Does high offshore petroleum activity affect the lipid composition in wild Atlantic Cod (*Gadus morhua*) from the North Sea?

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by

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Thesis for the degree of European Master in Quality in Analytical Laboratories

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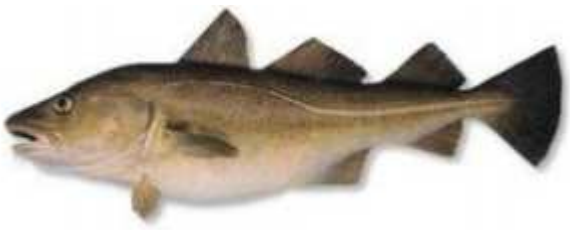

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List of Publication

[1] Mari, Bratgerg; **Li, Liu**; Sonnich, Meier, Pitfalls in the use of polyethylene aminopropyl-coated columns for solid phase extraction (SPE) separation of lipids. *Journal of Chromatography A* (Submitted)

[2] Sonnich, Meier; Eirin, Kalsteveit; **Li, Liu**; Mari, Bratgerg; Svein A. Mjøs, Improved solid phase analysis of phospholipids from extreme fatty fish samples using fractionally extraction with a combination of hexane: methanol and chloroform: methanol. (Manuscript)

List of Abbreviation

ALA	α-linolenic acid
AP	Alkylphenols
ATP	Adenosine triphosphate
DHA	Docosahexaenoic acid
PAH	Polyaromatic hydrocarbons
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FFA	Free fatty acid
GC	Gas chromatography
HPLC	High-performance liquid chromatography
LA	Linoleic acid
LPC	Lysophosphatidylcholine
MUFA	Monounsaturated fatty acid
NL	Neutral lipid
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
SPE	Solid phase extraction
TAG	Triacylglycerol
TLC	Thin-layer chromatography

Abstract

A combined two step extraction, using hexane/methanol to extract neutral lipids and chloroform/methanol to extract polar lipids, has been developed and validation. Four times methanol wash were done to hexane/methanol extract and merged with chloroform/methanol extract to ensure optimized lipid classes distribution between two solvent systems. Quick freeze of hexane/methanol solvent system by dry ice was adopted to avoid adverse effect of generation of micelle. The glass aminopropyl bonded column was used to fractionate the lipid classes into: neutral lipids (NL), free fatty acids (FFA), phosphatidylcholine/phosphatidylethanolamine (PC/PE) and phosphatidylserine/phosphatidylinositol (PS/PI). The results show that 99% of neutral lipids were extracted into hexane while the chloroform/methanol remained 95 % of PC/PE and 88% of PS/PI. According to verification of thin-layer chromatography (TLC) and gas chromatography (GC) results, the solid phase extraction (SPE) separated the lipid classes effectively with good recoveries. The fatty acids profiles were compared with Folch extraction. There were some differences between these two methods. However, the two-step extraction showed very good repeatability and provided higher concentration of phospholipids in SPE eluates, which make it suitable for analysis of livers from wild Atlantic cod fish.

Forty-three of wild Atlantic cod fish (*Gadus morhua*) were analyzed by the two-step extraction we proposed. The lipid distribution and fatty acid composition of different lipid classes were studied. High level of 22:1 *n-11* was detected in neutral lipids as an indicator of diet. Higher amount of 18:1 *n-9* and lower amount of 20:1 *n-9* were found in phospholipids fractionated from samples in high offshore oil activity field. Lower level of *n-3*/*n-6* ratio was also observed in PC/PE fraction. These observations support the hypothesis of altered fatty acid metabolism proposed by Balk *et al.*[2]. However, the evidences were limited by the small sample size and larger biological variance. The Principal Components Analysis (PCA) did not find obvious difference between samples collected in two areas. No effect of offshore petroleum activity on fatty acid composition in liver of Atlantic cod (*Gadus morhua*) can be concluded based on present study.

