Few studies have been done on commercial phospholipid samples such as krill oil and raw extract intended for commercial use. The present study is a contribution to the evaluation of marine oils used as dietary supplements and weather the content of the supplements match the labelled ingredients.

The next step of the method development would be to use the established methods to analyse more PLs standards and also other more complicated lipid samples. Another natural step would be to verify the regioisomerism by conducting experiments with phospholipases, and particularly phospholipase A2, for both standards and complex lipid mixtures. To gain more knowledge about dissolution and absorption of phospholipids the use an in vitro dissolution testing system, such as a Sotax, to imitate the conditions of the intestine would be beneficial. This will aid in the evaluation of the importance of different regioisomers of the phospholipids.

References

1. Norwegian Food Authorities Nordic Council. Food Supplements in the Nordic Countries. 2009.
2. Norwegian Food and Safety Authority. Kosttilskudd - en tilstandsbeskrivelse. 2013.
3. Directorate-General for Health and Consumers of the European Commission. The Use of Substances with Nutritional or Physiological Effects other than Vitamins and Minerals in Food Supplements 2006 Contract No.: SANCO/2006/E4/018.
4. Rossi M. Use of Lecithin and Lecithin Fractions. In: Huopalahti R, López-Fandiño R, Anton M, Schade R, editors. Bioactive Egg Compounds: Springer Berlin Heidelberg; 2007. p. 229-39.
5. Norwegian Food Safety Authority. Kosttilskudd [06.12.2013]. Available from: http://www.mattilsynet.no/mat_og_vann/spesialmat_og_kosttilskudd/kosttilskudd/.
6. Minestry of Healt and Care Norway. Forskrift om legemidler (legemiddelforskriften), FOR-2009-12-18-1839 (2010).
7. European Food and Safety Authority. Scientific Opinion on Fish Oil for Human Consumption and Food Hygiene, including Rancidity. EFSA Journal. 2010;8(10).
8. Bransjerådet for Naturmidler. Markedsstatistikk 2010 [10.12.2013]. Available from: http://brn.no/brn.no/brnno/Bransjen/Markedsdata/Statistikk/.
9. Ministry of Health and Care Services. Forskrift om ernærings- og helsepåstander om næringsmidler, FOR-2010-02-17-187 (2010).
10. Christie WW. Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids: Amer Oil Chemists Society; 2000.
11. Food Lipids: Chemistry, Nutrition, and Biotechnology, Third Edition: CRC Press; 2008.
12. Fahy E, Subramaniam S, Brown HA, Glass CK, Merrill AH, Murphy RC, et al. A comprehensive classification system for lipids. Journal of Lipid Research. 2005 May 1, 2005;46(5):839-62.
13. Fahy E, Subramaniam S, Murphy RC, Nishijima M, Raetz CRH, Shimizu T, et al. Update of the LIPID MAPS comprehensive classification system for lipids. Journal of Lipid Research. 2009;50(Supplement):9-14.
14. Gunstone FD, Harwood JL, Dijkstra AJ. The Lipid Handbook. Third ed: CRC Press; 2007.
15. Moe MK, Jensen E. Structure elucidation of unsaturated fatty acids after vicinal hydroxylation of the double bonds by negative electrospray ionisation low-energy tandem mass spectrometry. European journal of mass spectrometry. 2004;10(1):47-55.
16. Handbook of Functional Lipids. Second ed: CRC Press; 2005.
17. Loef M, Walach H. The Omega-6/Omega-3 Ratio and Dementia or Cognitive Decline: A Systematic Review on Human Studies and Biological Evidence. Journal of Nutrition in Gerontology and Geriatrics. 2013 2013/01/01;32(1):1-23.
18. Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Experimental biology and medicine 2008;233(6):674-88. PubMed PMID: 18408140. eng.
19. Chang CY, Ke DS, Chen JY. Essential fatty acids and human brain. Acta neurologica Taiwanica. 2009;18(4):231-41.
20. Essensielle Fettsyrer. Store Norske Leksika. http://snl.no/essensielle_fettsyrer: SNL AS; 2009.
21. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Lancet. 1999;354(9177):447-55.

22. Burr ML, Fehily AM, Gilbert JF, Rogers S, Holliday RM, Sweetnam PM, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). Lancet. 1989;2(8666):757-61.
23. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, et al. Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet. 2007;369(9567):1090-8.
24. Saravanan P, Davidson NC, Schmidt EB, Calder PC. Cardiovascular effects of marine omega-3 fatty acids. The Lancet.376(9740):540-50.
25. Hooper L, Thompson RL, Harrison RA, Summerbell CD, Ness AR, Moore HJ, et al. Risks and benefits of omega 3 fats for mortality, cardiovascular disease, and cancer: systematic review. British Medical Journal. 2006 2006-03-30 23:57:53;332(7544):752-60.
26. Burr ML, Ashfield-Watt PA, Dunstan FD, Fehily AM, Breay P, Ashton T, et al. Lack of benefit of dietary advice to men with angina: results of a controlled trial. European journal of clinical nutrition. 2003;57(2):193-200.
27. Kris-Etherton PM, Harris WS, Appel LJ, Committee ftN. Fish Consumption, Fish Oil, Omega-3 Fatty Acids, and Cardiovascular Disease. Arteriosclerosis, Thrombosis, and Vascular Biology. 2003;23(2):20-30.
28. The Norwegian Directorate of Health. Kostråd for å fremme folkehelsen og forebygge kroniske sykdommer. 2011 Contract No.: IS-1881
29. Association BCSBHABHSebtBD. Joint British recommendations on prevention of coronary heart disease in clinical practice. Heart. 1998;80(suppl 2):1-29.
30. Cohn J, Kamili A, Wat E, Chung RW, Tandy S. Dietary Phospholipids and Intestinal Cholesterol Absorption. Nutrients. 2010;2(2):116-27.
31. Lagarde M, Bernoud N, Brossard N, Lemaitre-Delaunay D, Thies F, Croset M, et al. Lysophosphatidylcholine as a preferred carrier form of docosahexaenoic acid to the brain. Journal of molecular neuroscience : MN. 2001;16(2-3):201-4; discussion 15-21.
32. Borgström B. Phospholipid Absorption. In: Rommel K, Goebell H, Böhmer R, editors. Lipid Absorption: Biochemical and Clinical Aspects: Springer Netherlands; 1976. p. 65-72.
33. Guo Z, Vikbjerg AF, Xu X. Enzymatic modification of phospholipids for functional applications and human nutrition. Biotechnology Advances. 2005;23(3):203-59.
34. Zeisel SH, DaCosta KA. Choline: an essential nutrient for public health. Nutritional Review. 2009 Nov;67(11):615-23.
35. Tou JC, Jaczynski J, Chen Y-C. Krill for Human Consumption: Nutritional Value and Potential Health Benefits. Nutrition Reviews. 2007;65(2):63-77.
36. Phleger CF, Nelson MM, Mooney BD, Nichols PD. Interannual and between species comparison of the lipids, fatty acids and sterols of Antarctic krill from the US AMLR Elephant Island survey area. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2002;131(4):733-47.
37. Nicol S. Krill, Currents, and Sea Ice: Euphausia superba and Its Changing Environment. BioScience. 2006;56(2):111-20.
38. Fricke H, Gercken G, Schreiber W, Oehlenschläger J. Lipid, sterol and fatty acid composition of antarctic krill (Euphausia superba Dana). Lipids. 1984 1984/11/01;19(11):821-7. English.
39. Ulven S, Kirkhus B, Lamglait A, Basu S, Elind E, Haider T, et al. Metabolic Effects of Krill Oil are Essentially Similar to Those of Fish Oil but at Lower Dose of EPA and DHA, in Healthy Volunteers. Lipids. 2010;46:37 - 46.
40. Ramírez M, Amate L, Gil A. Absorption and distribution of dietary fatty acids from different sources. Early Human Development. 2001;65, Supplement 2(0):95-101.
41. Schuchardt J, Schneider I, Meyer H, Neubronner J, von Schacky C, Hahn A. Incorporation of EPA and DHA into plasma phospholipids in response to different omega-3

fatty acid formulations - a comparative bioavailability study of fish oil vs. krill oil. Lipids in Health and Disease. 2011;10(1):145.
42. Maki KC, Reeves MS, Farmer M, Griinari M, Berge K, Vik H, et al. Krill oil supplementation increases plasma concentrations of eicosapentaenoic and docosahexaenoic acids in overweight and obese men and women. Nutrition Research. 2009;29(9):609-15.
43. Graf B, Duchateau G, Patterson A, Mitchell E, van Bruggen P, Koek J, et al. Age dependent incorporation of 14C-DHA into rat brain and body tissues after dosing various 14C-DHA-esters. Prostaglandins, Leukotrienes and Essential Fatty Acids. 2010;83(2):89-96.
44. Wijendran V, Huang MC, Diau GY, Boehm G, Nathanielsz PW, Brenna JT. Efficacy of dietary arachidonic acid provided as triglyceride or phospholipid as substrates for brain arachidonic acid accretion in baboon neonates. Pediatric research. 2002;51(3):265-72.
45. Frega NG, Pacetti D, Boselli E. Characterization of Phospholipid Molecular Species by Means of HPLC-Tandem Mass Spectrometry, Tandem Mass Spectrometry - Applications and Principles. 2012.
46. Hansen S, Pedersen-Bjergaard S, Rasmussen K. High Performance Liquid Chromatography. Introduction to Pharmaceutical Chemical Analysis: John Wiley & Sons, Ltd; 2011.
47. HPLC for Pharmaceutical Scientists: WILEY-Interscience; January 2007.
48. Pulfer M, Murphy RC. Electrospray mass spectrometry of phospholipids. Mass Spectrometry Reviews. 2003;22(5):332-64.
49. Rainville PD, Stumpf CL, Shockcor JP, Plumb RS, Nicholson JK. Novel Application of Reversed-Phase UPLC-oaTOF-MS for Lipid Analysis in Complex Biological Mixtures: A New Tool for Lipidomics. Journal of Proteome Research. 2006 2007/02/01;6(2):552-8.
50. Gross JH. Mass Spectrometry: A Textbook. 2nd ed: Springer; 2011.
51. Murphy RC, Jessica Krank, Barkley RM. LC/MS Methodology in Lipid Analysis and Structural Characterization of Novel Lipid Species (Chapter 2), Functional Lipidomics: CRC Press; 2005.
52. Winther B, Hoem N, Berge K, Reubsaet L. Elucidation of Phosphatidylcholine Composition in Krill Oil Extracted from Euphausia superba. Lipids. 2011;46(1):25-36.
53. Harrison KA, Murphy RC. Negative electrospray ionization of glycerophosphocholine lipids: Formation of [M – 15]− ions occurs via collisional decomposition of adduct anions. Journal of Mass Spectrometry. 1995;30(12):1772-3.
54. Han X, Gross RW. Structural determination of picomole amounts of phospholipids via electrospray ionization tandem mass spectrometry. Journal of the American Society for Mass Spectrometry. 1995;6(12):1202-10.
55. Moe MK, Anderssen T, Strøm MB, Jensen E. Vicinal hydroxylation of unsaturated fatty acids for structural characterization of intact neutral phospholipids by negative electrospray ionization tandem quadrupole mass spectrometry. Rapid Communications in Mass Spectrometry. 2004;18(18):2121-30.
56. Murphy RC, Axelsen PH. Mass spectrometric analysis of long-chain lipids. Mass Spectrometry Reviews. 2011;30(4):579-99.
57. Moe MK, Anderssen T, Strom MB, Jensen E. Total structure characterization of unsaturated acidic phospholipids provided by vicinal di-hydroxylation of fatty acid double bonds and negative electrospray ionization mass spectrometry. Journal of the American Society for Mass Spectrometry. 2005;16(1):46-59.

Appendix 1

Lipoid

ANALYTICAL DATA

Product: LIPOID E 80

University of Tromso

Date of production: 09/2011
Retest date: 09/2014
recommended storage temperature: -20°C ± 5°C
Delivery: 21.11.2011
Quantity: 1.0 kg

Lot No.: 510300-2113044-05

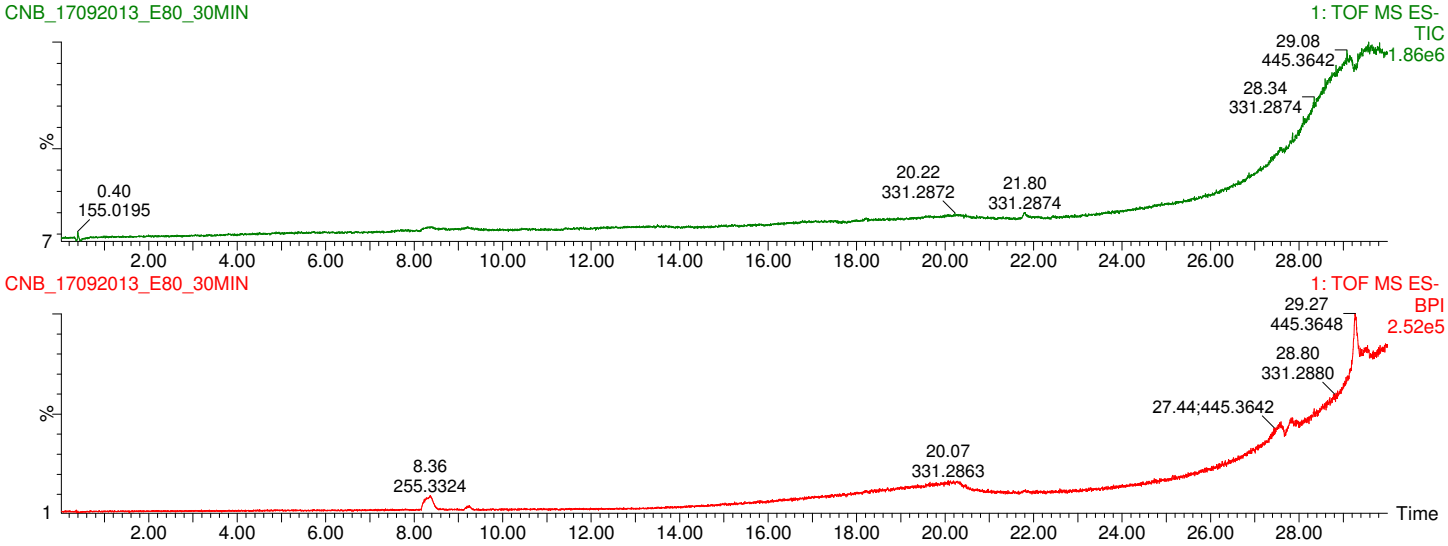
	Specification	Result	Method
Phosphatidylcholine (+ LPC)	80.0 - 85.0 %	83.1 %	PC3
Phosphatidylethanolamine	7.0 - 9.5 %	8.0 %	DE-PE
Lysophosphatidylcholine	n.m.t. 3.0 %	1.8 %	ADC3
Lysophosphatidylethanolamine	n.m.t. 0.5 %	0.4 %	ADC3
Sphingomyelin	2.0 - 3.0 %	2.5 %	ADC3
Phosphorus	3.7 - 4.0 %	3.8 %	TP
Triglycerides	n.m.t. 3.0 %	1.9 %	ADC5
Cholesterol	n.m.t. 1.0 %	< 1.0 %	ADC5
Free fatty acids	n.m.t. 0.05 %	< 0.05 %	ADC5
Dl-a-Tocopherol	0.05 - 0.1 %	0.07 %	TO
Water (KF)	n.m.t. 2.0 %	0.6 %	USP <921>Ia
Ethanol	n.m.t. 0.2 %	< 0.2 %	ET
Acid value	7 - 10	8.5	SZ
Peroxide value	n.m.t. 3	0	POZ
Iodine value	65 - 69	69	JZ