1. Introduction

1.1 Petroleum Inputs to the Sea

There is no argument that petroleum (crude oil and the products refined from it) plays a pervasive role in modern society. A fluctuation of 20 percent in liquid petroleum's price could influence automotive sales, interest rates, holiday travel decision, stock market trends, and even the gross national product of a country. The fundamental impact access to crude oil can be easily revealed by a quick examination of world history over the last century. Fortunes are made and lost over it; wars have been fought over it. However, widespread use of petroleum is changing the environment as well as the human civilization.

A report from a variety of sources, including industry, government, and academic sources, indicated that the sources of inputs to the sea can be categorized into four major groups: natural seeps, petroleum extraction, petroleum transportation and petroleum consumption.[1] Natural seeps occur when crude oil seeps from the geologic strata beneath the seafloor to the overlying water column. Yet these seeps release are at a rate low enough that the surrounding ecosystem can adapt and even thrive in their presence. Petroleum extraction is sorts of human activities associated with efforts to explore for and produce petroleum, which can result in releases of crude oil and refined products. Petroleum transportation could be either major spill associated with tanker accidents or relatively small operational releases that occur regularly. Petroleum consumption can result in releases as variable as the activities that consume petroleum. Obviously, the petroleum industry is the primary source of contaminants in the sea. Estimate of the annual worldwide release of petroleum into the ocean is up to 1,300,000 metric tons (about 380,000,000 gallons). [1] The corresponding estimate for the North Sea was a total of approximately 8,200 tons according to estimations form Norway, Denmark, the Netherlands and the United Kingdom.[1] In 1992, the discharge of oil-based drilling fluids, also known as muds, to the Norwegian continental shelf was banned. These muds must be reinjected into reservoir or brought to shore for cleaning and storage.[2] Hence, in recently years the petroleum contaminants released into sea have been produced water, which is primarily from fossil water present in the reservoir and seawater injected into the reservoir to maintain pressure. In 2010, 131 million cubic meters of produced water was discharged on the Norwegian shelf.[3] The discharged volume

is reduced since 2001, with the decline mostly due to reduced oil production.[3]

1.2 Components of Produced Water

Produced water is typically discharged to the ocean in offshore petroleum production operations. In an effort to allay the contamination of environment, the components of produced water and their effects of ocean have been thoroughly investigated by a large number of government and industry studies. These studies focus on the following groups: oil, heavy metal and radionuclides.

Oil

Oil is a term applied to organic material that is dispersed or dissolved in produced water at the time of discharge. It can be in form of dispersed oil or hydrocarbon organic material. Dispersed oil is small oil droplets suspended in the water by interfacial tension between the oil and the produced water. It is reported that an average of 46.3 metric tons oil per platform discharged into the North Sea in 1989.[4] The hydrocarbon organic materials can be phenols, carboxylic acid and low-molecular weight aromatic compounds. Large quantities of oil were discharged into North Sea (Table 1-1) in 2009[5]. Over 27,000 tons organic acids were discharged into north see which contributed the largest part of pollutant. This result is consistent with Somerville's reported[6] that the acetic acid in produced water was found up to 700 mg/L. In 2010, the average oil concentration in the discharges of produced water was 11 mg/L when the regulatory requirement stipulates a maximum of 30 mg/L.[3] The concentration of oil in water was under control however the volume was huge and up to 1157 tons[5]. Meanwhile, organic compounds, such as benzene/toluene/ethylbenzene/xylenes (BTEX), alkylphenols (AP) and polycyclic aromatic hydrocarbon (PAH), were also response for main pollution of ocean.

Metal

Produced water may contain several metals in solution. Since produced water is thought to be a concentrate of ancient seawater or fresh water, it is not surprise that the metals present in seawater also exist in produced water, such as copper, lead, zinc. However, a few metals may be present much higher concentrations than that in clean seawater. For instance, the produced waters in North Sea contain high concentrations of nickel, possibly derived from biodegradation of nickel porphyrins.[6] Besides, zinc is another metal need to be concerned as the annual discharge of 2009 is much larger than others.

Table 1-1 Annual discharges of organic compounds and heavy metal in produced water from the Norwegian sector of the North Sea[5]. Unit is ton.

Compounds	Annual discharges 2009
Oil in water	1157
BTEX	1903
Alkylphenols (C_1-C_9)	323
PAH	102
Organic acids	27204
Cupper (Cu)	0.1
Lead(Pb)	0.3
Mercury(Hg)	0.009
Nickel (Ni)	0.1
Zinc(Zn)	7.1

Radionuclide

Radionuclides found in oilfield production are often referred to as naturally occurring radioactive material (NORM). The most abundant usually are radium-226 and radium-228 (^{226}Ra and ^{228}Ra).[6] Produced water from 153 oil and gas wells in Texas, U.S.A., contains 0.1 picocurie/L (pCi/L) to 5,150 pCi/L ^{226}Ra, and possibly a similar activity of ^{228}Ra.[7] Several other radionuclides have been identified in the NORM of produced water, including ^{89}Sr, ^{212}Bi, ^{228}Ac, ^{210}Pb. However, activities of these radionuclides are much lower than those of radium.

1.3 Biological Effects of Petroleum Releases

Petroleum input from anthropogenic sources, whether from spills or chronic release, is perceived as a major environmental problem. Major oil spills occur occasionally and receive considerable public attention because of the obvious attendant environmental damage, including oil coated shorelines and dead or moribund wildlife, especially oiled seabirds and marine mammals. Meanwhile small amounts of oil released over long periods also create chronic exposure of organisms in the sea. Sources of chronic oil release can be natural seeps, leaking pipelines, offshore production discharges, and non-point runoff from land based facilities.

Oil can kill marine organism, reduce their fitness through sublethal effects, and disrupt the structure and function of marine communities and ecosystems. Multiple temporal and spatial variables make deciphering the effects extremely difficult, especially when considering the time and space scales at which marine populations and ecosystems change. The quantification of both

effects and recovery are difficult, particularly when they must be measured against a changing marine environment. Determining its significance is more difficult than detecting an effect (Figure 1-1). Besides, assessing recovery after a pollution event is perhaps even more challenging than assessing initial damage. However, some conclusions have been obtained recent years.