- 2 -

LIPOID GMBH · FRIGENSTRASSE 4 · D-67065 LUDWIGSHAFEN · TELEFON 0621-5 38 19-0 · TELEFAX 0621-55 35 59

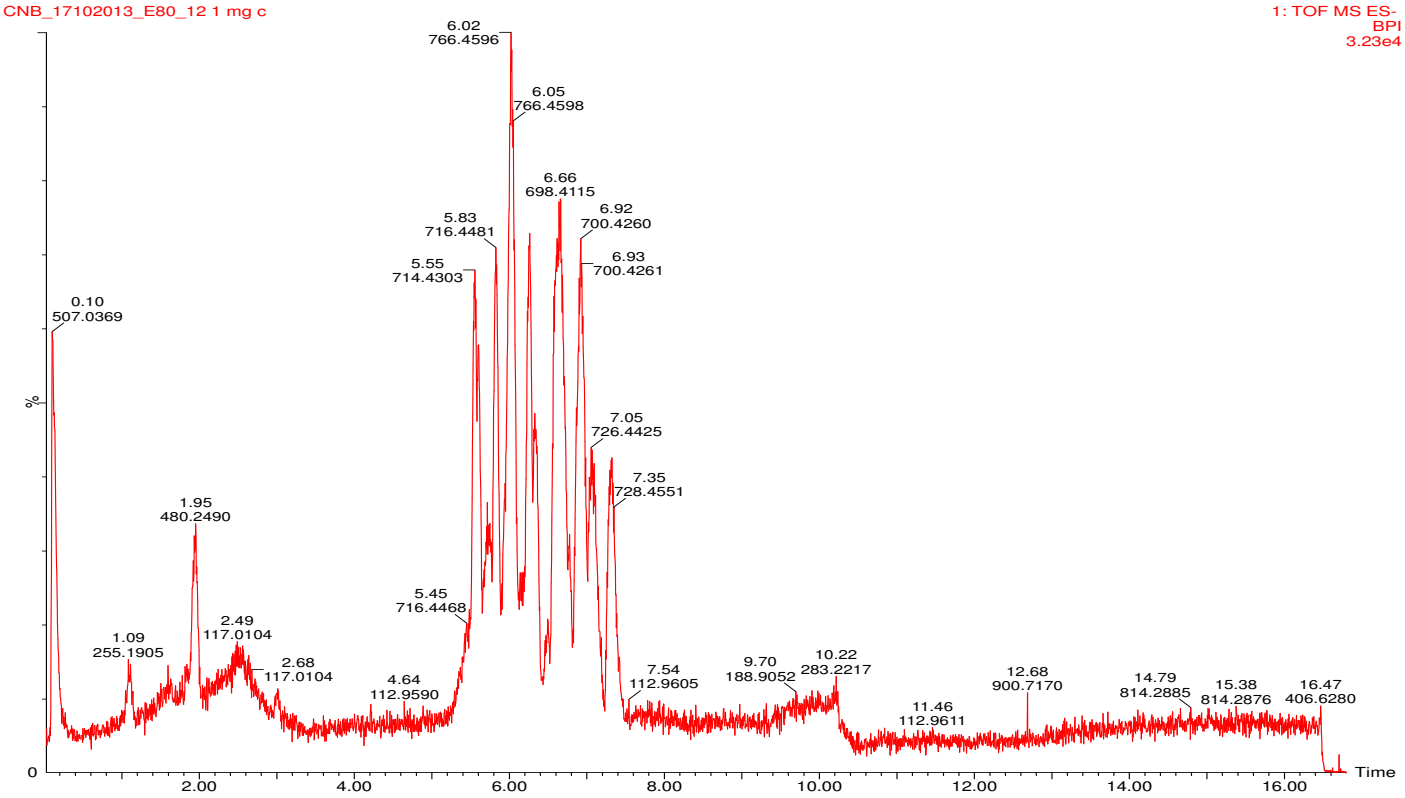
Appx. Figure 1: Analysis certificate for standard E80 fro Lipoid GmbH (Ludwigshafen, Germany).

Appendix 2



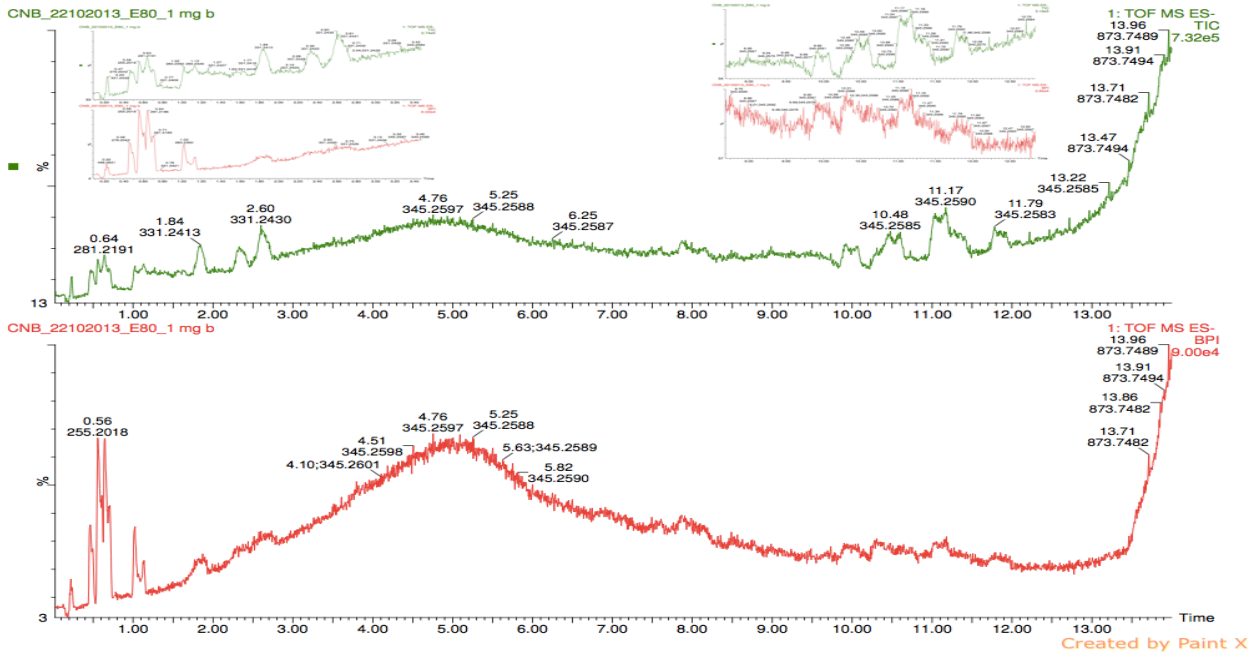
Appx. Figure 2: E80 from Lipoid analysed on Acquity UPLC BEH C18 column 100 mm with mobile phase composition 1. The gradient profile started at 70% mobile phase A and 30 % mobile phase B and ended at 5% mobile phase A and 95% mobile phase B after 30 minutes.

Appendix 3



Appx. Figure 3: The first sign of separation of lipids was achieved on Acquity UPLC BEH C18 VanGuard Pre-column with the E80 standards from Lipoid (100 mcg/ml) with mobile phase composition 2.

Appendix 4



Appx. Figure 4: E80 standard analysed on Acquity UPLC BEH C18 column 50 mm with mobile phase composition 2 resulted in the best separation.

Gradient profiles tried on Acquity UPLC BEH C18 column 50mm with standard E80 (100 µg/ml)

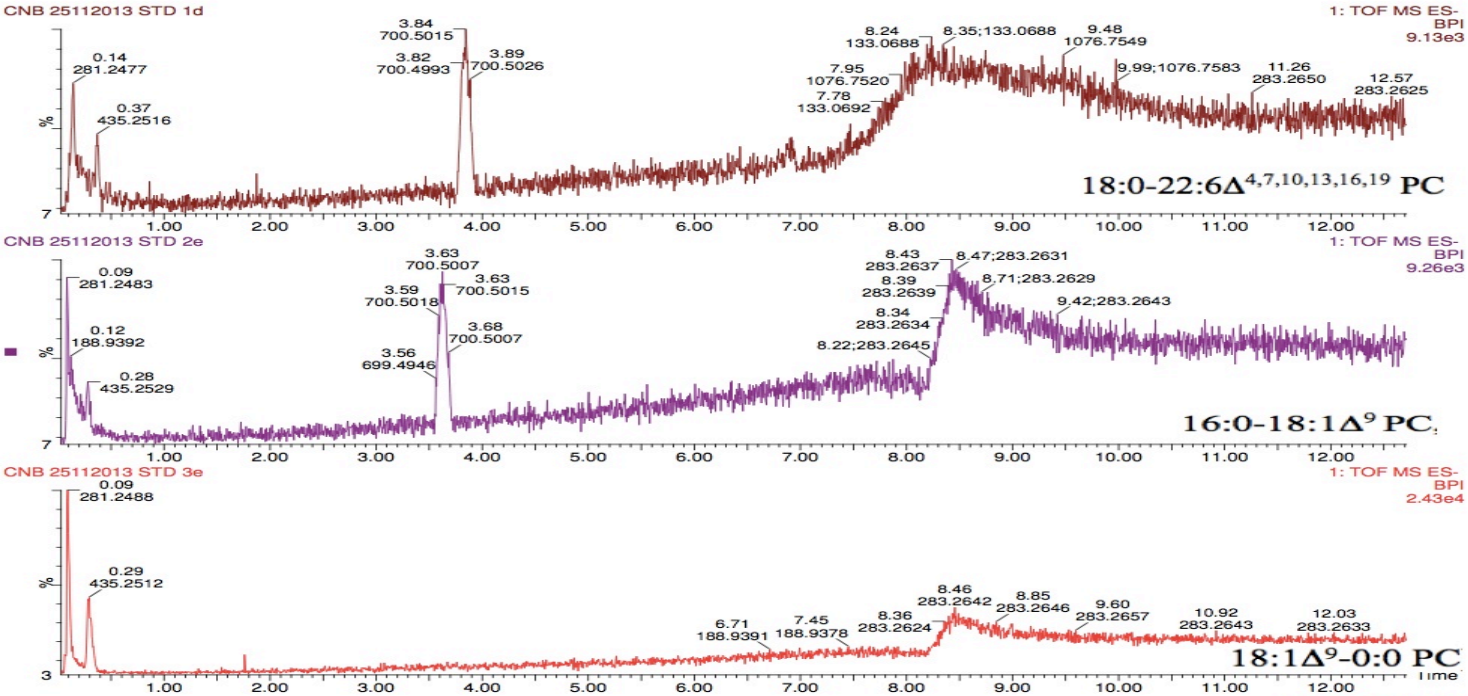
Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	30 %	70 %
14	0.6	0 %	100 %

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	20 %	80 %
10	0.6	0 %	100 %

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	10 %	90 %
60	0.6	0 %	100 %

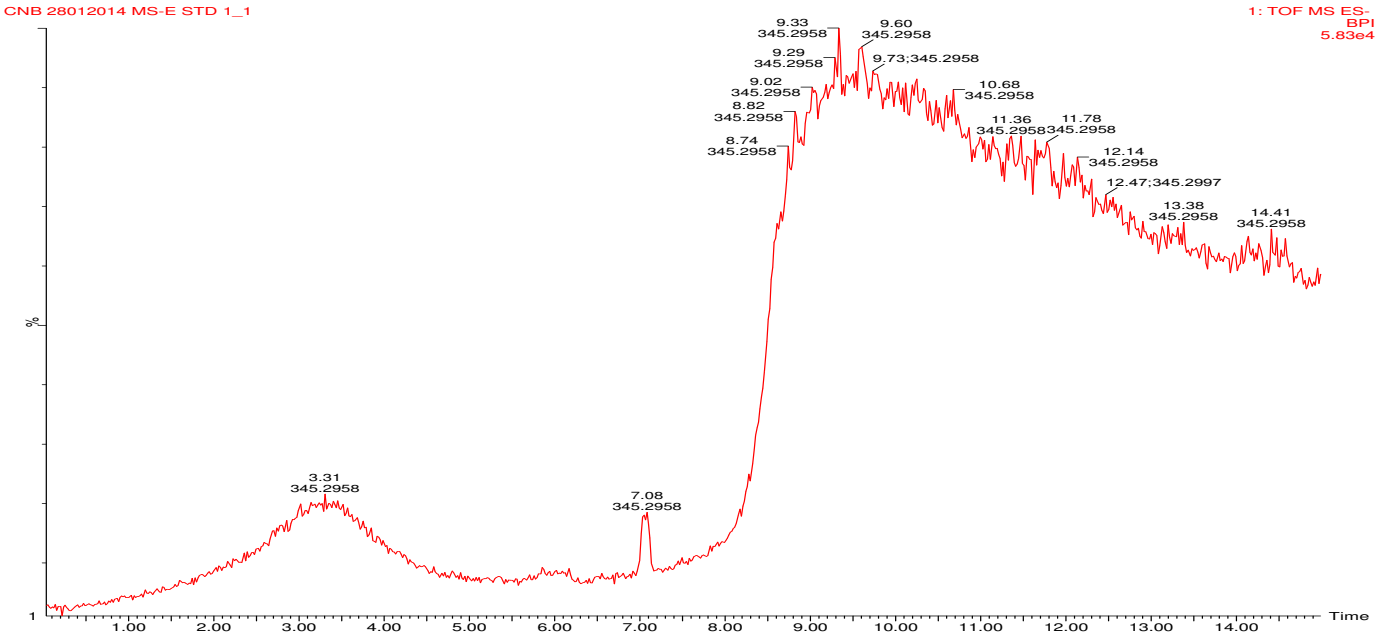
Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	5 %	95 %
30	0.6	0 %	100 %

Appendix 5

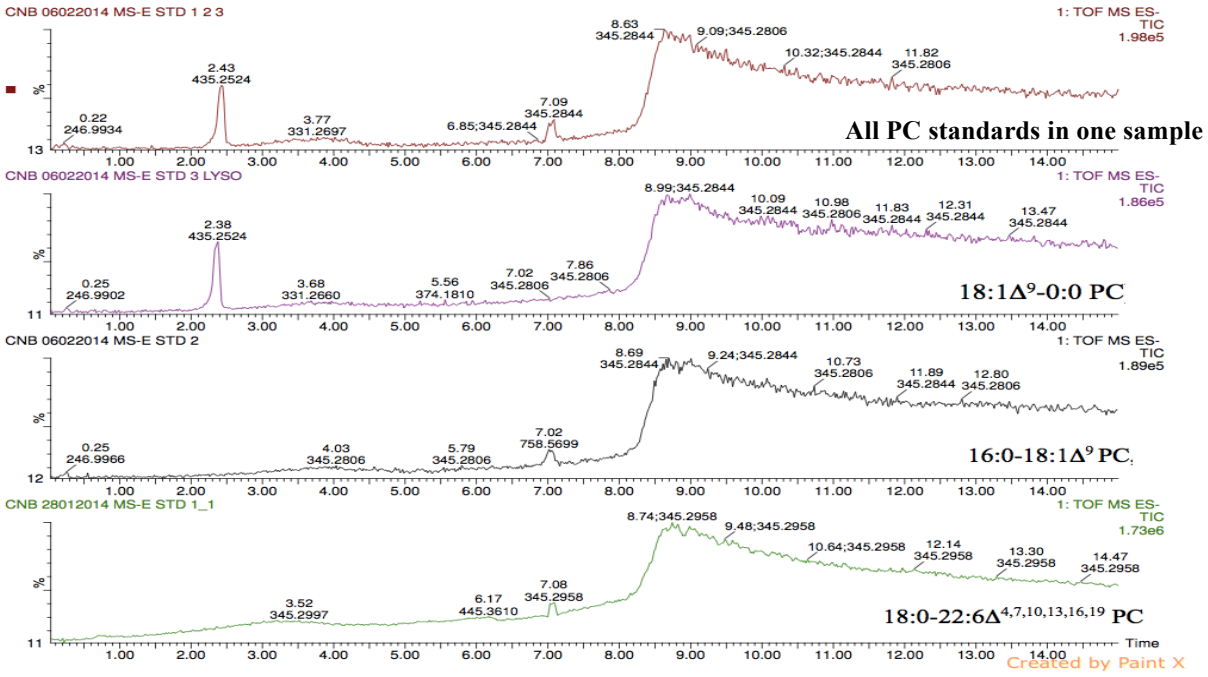


Appx. Figure 5: Chromatogram obtained from full scan MS of standards 18:0-22:6Δ^{4,7,10,13,16,19} PC, 16:0-18:1Δ9 PC and 16:0-0:0 I analysed on Acquity UPLC® BEH C18 VanGuard 5mm column

Appendix 6

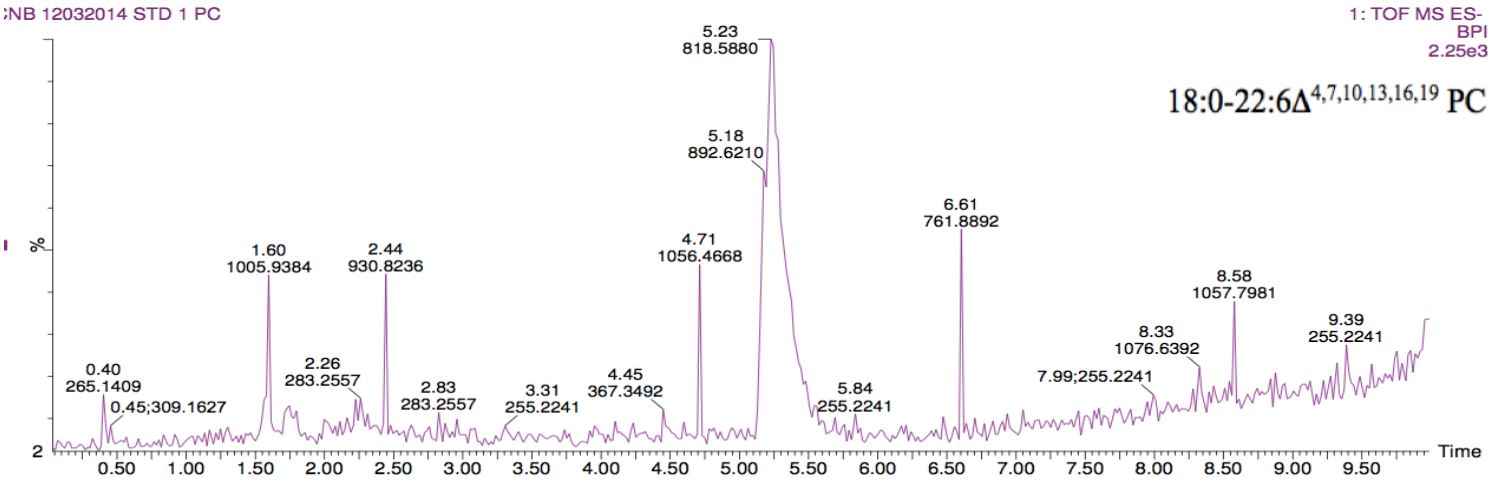


Appx. Figure 6: Standard 18:0-22:6Δ^{4,7,10,13,16,19} PC analysed on Acquity UPLC BEH C18 column 50 mm with mobile phase composition 2.

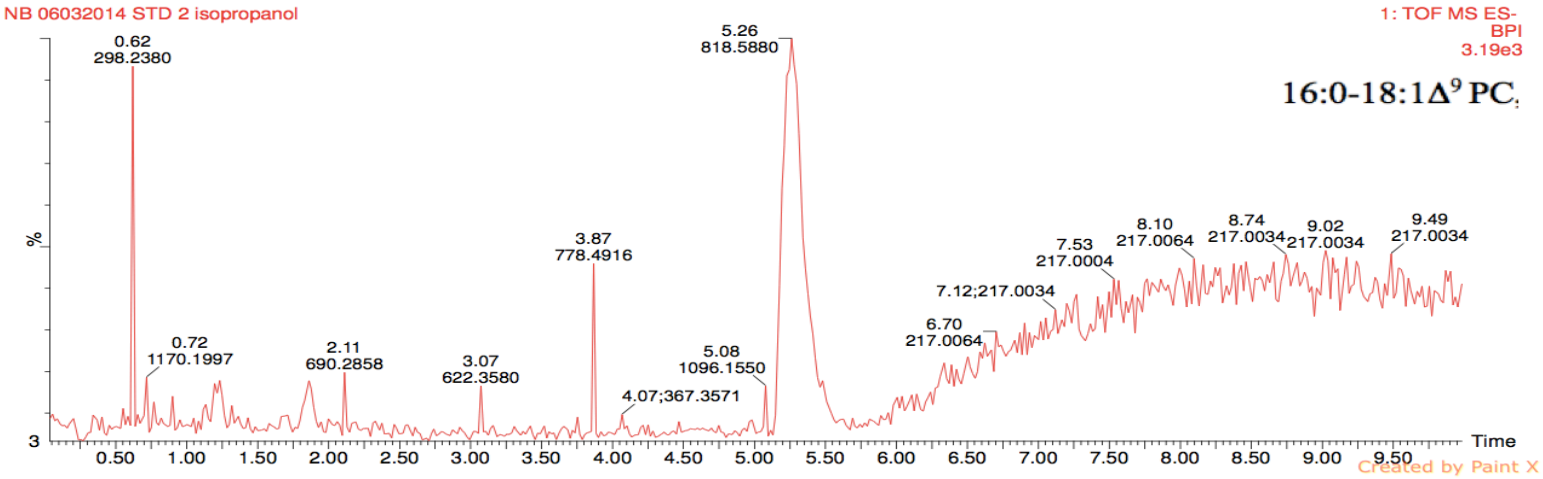


Appx. Figure 7: All three PC standards analysed on Acquity UPLC BEH C18 column 50 mm with mobile phase composition 2 displayed in the top chromatogram. Below are the individual chromatograms for the different PC standards.

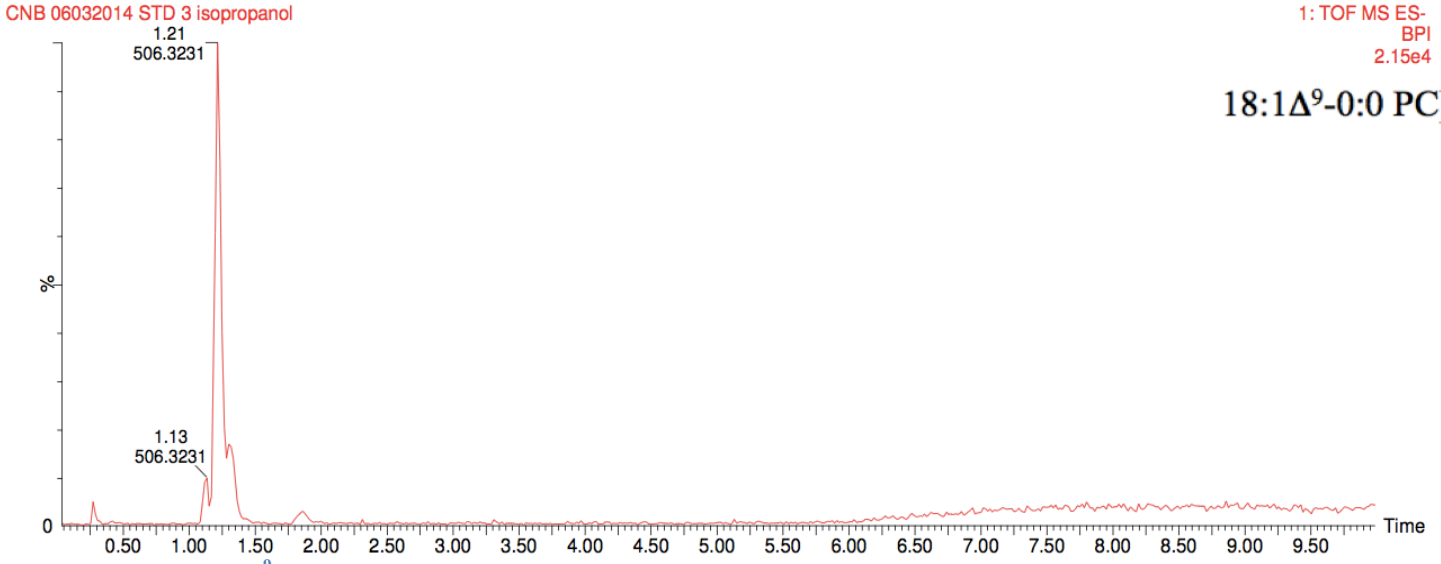
Appendix 7



Appx. Figure 8: 18:0-22:6Δ^{4,7,10,13,16,19} PC analysed on Acquity UPLC BEH C18 column 50 mm with mobile phase composition 3.

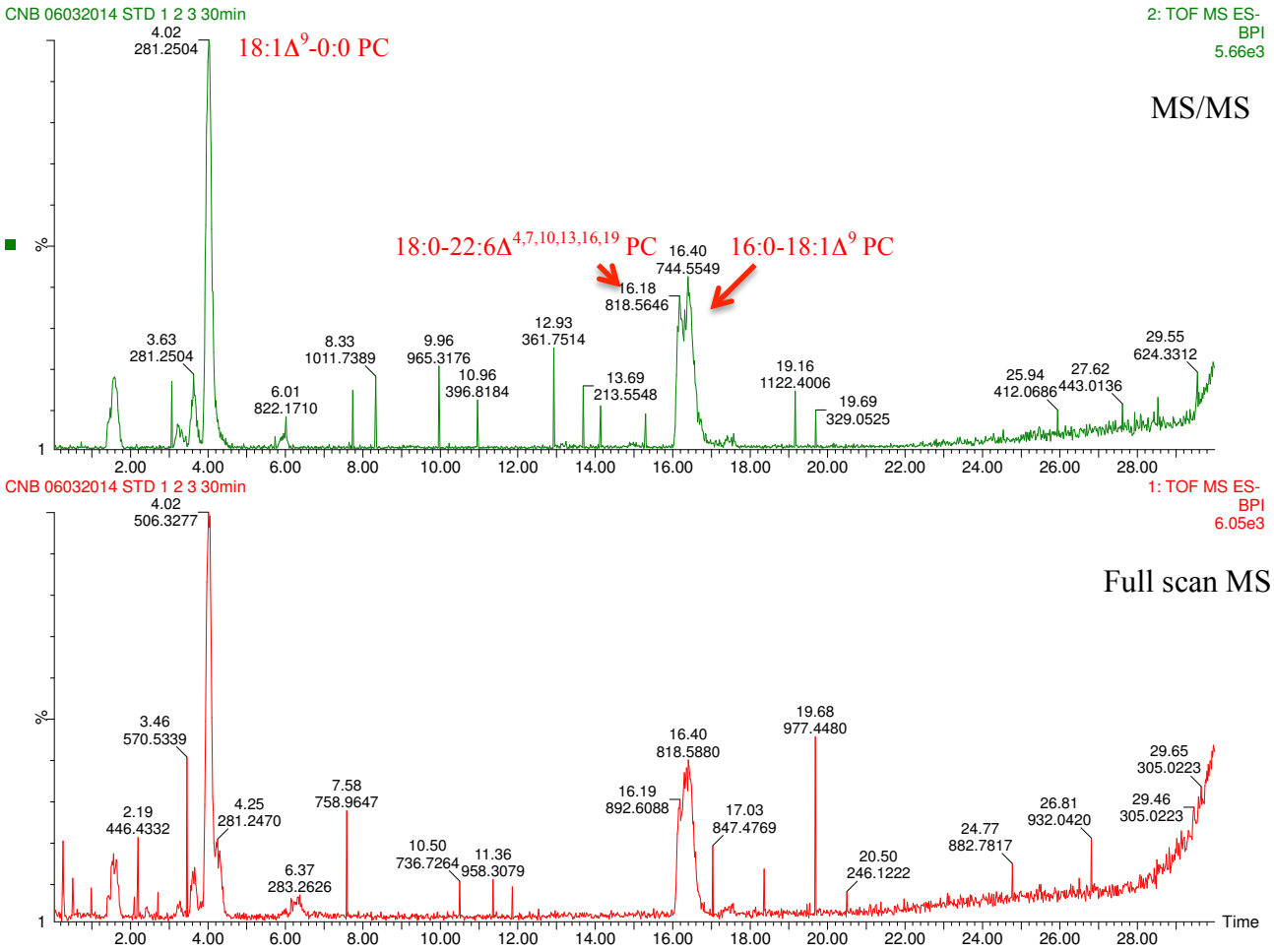


Appx. Figure 9: 16:0-18:1Δ^{9} PC analysed on Acquity UPLC BEH C18 column 50 mm with mobile phase composition 3.



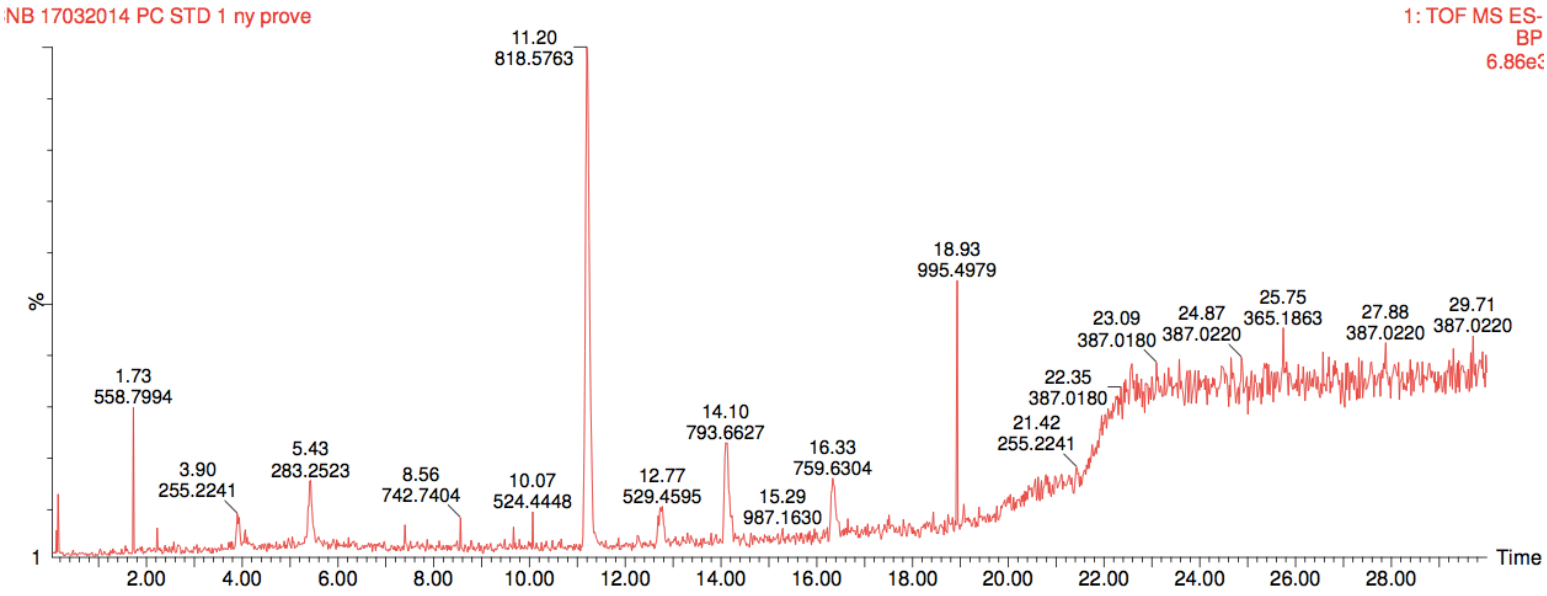
Appx. Figure 10: 18:1Δ^{9}-0:0 PC analysed on Acquity UPLC BEH C18 column 50 mm with mobile phase composition 3.

Appendix 8



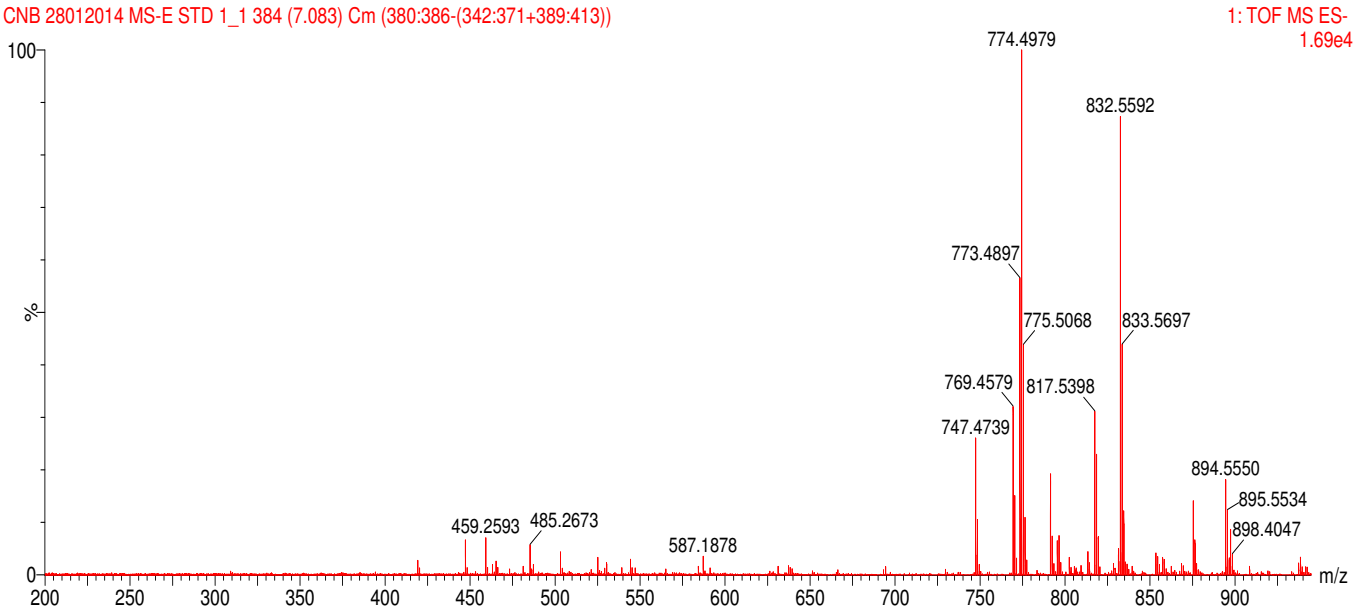
Appx. Figure 11: MS^E analysis of all PC standards on Acquity UPLC BEH C18 50mm column using mobile phase composition 3