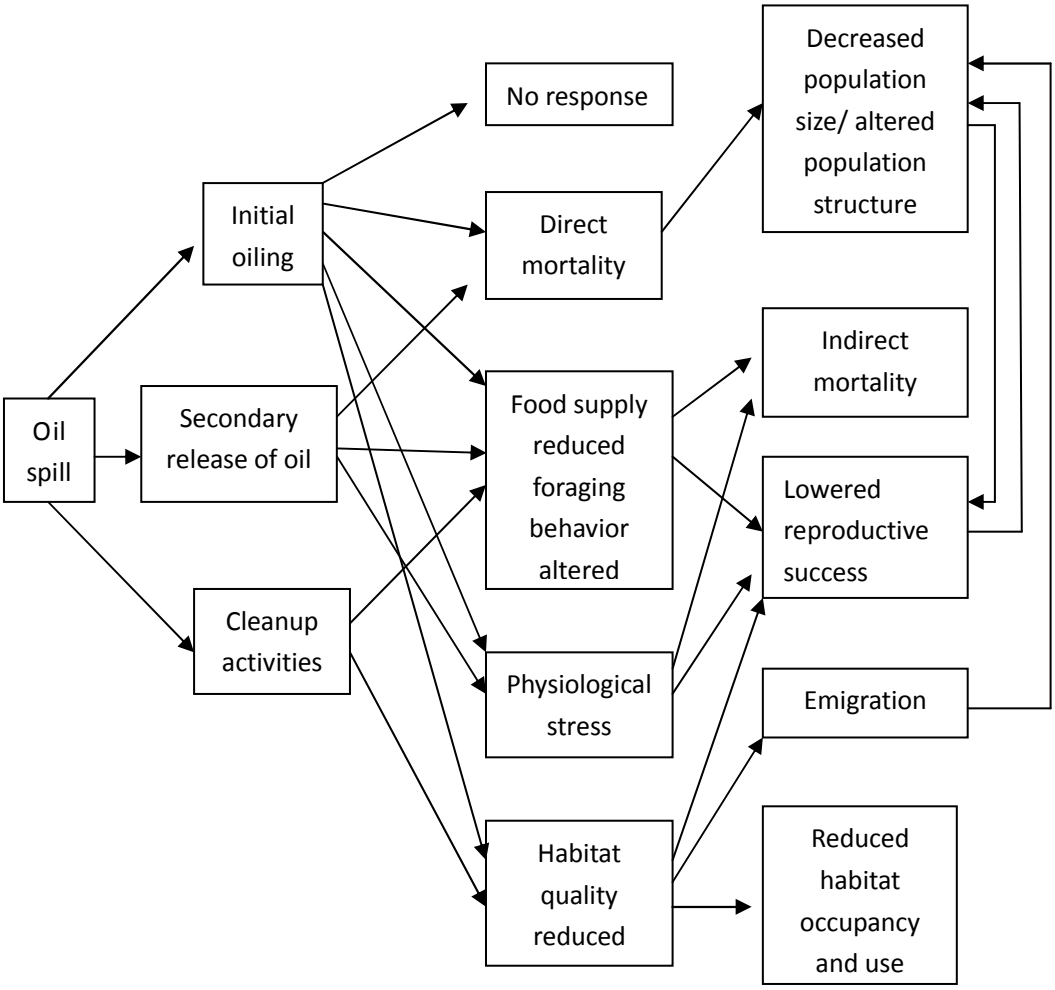

Figure 1-1 Schematic representation of oil spill influences on seabirds. (from Wiens, 1995, American Society for Testing and Materials).

Through the past three decades, contaminant level and its potential effect to the coastal ecosystems of North Sea have been monitored by all surrounding counties. The fish stocks declined over the past decades. The main reason for declining fish stocks is nearly certainly overfishing, but other environmental pressures also affect fish populations, such as exposure to metals and organic pollutions including PAHs, APs and organochlorine compounds. Produced water is one of the main sources for these pollutants. A studies conducted by an international workshop (*BECPELAG*) demonstrated that components in offshore effluents may affect fish

reproduction and that tissues of fish near oil rigs are structurally different to tissues of fish from reference areas.[8] Meier *et al.*[9] claimed APs in produced water could alter the fatty acid in the liver and brain of Atlantic cod. Their research group also found that juvenile cod (from 3 to 6 months of age) appeared able to effectively metabolize short chain APs after exposed to produced water. 1% produced water clearly interfered with the development of normal larval pigmentation and lead to inability of feed. The reason for the inability may be linked to the increased incidence of jaw deformities.[10] Balk *et al.*[2] reported that there is a general relationship between the intensity of oil production and the biomarker responses, such as induction of biotransformation enzymes, oxidative stress, altered fatty acid composition and genotoxicity, in haddock and Atlantic cod in North Sea. Our research is focusing on the fatty acid composition. Instead of measuring chosen fatty acids (8 acids were measured by Balk *et al.*[2]), a full scan of fatty acid compositions will be done to provide more evidence about effects of offshore petroleum activity on lipid composition in Atlantic cod's liver. Moreover, the lipids in liver will be analyzed by classes (storage lipids and membrane lipids) instead of investigating the total lipids. The present work is included in a comprehensive investigation; the brain, muscle and liver of wild Atlantic cod and haddock will be researched to discuss the potential effects of petroleum activity on ecology environment at North Sea.