Appendix 9

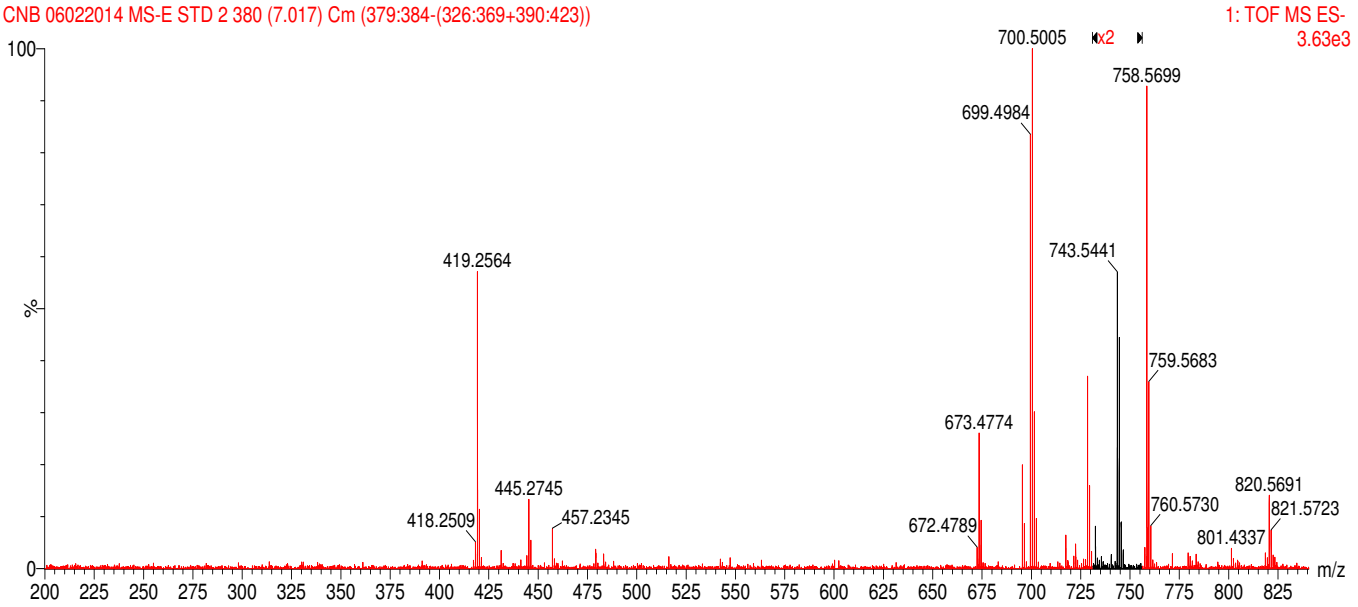


Appx. Figure 12: Standard 18:0-22:6Δ^{4,7,10,13,16,19} PC analysed with mobile phase composition 3 on Acquity UPLC® BEH C18 150 mm column.

Appendix 10

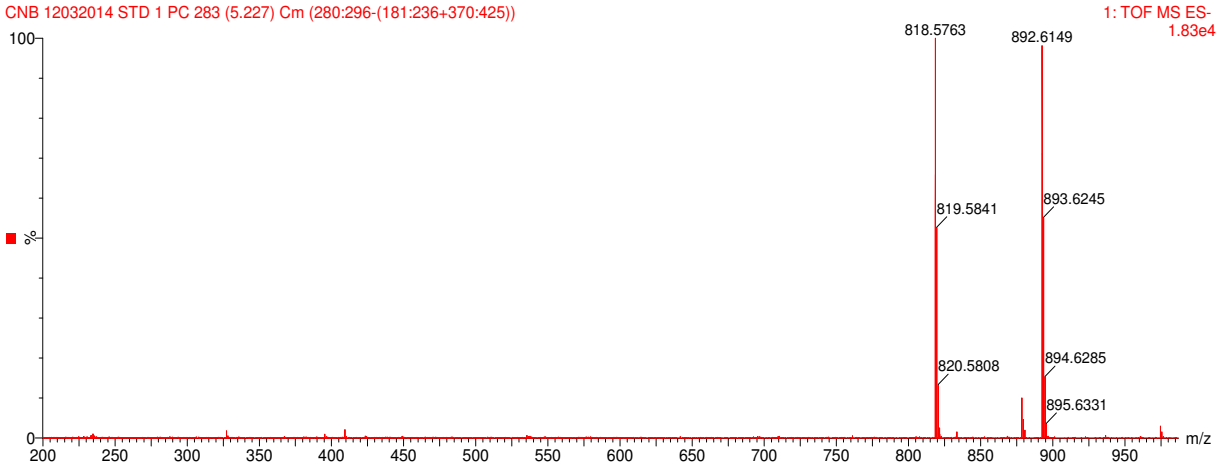


Appx. Figure 13: MS^{E} full scan spectra recorded for 18:0-22:6Δ^{4,7,10,13,16,19} PC upon CID. Analysed on Acquity UPLC BEH C18 column 50 mm using mobile phase gradient 2.

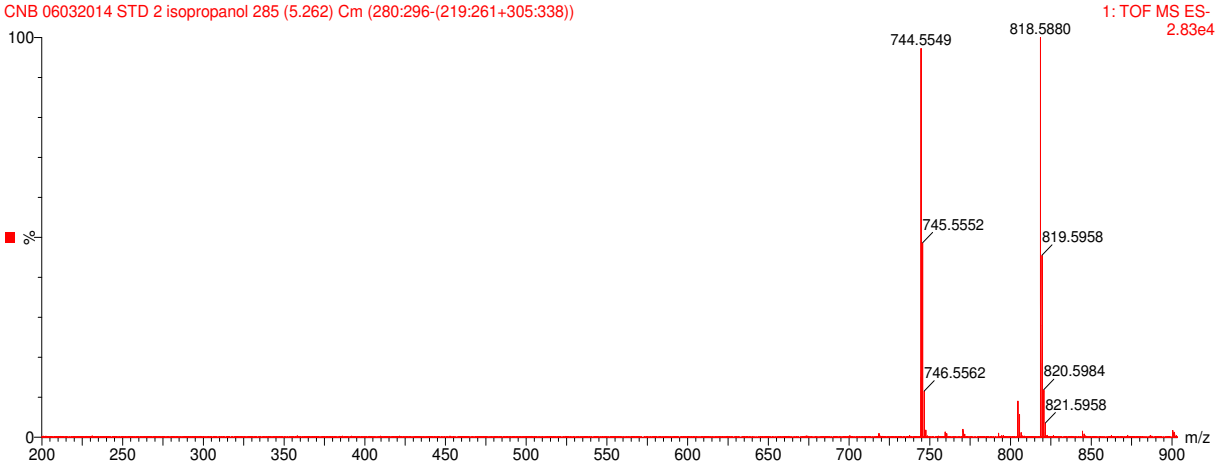


Appx. Figure 14: MS^{E} full scan spectra recorded for 16:0-18:1Δ^{9} PC upon CID. Analysed on Acquity UPLC BEH C18 column 50 mm using mobile phase gradient 2.

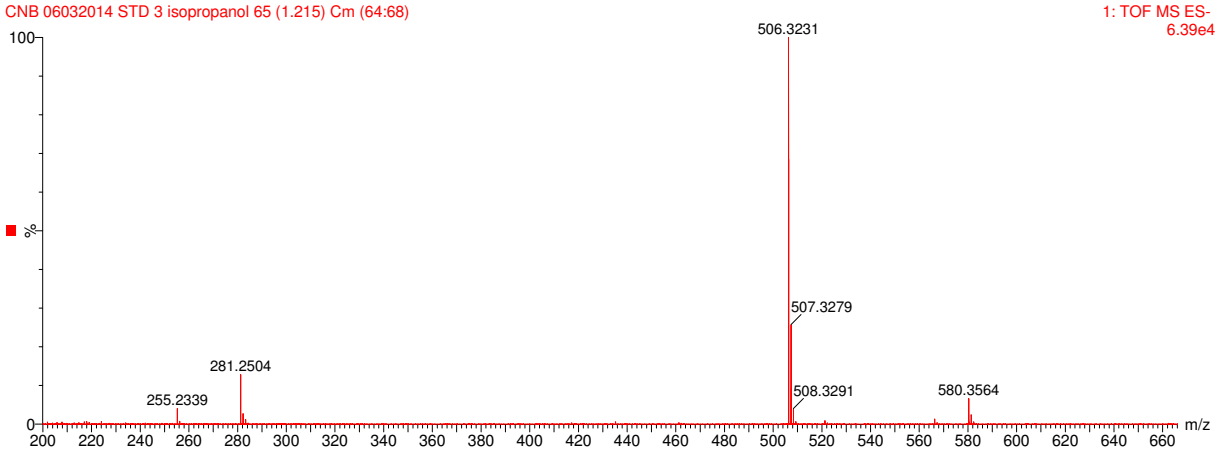
Appendix 11



Appx. Figure 15: MS^E full scan spectra recorded for 18:0-22:6$\Delta^{4,7,10,13,16,19}$ PC upon CID. Analysed on Acquity UPLC BEH C18 column 50 mm using mobile phase gradient 3.

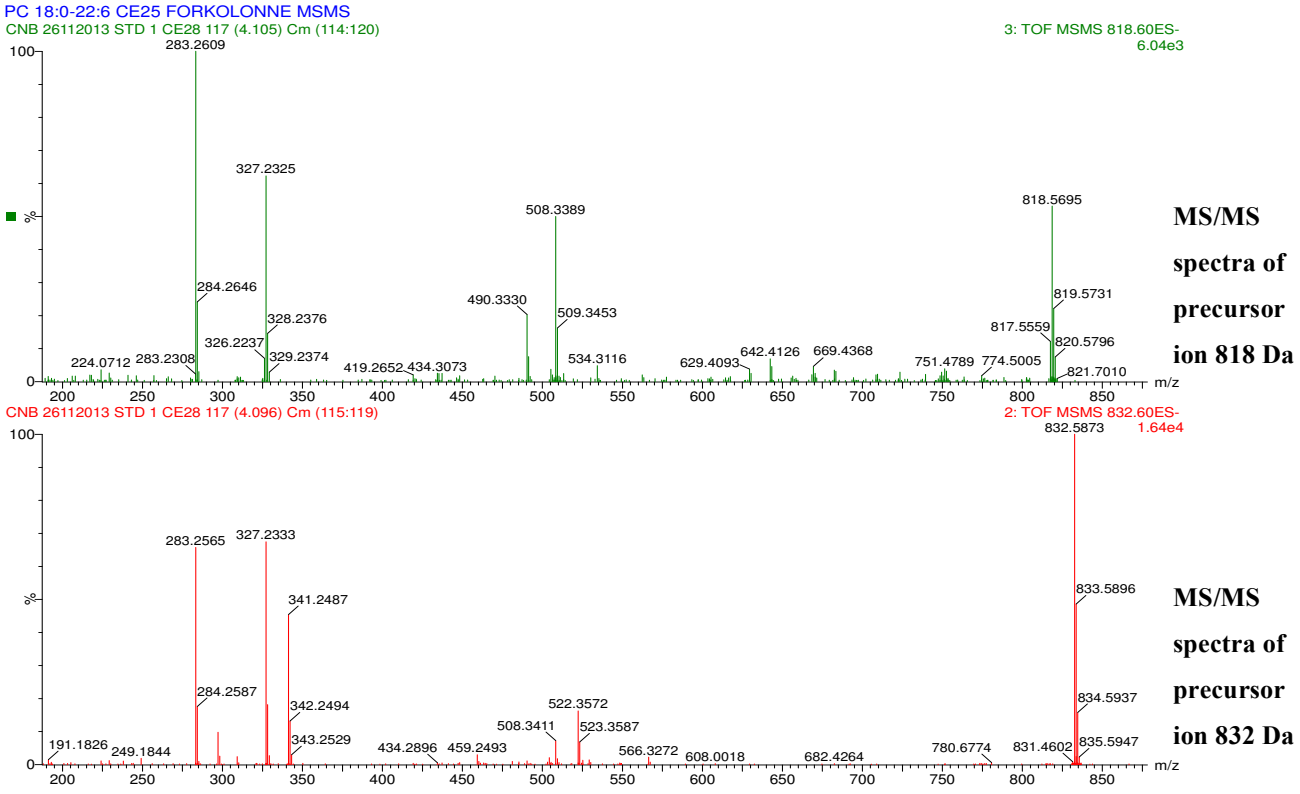


Appx. Figure 16: MS^E full scan spectra recorded for 16:0-18:1Δ^9 PC upon CID. Analysed on Acquity UPLC BEH C18 column 50 mm using mobile phase gradient 3.

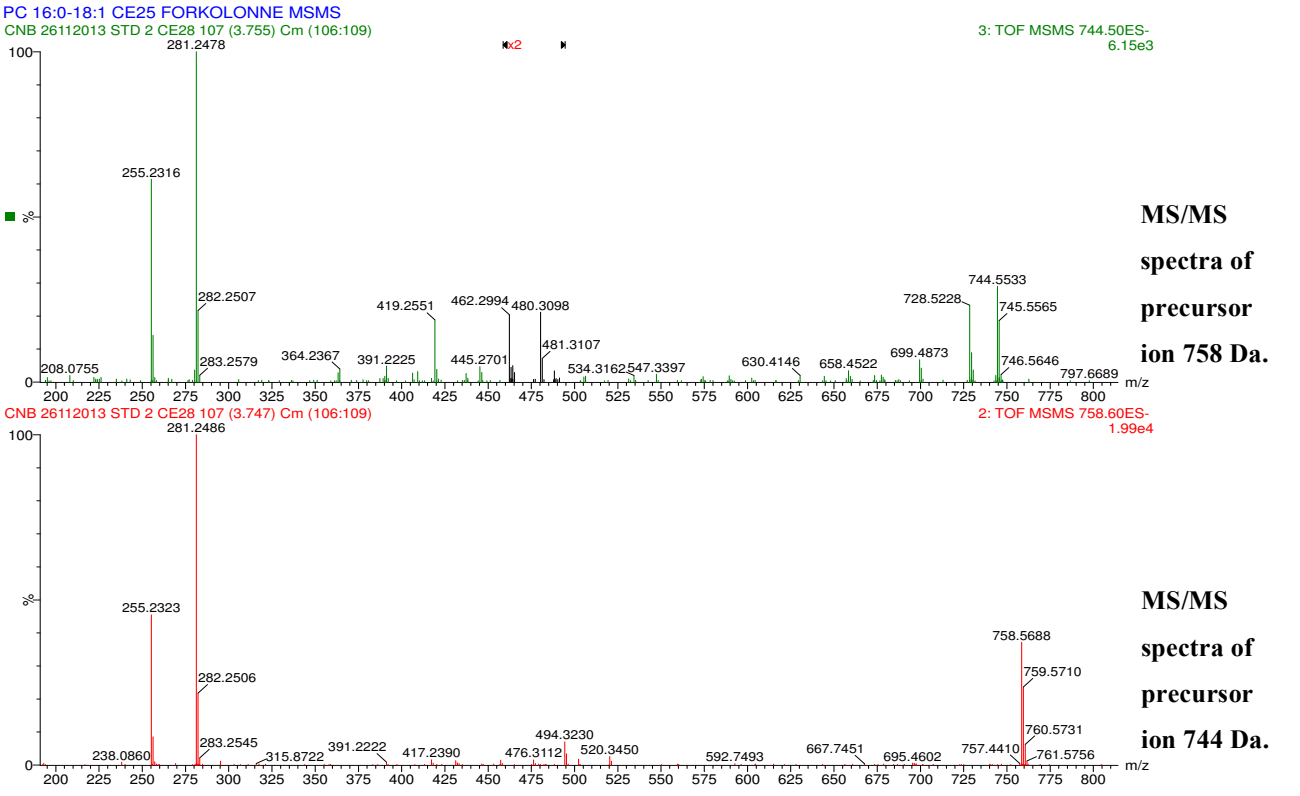


Appx. Figure 17: MS^E full scan spectra recorded for 18:1Δ^9-0:0 PC upon CID. Analysed on Acquity UPLC BEH C18 column 50 mm using mobile phase gradient 3.

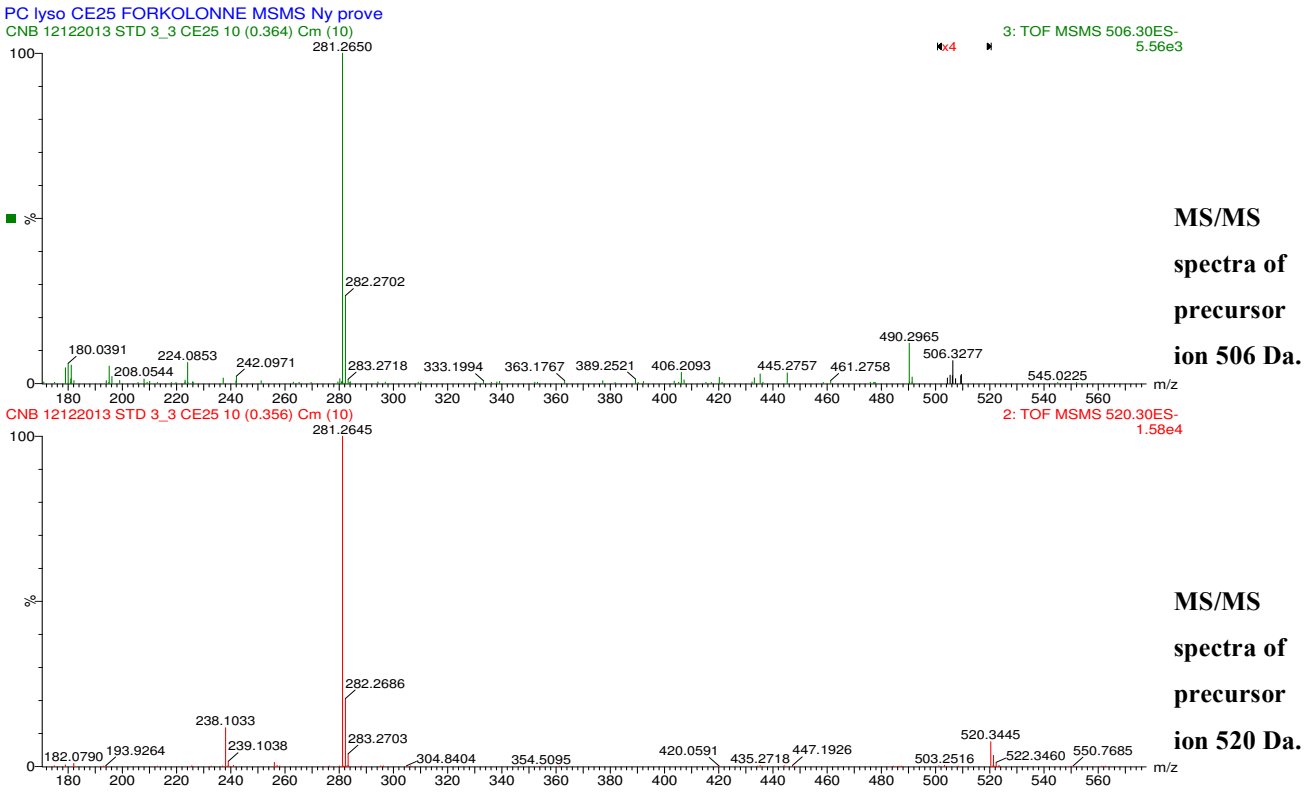
Appendix 12



Appx. Figure 18: MS/MS spectra of 18:0-22:6Δ^{4,7,10,13,16,19} PC, CE 28V, centroid mode.



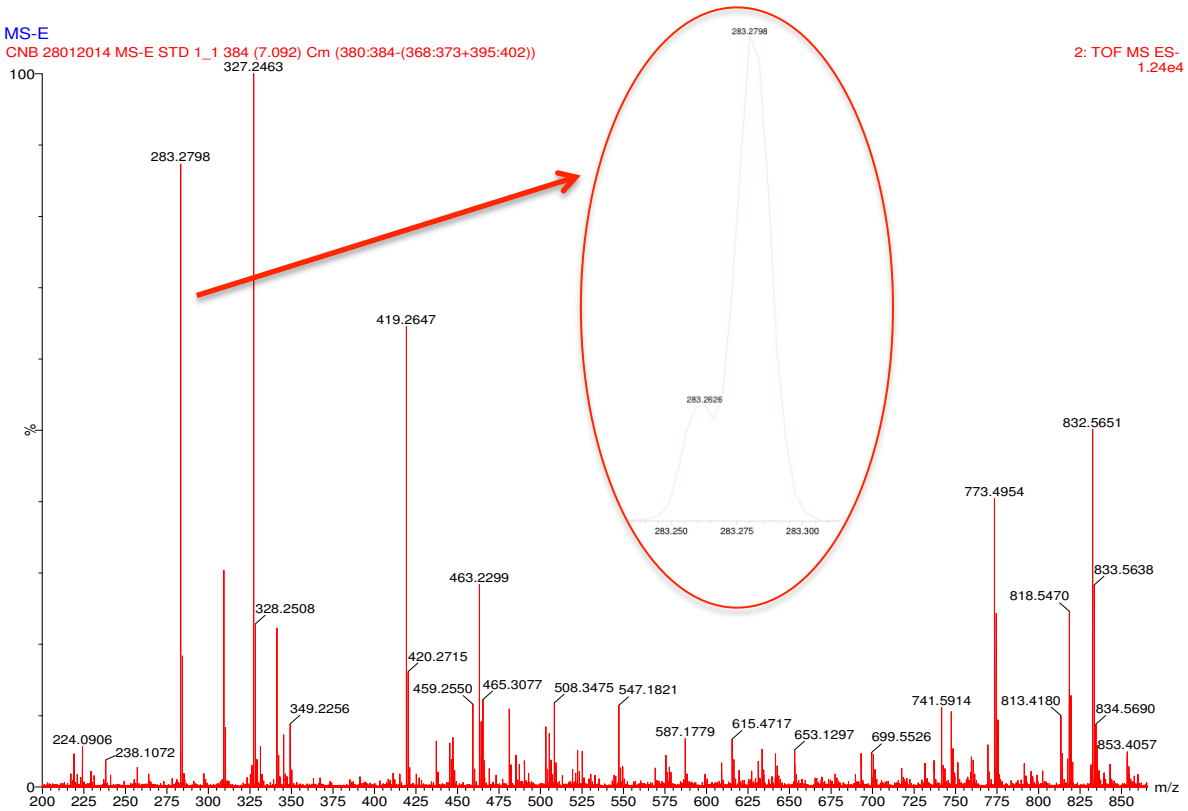
Appx. Figure 19: MS/MS spectra of 16:0-18:1Δ^{9} PC, CE 28V, centroid mode.



MS/MS spectra of precursor ion 506 Da.

MS/MS spectra of precursor ion 520 Da.

Appx. Figure 20: MS/MS spectra of 18:1Δ^{9} – 0:0 PC, CE 28V.



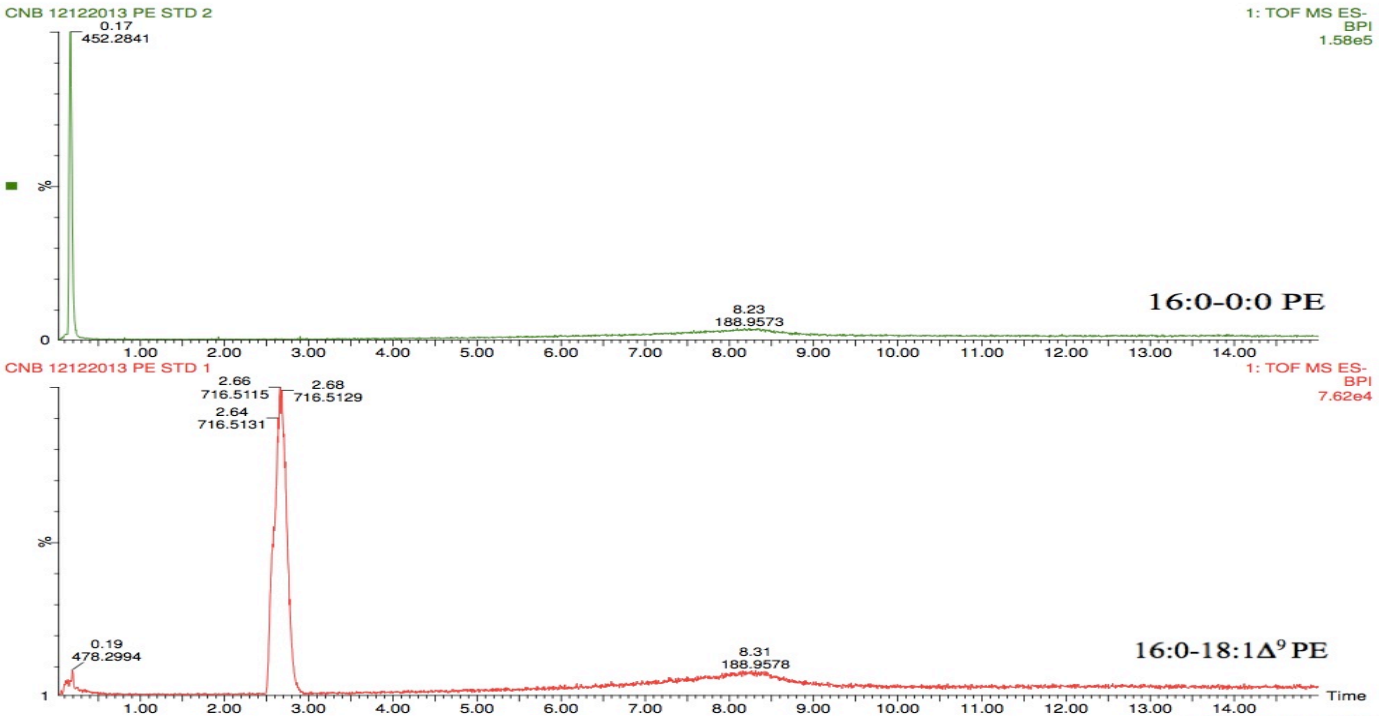
Appx. Figure 21: Data acquired in continuum mode to be able to differentiate between the signals 283.2426 (22:6) and 283.2637 (18:0)

Appendix 13

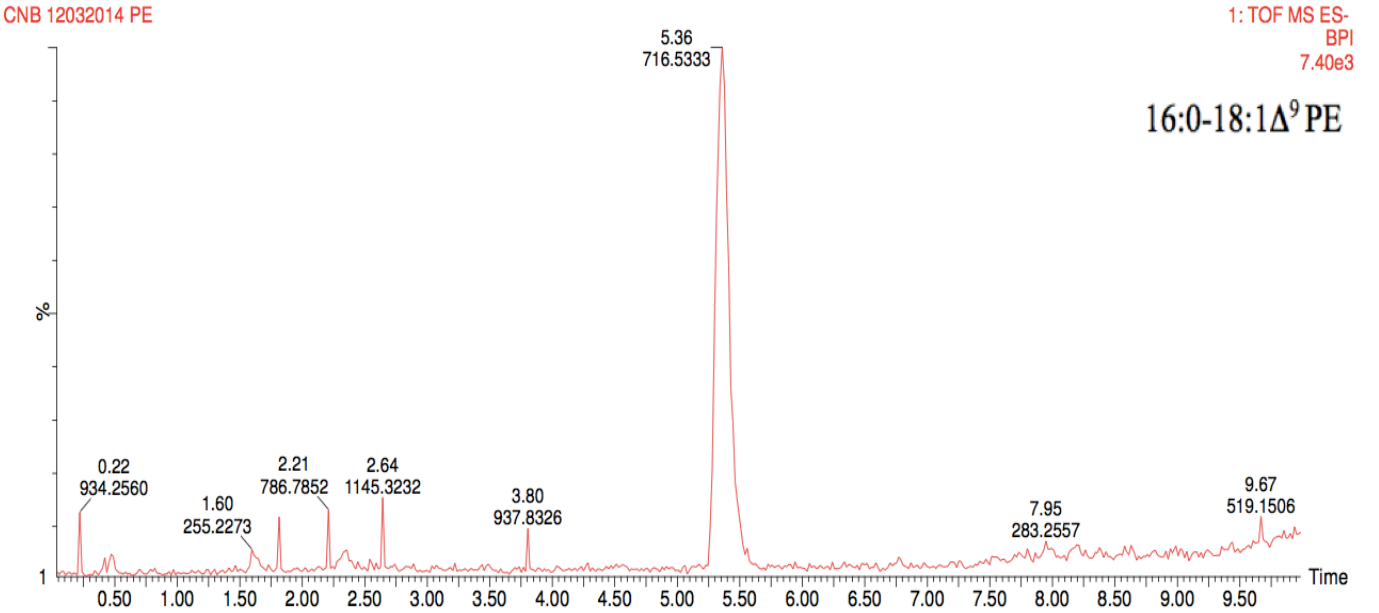
MS/MS experiments of 16:0-18:1Δ^9 PC (CE 28V) and the ratio between R_2COO^-/ R_1COO^- fragment ions

	$[M-H]^-$ = 758			$[M-CH_3]^-$ = 744		
Experiment	**Intensity of FA 281 (R_2COO^-)**	**Intensity of FA 255 (R_1COO^-)**	**Ratio R_2COO^-/ R_1COO^-**	**Intensity of FA 281 (R_1COO^-)**	**Intensity of FA 255 (R_1COO^-)**	**Ratio R_2COO^-/ R_1COO^-**
1	100	48	2,08	100	80	1,25
2	100	51	1,96	100	66	1,52
3	100	47	2,13	100	64	1,56
4	100	52	1,92	100	81	1,23
5	100	47	2,13	100	82	1,22
6	100	51	1,96	100	67	1,49
7	100	49	2,04	100	62	1,61
8	100	52	1,92	100	88	1,14
9	100	49	2,04	100	87	1,15
10	100	50	2,00	100	74	1,35

Appendix 14

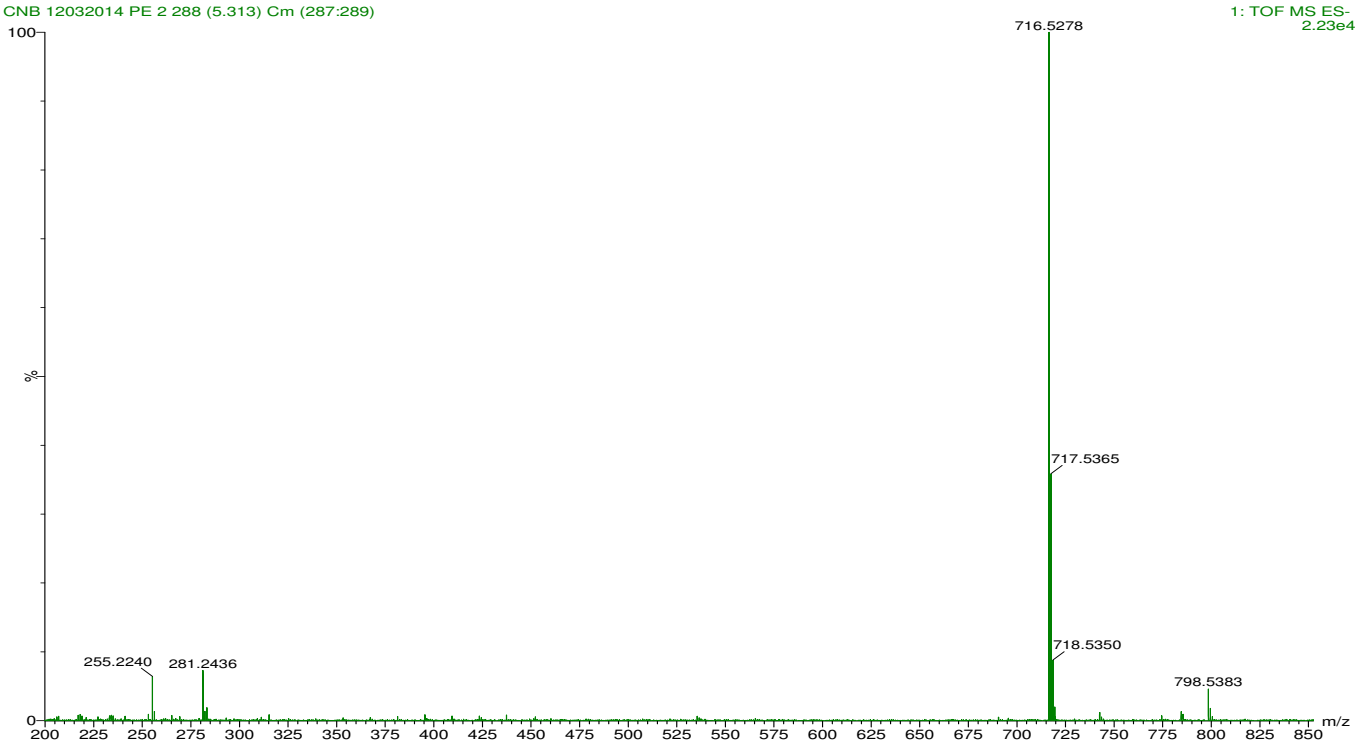


Appx. Figure 22: Chromatogram obtained from analysis of 16:0-0:0 PE and 16:0-18:1Δ^9 PE on analysed on Acquity UPLC® BEH C18 VanGuard 5mm column with mobile phase composition 2.