1.4 Lipid Generalities

There is no definition of lipid that has been accepted by any international body that recommends standards or comment on nomenclature issues. There are some useful online resources for an overview of these molecules and their structures, such as LIPID MAPS (LIPID Metabolites and Pathway Strategy; http://www.lipidmaps.org), Lipid Library (http://lipidlibrary.co.uk), and LIPIDAT (http://www.lipidat.chemistry.ohiostate.edu). One specific definition has been proposed by Christie[11] as "fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds". A comprehensive classification was defined lipids as hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters (fatty acids, polyketides, etc.) and or by carbocation-based condensations of isoprene units (prenols, sterols, etc.).[12] In this thesis, we discuss the lipids based on the function. The lipids are classified as storage and membrane lipids.

1.4.1 Storage lipids

Storage lipids are stored in tissues and form an alternate source of energy to glucose during emergency situations. The typical storage lipids are triacylglycerol (TAG) and waxes.

Triacylglycerol (TAG)

TAG is an ester derived from glycerol and the three fatty acids. It is resulted when all the three hydroxyl groups of the glycerol molecule are esterified by one each of fatty acid molecules (Figure 1-2). TAG is the most common type of storage lipid in plant and animal organisms. It is non-polar and hydrophobic molecules, essentially insoluble in water. There are many TAG, depending on the oil source, some are highly unsaturated, some less so. Unsaturated fatty acids (typically extracted from plants) have a lower melting point and are more likely to be liquid at body temperature while those from animals have saturated fatty acid and are usually semisolids at room temperature. Intake of unsaturated fatty acid could reduce the incidence of cardiac diseases, when they are made up of fat cells storing neutral fat and inter-cellular substance, together constituting the adipose tissue in human body.[13] Compared to other animal oil, the fish oil has high level of omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are high unsaturated fatty acid.

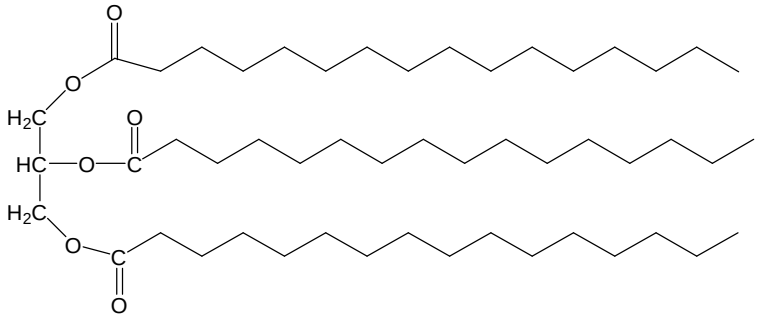

Figure 1-2 The structure of tripalmitin (example ofTAG.)

Waxes

Biological waxes are long chain (14-36 carbon atoms) saturated or unsaturated fatty acid with long chain (16-30 carbon atoms) alcohols (Figure 1-3).

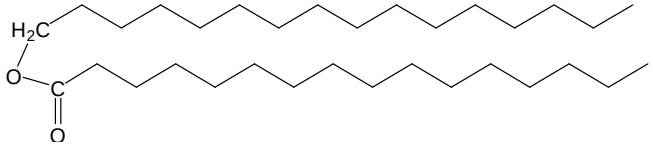

Figure 1-3 The structure of Hexadecyl palmitate (example of wax)

Wax esters generally occur on the surfaces of organisms to protect against water loss, but in aquatic animals they are used as metabolic energy reserves.[14,15] Wax esters are the major neutral lipids of deep-water zooplankton and fish, and of calanoida copepods occurring at all depths.[15]

1.4.2 Membrane lipids

The four major classes of membrane lipids are phospholipids, sphingolipids, glycolipids and cholesterol.

Phospholipids

Phospholipids are a class of lipids that are a major component of all cell membranes as they can form lipid bilayers. Most phospholipids contain a diglyceride, a phosphate group, and a simple organic molecule such as choline (Figure 1-4b). The head group is hydrophilic while the long fatty acid hydrocarbon chains are repelled by water. This special amphipathic character allows phospholipids to play an important role in the phospholipid bilayer. Lipid bilayers occur when hydrophobic tails line up against one another, forming a membrane hydrophilic heads on both sides facing the water.