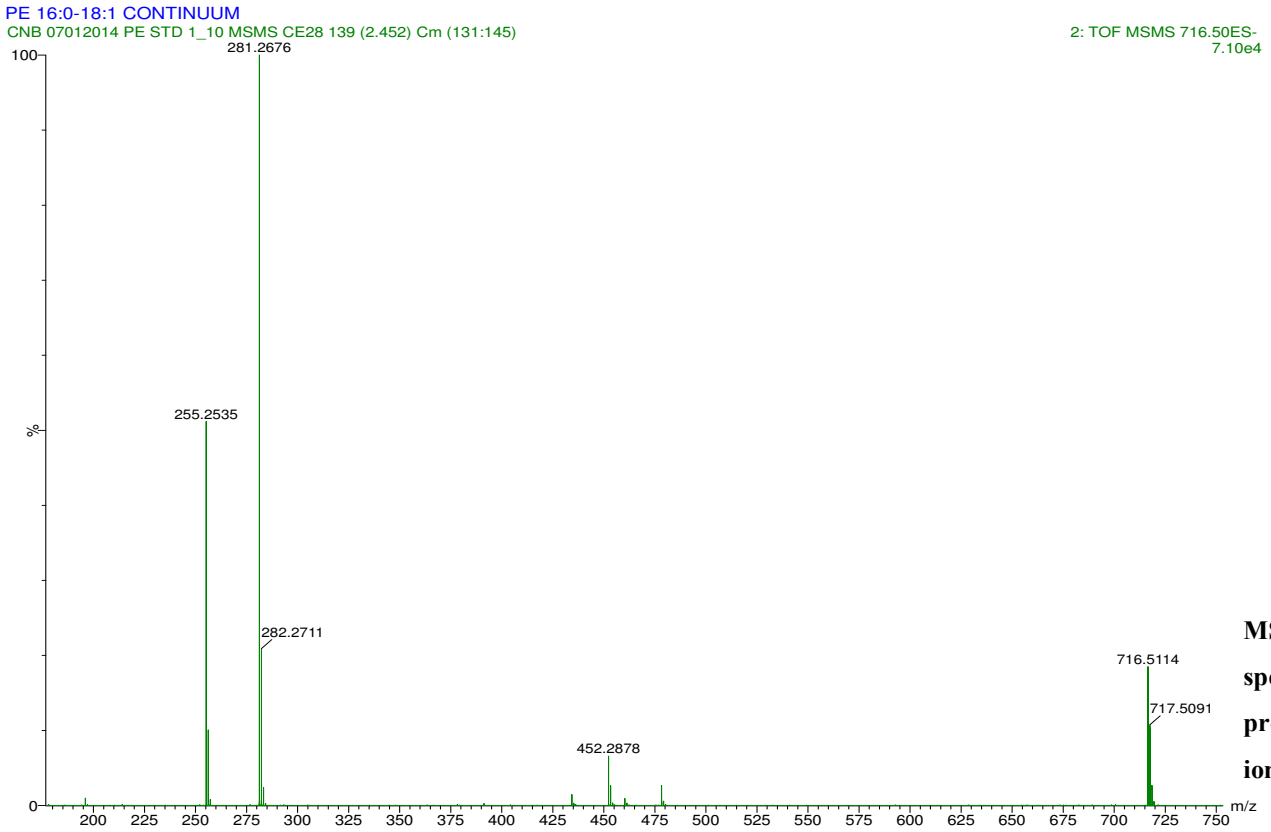
Appx. Figure 23: Chromatogram obtained from analysis of 16:0-18:1Δ^9 PE using on Acquity UPLC BEH C18 column 50 mm column and mobile phase composition 3.

Appendix 15

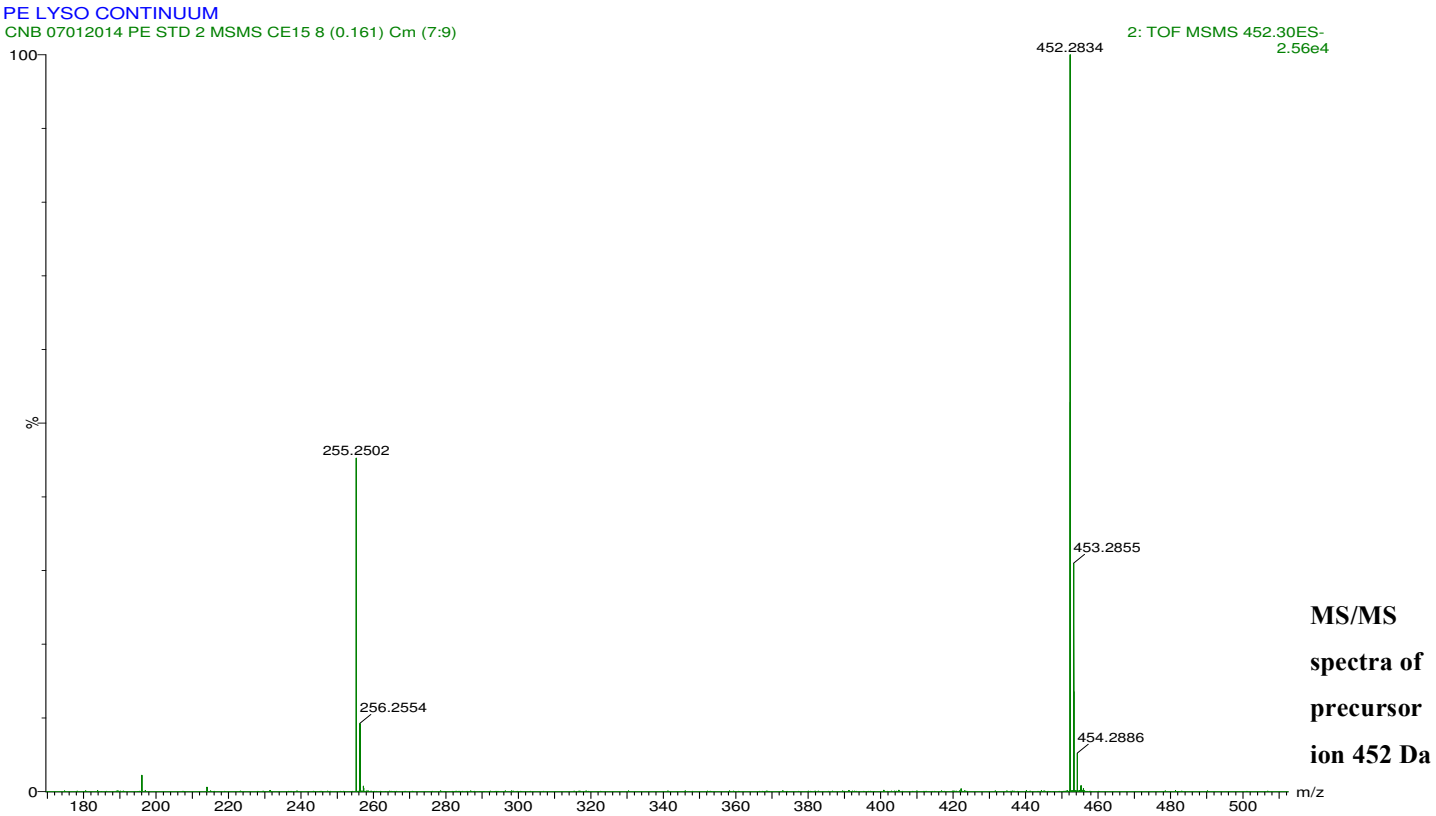


Appx. Figure 24: MS^{E} full scan spectra recorded for 16:0-18:1Δ^{9} PE upon CID. Analysed on Acquity UPLC BEH C18 column 50 mm using mobile phase gradient 3

Appendix 16

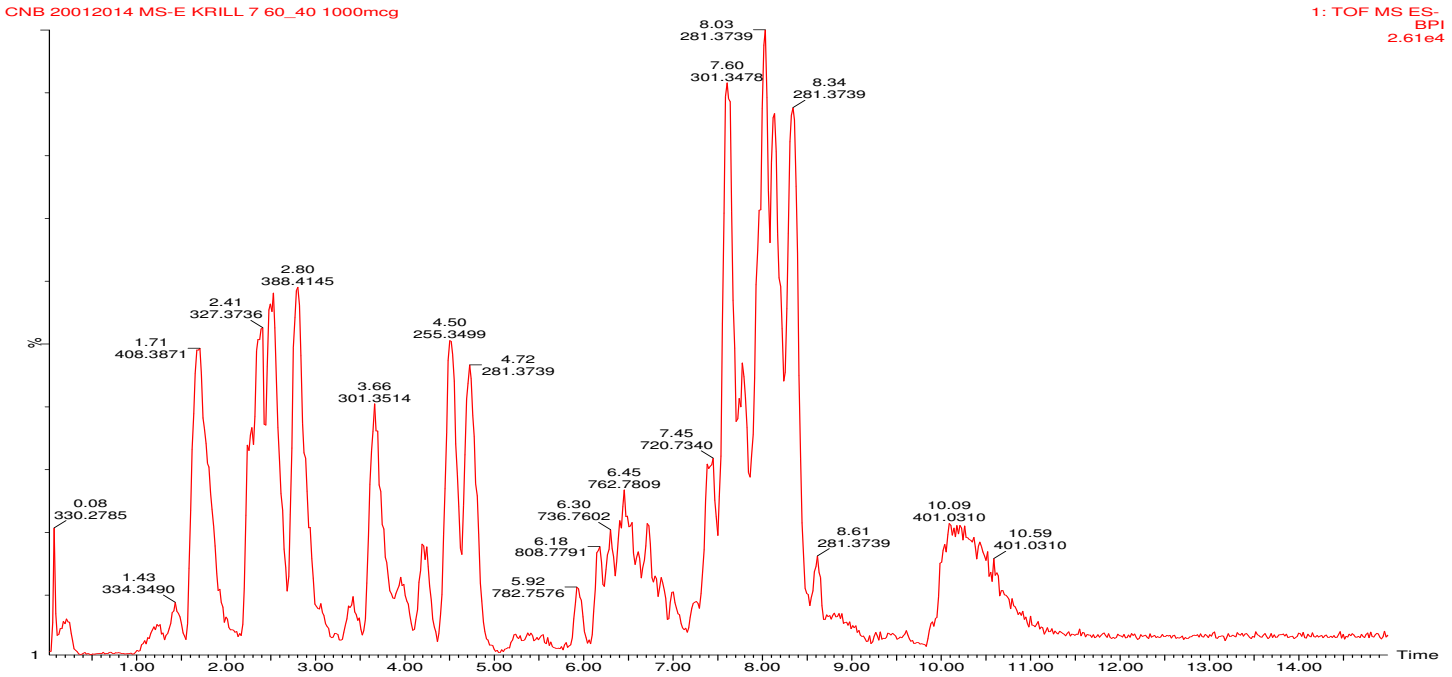


Appx. Figure 25: MS/MS spectra of 16:0-18:1Δ^9 PE, CE 28V.



Appx. Figure 26: MS/MS spectra of 16:0-0:0 PE, CE 28V.

Appendix 17



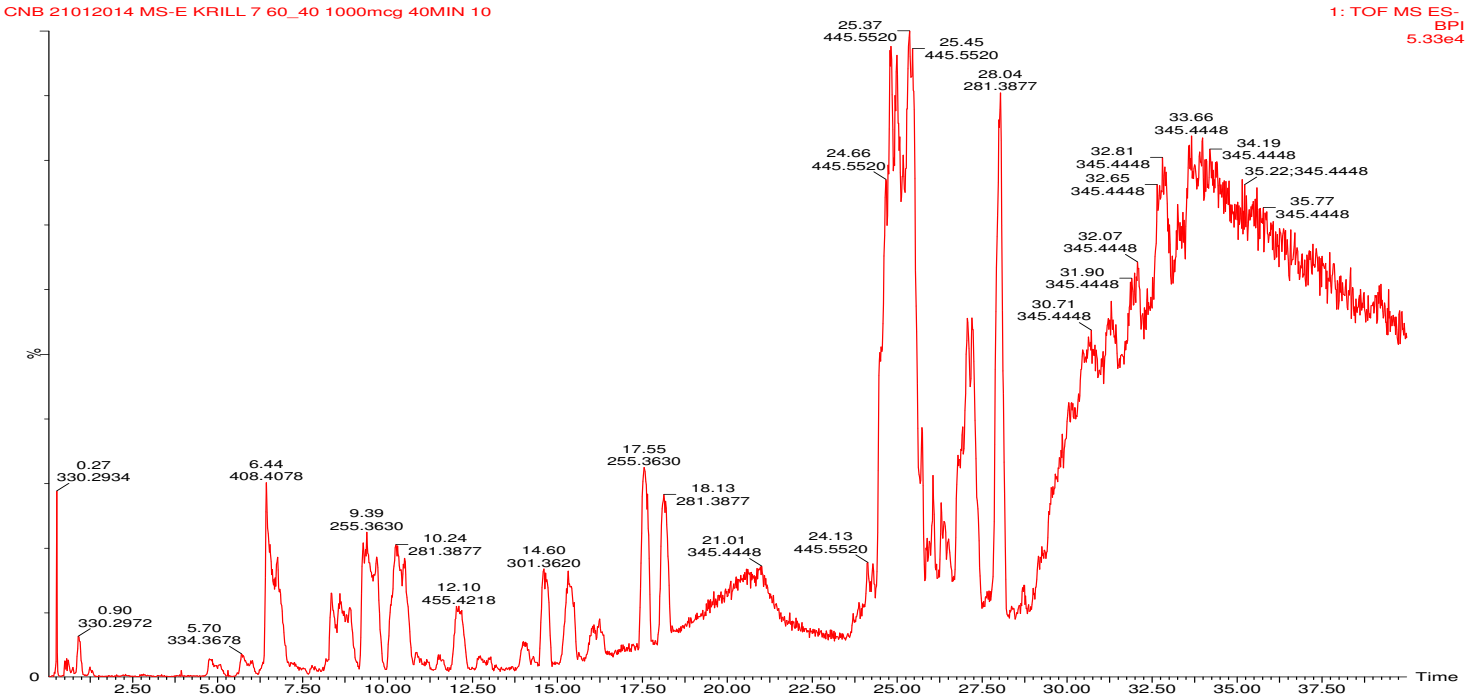
Appx. Figure 27: Life krill oil capsules (1000 µg/ml) analysed on Acquity UPLC® BEH C18 VanGuard 5mm column with mobile phase composition 2

Gradients tried when optimizing the method for analysing krill oil samples on Acquity UPLC BEH C18 VanGuard Pre-column

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	50 %	50 %
10	0.6	0 %	100 %
15	0.6	0 %	100%

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.6	70 %	30 %
10	0.6	0 %	100 %
15	0.6	0 %	100%

Appendix 18



Appx. Figure 28: Life krill oil capsules (1000 µg/ml) analysed on Acquity UPLC BEH C18 column 50 mm with mobile phase composition 2.

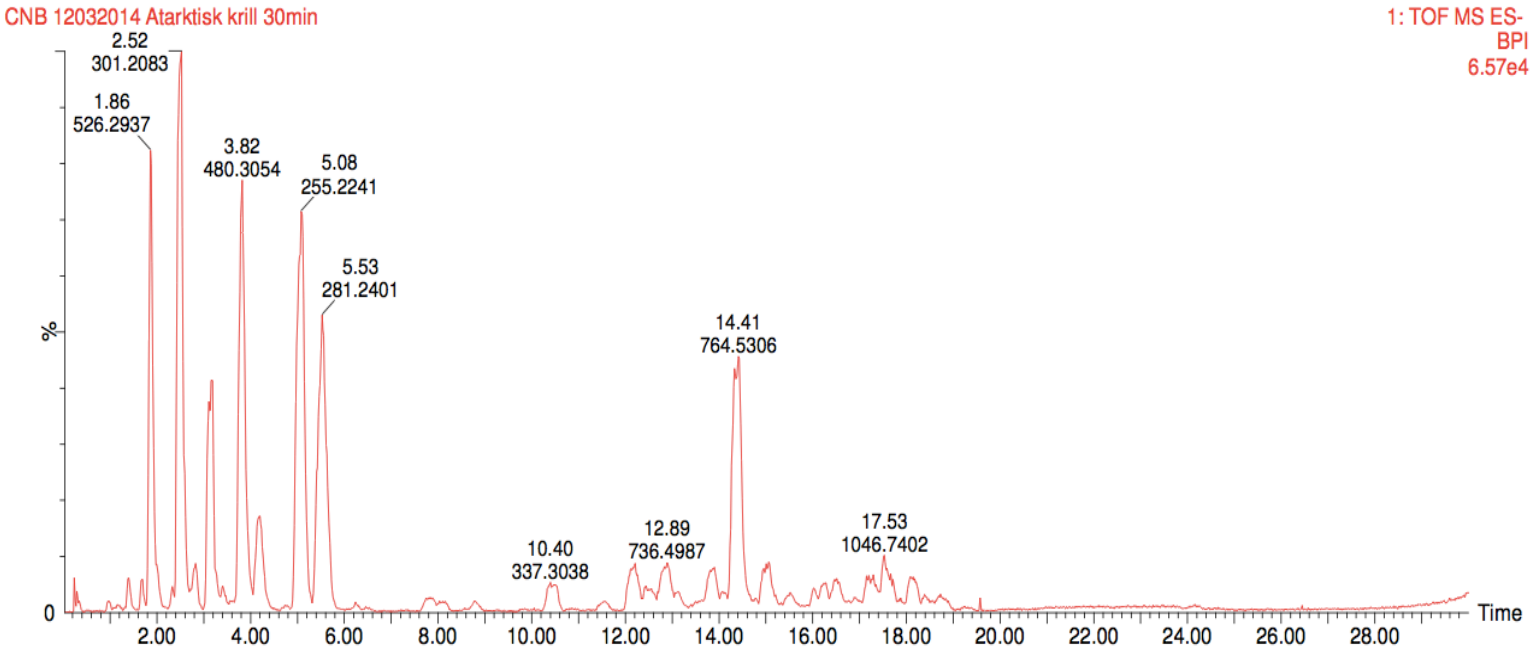
10-minute gradient with a 5 minute wash out period tested on Life Krill oil capsules

Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.5	60 %	40 %
15	0.5	0 %	100 %
30	0.5	0 %	100 %

30-minute gradient with a 10-minute wash out period tested on Life Krill oil capsules

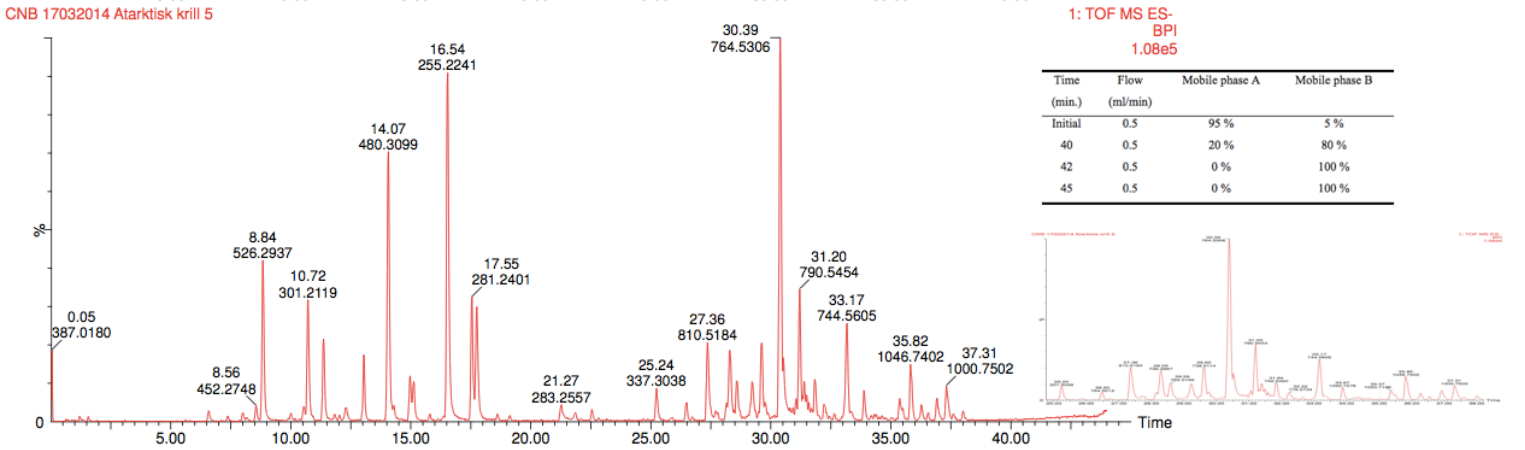
Time (min.)	Flow (ml/min)	Mobile phase A Water with 2% TEA	Mobile phase B Methanol with 2% TEA
Initial	0.5	60 %	40 %
30	0.5	0 %	100 %
40	0.5	0 %	100 %

Appendix 19



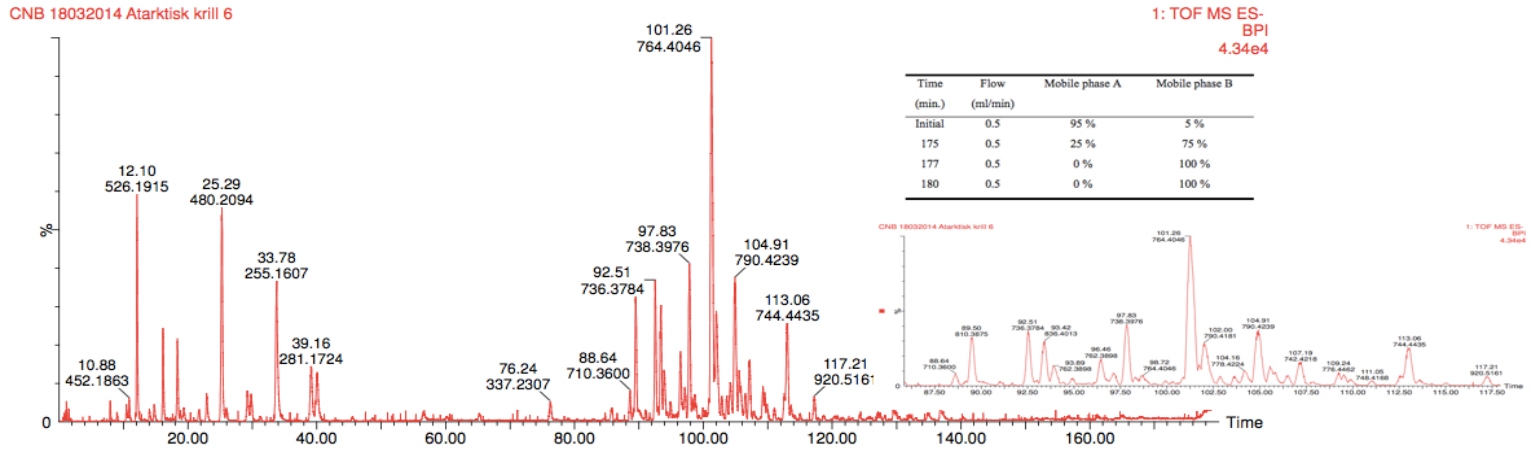
Appx. Figure 29: RIMFROST krill oil capsules (1000 µg/ml) analysed Acquity UPLC BEH C18 column 50 mm and mobile phase composition 3.

Appendix 20



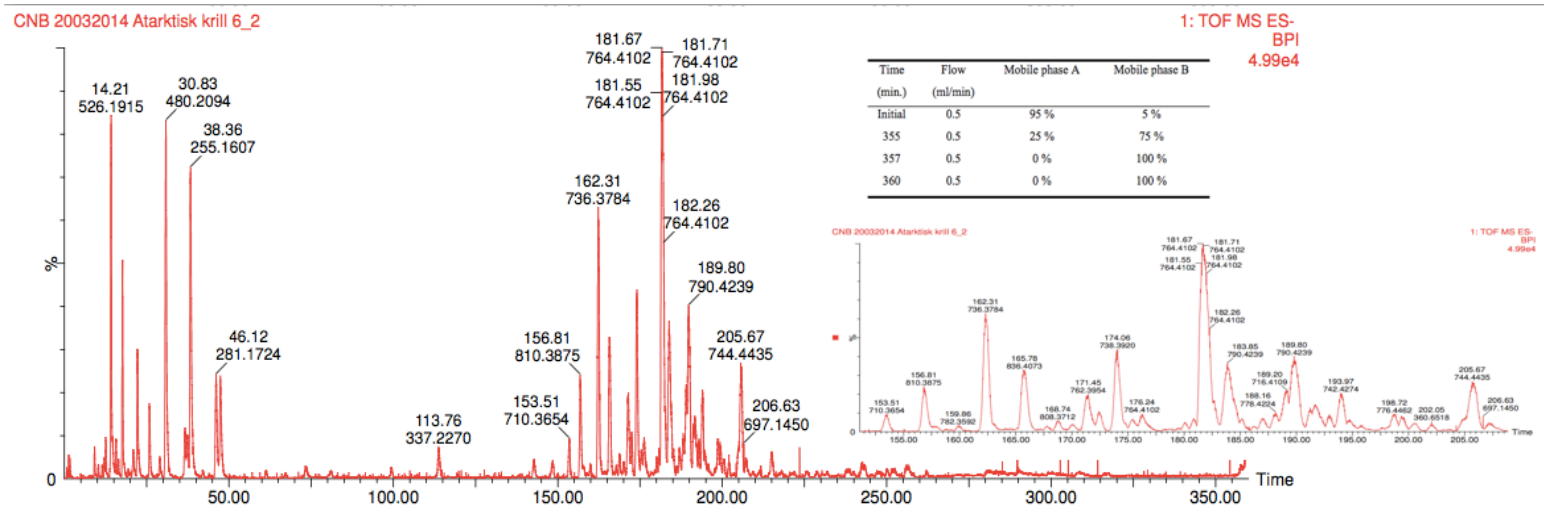
Time (min.)	Flow (ml/min)	Mobile phase A	Mobile phase B
Initial	0.5	95 %	5 %
40	0.5	20 %	80 %
42	0.5	0 %	100 %
45	0.5	0 %	100 %

Appx. Figure 30: Rimfrost Krill oil capsules (1000 µg/ml) analysed with mobile phase composition 3 on Acquity UPLC® BEH C18 column 150 mm using a 42 minute gradient.



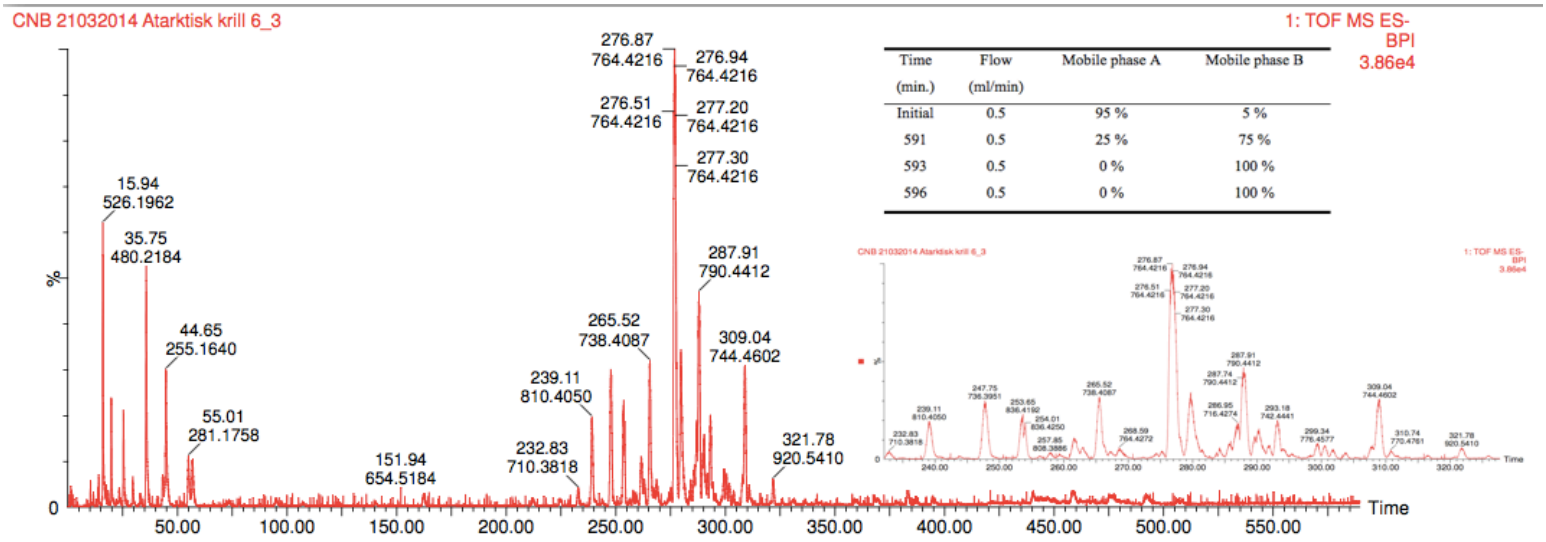
Time (min.)	Flow (ml/min)	Mobile phase A	Mobile phase B
Initial	0.5	95 %	5 %
175	0.5	25 %	75 %
177	0.5	0 %	100 %
180	0.5	0 %	100 %

Appx. Figure 31: Rimfrost Krill oil capsules (1000µg/ml) analysed with mobile phase composition 3 on Acquity UPLC® BEH C18 column 150 mm using a 177 minute gradient.



Time (min.)	Flow (ml/min)	Mobile phase A	Mobile phase B
Initial	0.5	95 %	5 %
355	0.5	25 %	75 %
357	0.5	0 %	100 %
360	0.5	0 %	100 %

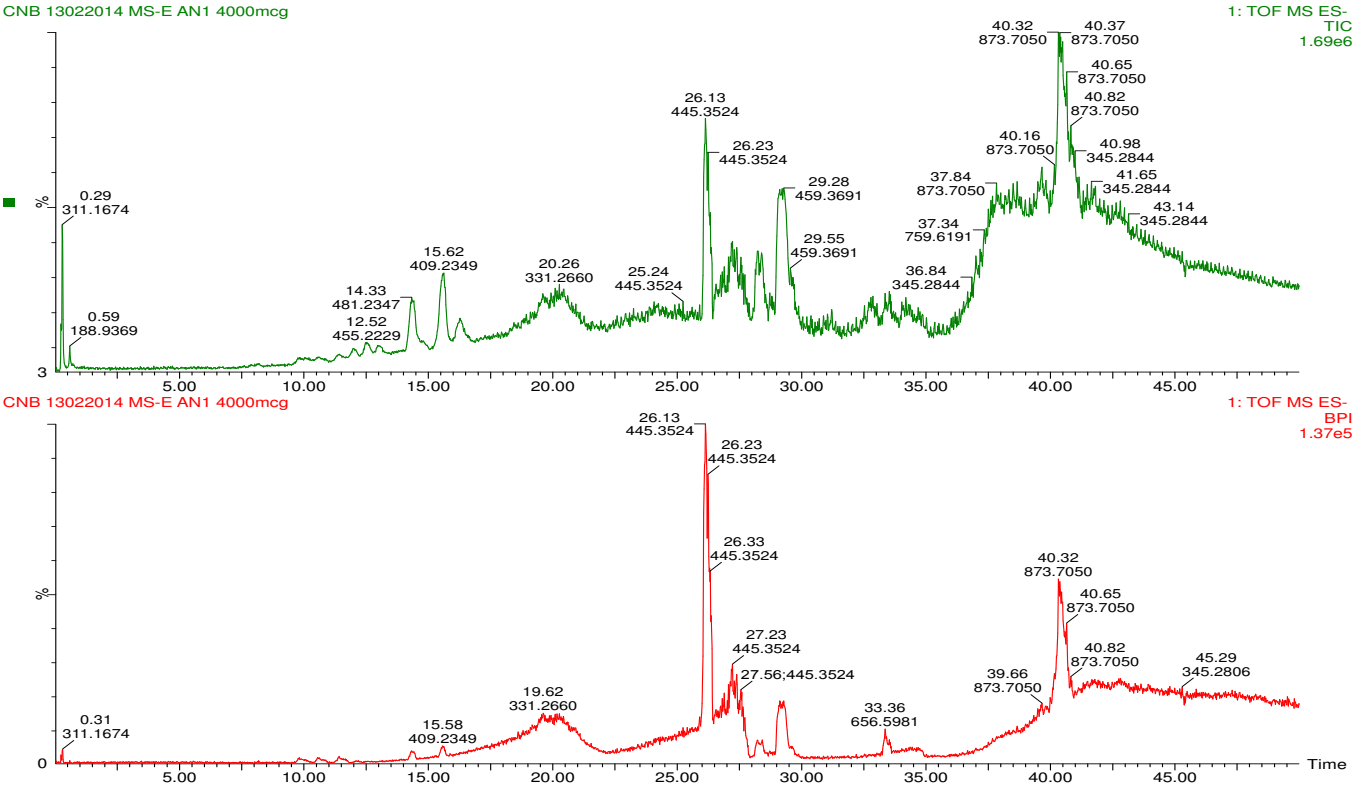
Appx. Figure 32: Rimfrost Krill oil capsules (1000µg/ml) analysed with mobile phase composition 3 on Acquity UPLC® BEH C18 column 150 mm using a 357 minute gradient.