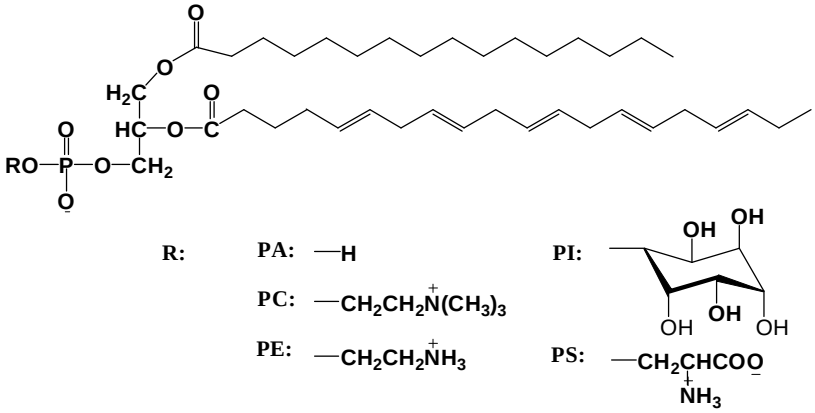

Figure 1-4 The structure of phospholipids. PA: Phosphatidic acid, PC: Phosphatidylcholine PE: Phosphatidylethanolamine, PI: Phosphatidylinositol, PS: Phosphatidylserine

Sphingolipids

Sphingolipids are a class of lipids containing a backbone of sphingoid bases, a set of aliphatic amino alcohols that includes sphingosine (Figure 1-5). These compounds play important roles in signal transmission and cell recognition.

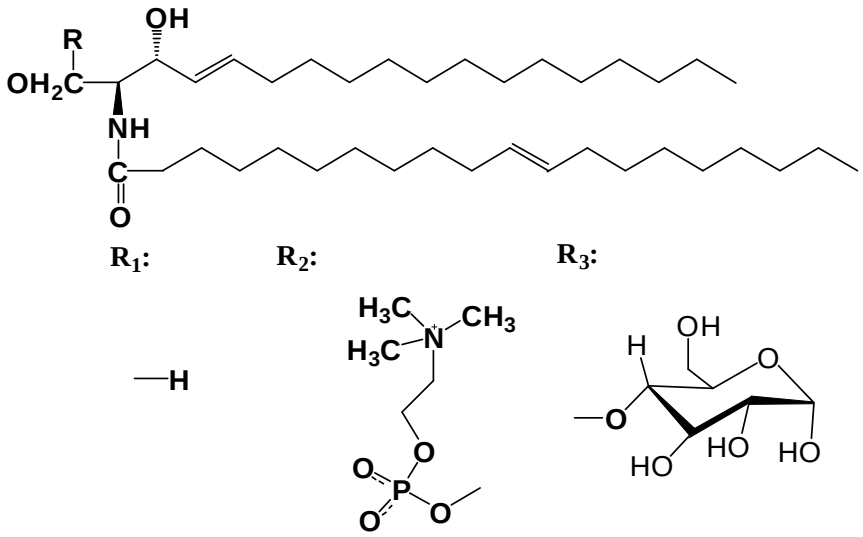

Figure 1-5 The structure of sphingolipids. R_1: ceramide; R_2: sphingomyelin; R3: glycosphingolipids.