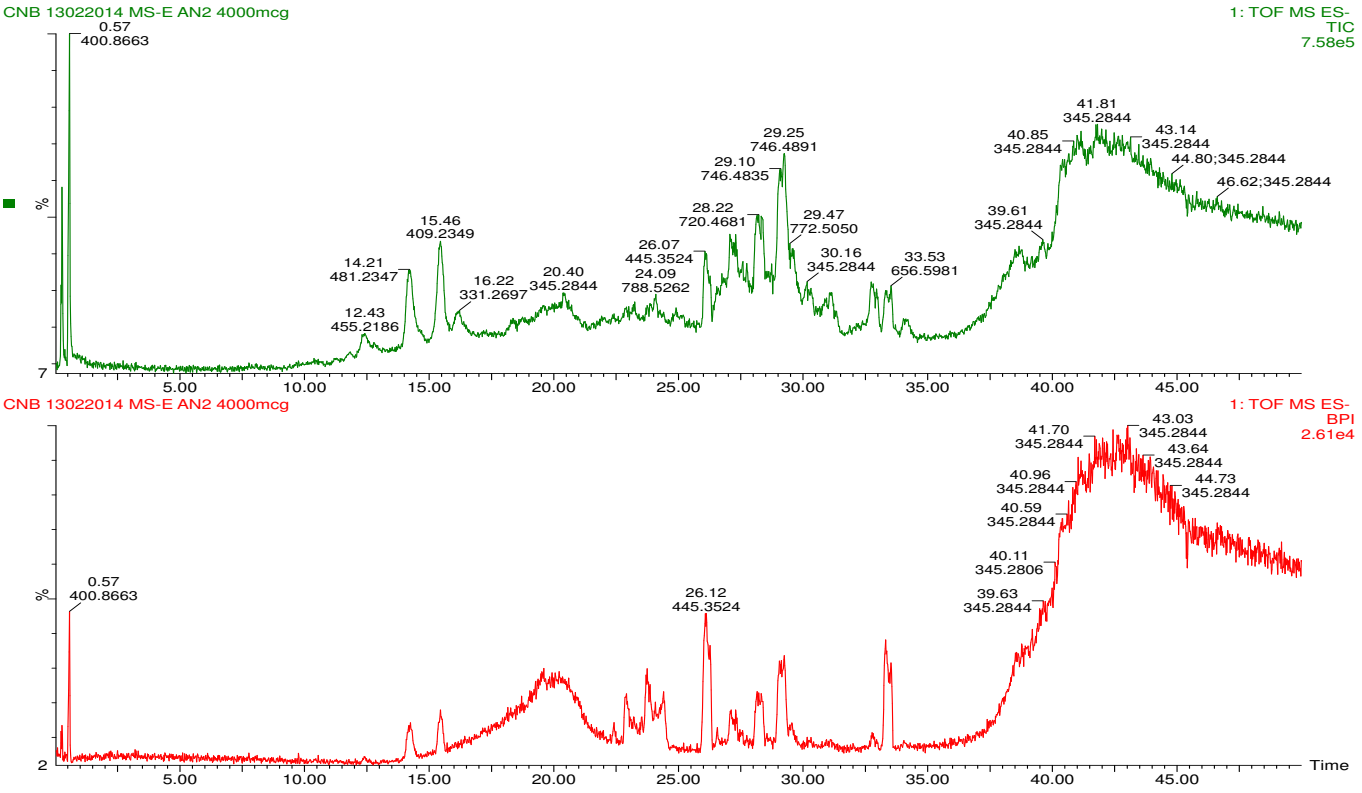
Time (min.)	Flow (ml/min)	Mobile phase A	Mobile phase B
Initial	0.5	95 %	5 %
591	0.5	25 %	75 %
593	0.5	0 %	100 %
596	0.5	0 %	100 %

Appx. Figure 33: Rimfrost Krill oil capsules (1000 µg/ml) analysed with mobile phase composition 3 on Acquity UPLC® BEH C18 column 150 mm using a 593 minute gradient.

Appendix 21

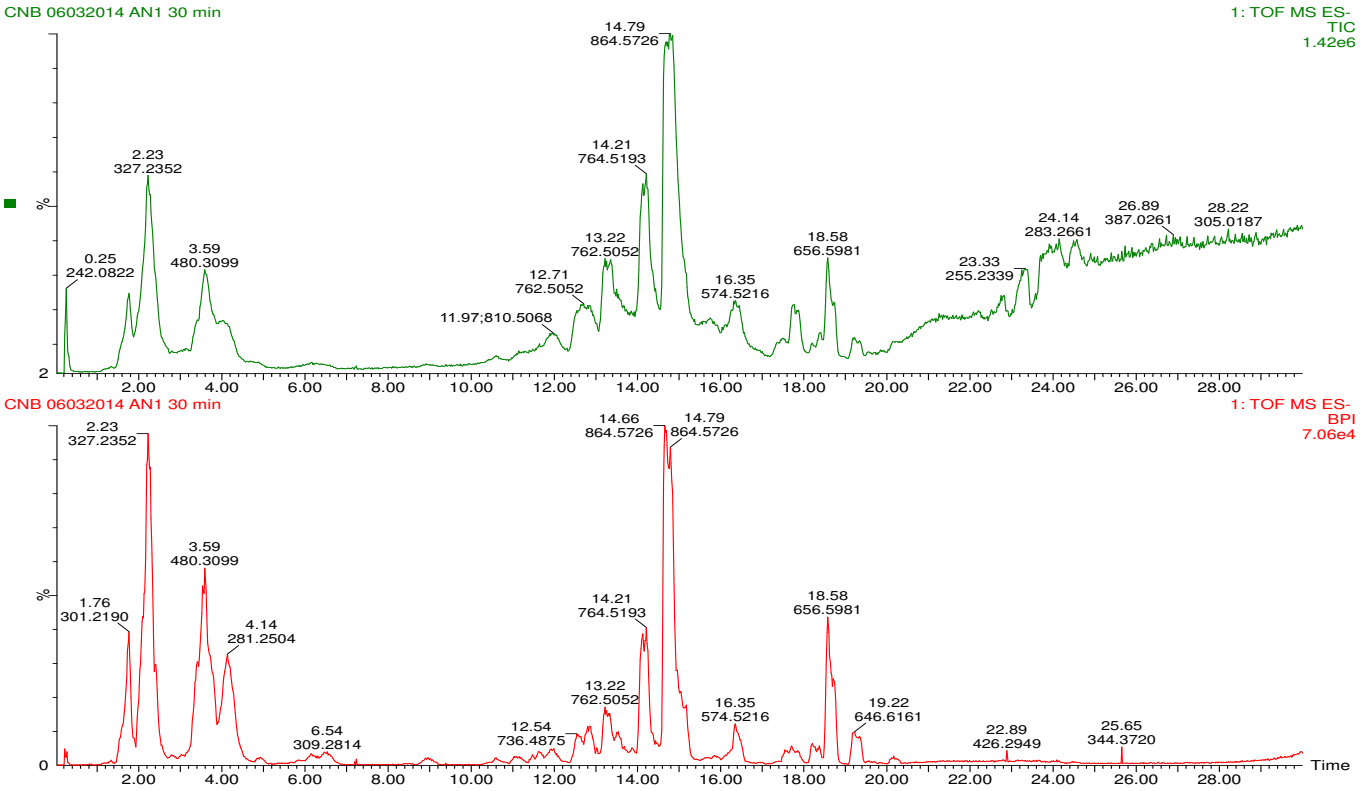


Appx. Figure 34: Full scan MS of AN1 raw extract (4000 µg/ml) were analysed using the Acquity UPLC BEH C18 column 50 mm and mobile phase composition 2

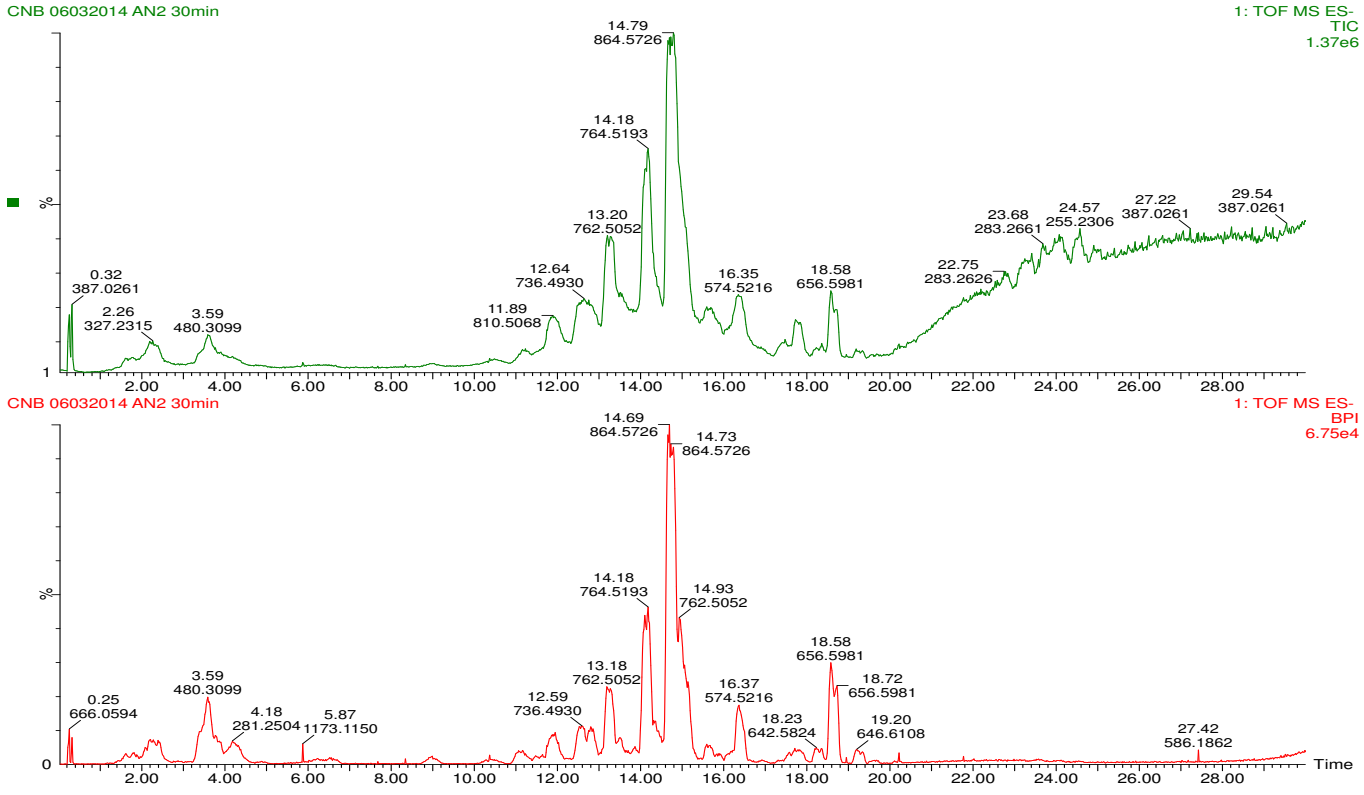


Appx. Figure 35: Full scan MS of AN2 raw extract (4000 µg/ml) were analysed using the Acquity UPLC BEH C18 column 50 mm and mobile phase composition 2

Appendix 22

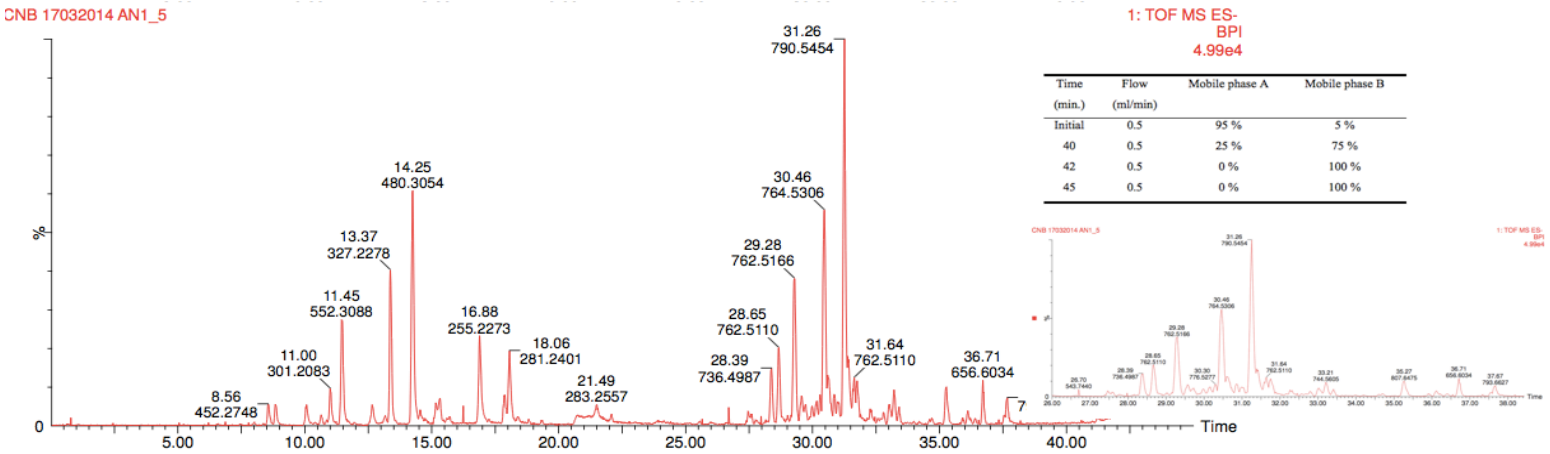


Appx. Figure 36: Full scan MS of AN1 raw extract (4000 µg/ml) were analysed using the Acquity UPLC BEH C18 column 50 mm and mobile phase composition 3.



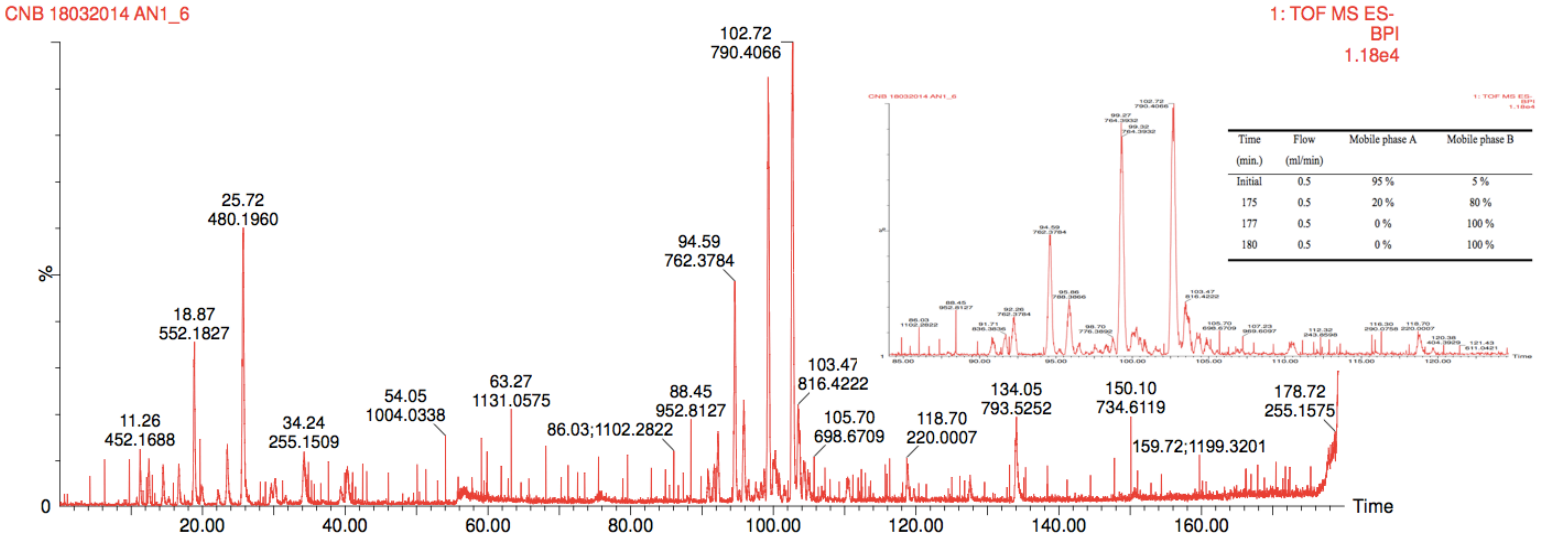
Appx. Figure 37: Full scan MS of AN2 raw extract (4000 µg/ml) were analysed using the Acquity UPLC BEH C18 column 50 mm and mobile phase composition 3.

Appendix 23



Time (min.)	Flow (ml/min)	Mobile phase A	Mobile phase B
Initial	0.5	95 %	5 %
40	0.5	25 %	75 %
42	0.5	0 %	100 %
45	0.5	0 %	100 %

Appx. Figure 38: Full scan MS of AN1 raw extract (4000 µg/ml) analysed with mobile phase composition 3 on Acquity UPLC® BEH C18 column 150 mm using a 42 minute gradient.



Time (min.)	Flow (ml/min)	Mobile phase A	Mobile phase B
Initial	0.5	95 %	5 %
175	0.5	20 %	80 %
177	0.5	0 %	100 %
180	0.5	0 %	100 %

Appx. Figure 39: Full scan MS of AN1 raw extract 4000 µg/ml) analysed with mobile phase composition 3 on Acquity UPLC® BEH C column 150 mm using a 177 minute gradient.