Glycolipids

Glycolipids are lipids with a carbohydrate attached (Figure 1-6). They provide energy and also serve as markers for cellular recognition. Glycolipids metabolites molecules are involved in diverse cellular processes.[16]

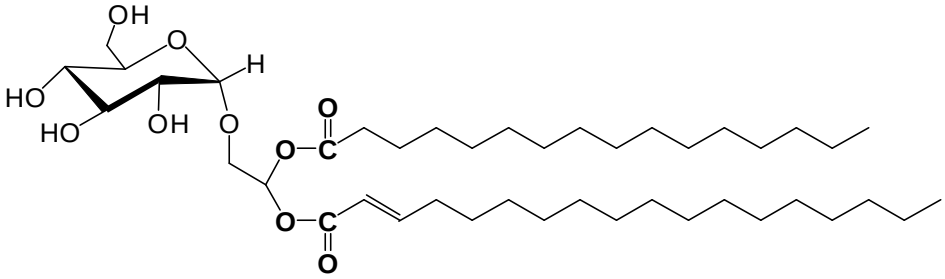

Figure 1-6 The structure of glycolipid

Cholesterol

Cholesterol is an organic chemical substance classified as a waxy steroid of fat (Figure 1-7). It is an essential structural component of mammalian cell membranes and is required to establish proper membrane permeability and fluidity. Cell membranes require high level (typically an average of 20%) cholesterol molecular in the whole membrane, increasing locally in raft areas up to 50% cholesterol.[17] Within the cell membrane, cholesterol also functions in intracellular transport, cell signaling and nerve conduction. In the liver, it is the precursor molecule in several biochemical pathways.[18]

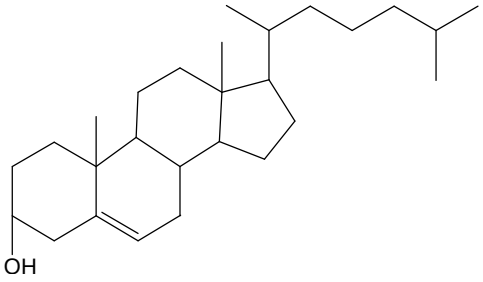

Figure 1-7 The structure of cholesterol

1.5 Fatty Acid

Fatty acid is a carboxylic acid with a long aliphatic tail (chain), which is either saturated or unsaturated. Most naturally occurring fatty acids have a chain of an even number of carbon atoms, from 4 to 28. They are usually derived from TAG or phospholipids. When they are not attached to other molecules, they are called as “free” fatty acids.

Saturated fatty acid (SFA)

Saturated fatty acids have no double bonds between the individual carbon atoms of the fatty acid chain. There are many kinds of naturally occurring SAF, which differ mainly in number of carbon atoms from 3 to 36. The dominating SFA in marine fish is palmitic acid (16:0) and stearic acid (18:0).[19]

Monounsaturated Fatty Acid (MUFA)

Monounsaturated fatty acids are fatty acids that have one double bond in the fatty acid chain and all of the remainder of the carbon atoms in the chain is single bonded. MUFAs are liquid at room temperature and semisolid or solid when refrigerated. The most abundant MUFA in tissue is *cis*-9-octadecenoic acid (18:1 *n-9*).

Polyunsaturated Fatty Acid (PUFA)

Polyunsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bond in their back bone. It includes many important compounds, such as essential fatty acids, which is fatty acid that humans and other animals must ingest because the body requires them for good health but cannot synthesized them.[20] Mammals lack the ability to introduce double bonds in fatty acids beyond 9 and 10, hence the *cis*-*cis*-9,12-Octadecatrienoic acid (18:2 *n-6*), also termed “linoleic acid (LA)”, and all *cis*-9,12,15-Octadecatrienoic acid (18:3 *n-3*), also termed “α-linolenic acid (ALA)” are essential for humans in the diet. In addition, the human body can make some long chain omega-3 PUFAs (EPA and DHA) from the tow essential fatty acids

aforementioned. Good dietary source of LA and ALA are seeds and vegetable oil, such as flax seeds, flaxseed oil, Canola oil, soybeans. EPA and DHA can be obtained from marine fish, such as salmon, mackerel, halibut, sardines and herrings etc.[21] Although the conversion of ALA to EPA and subsequently DHA occurs in human body, this requires more metabolic work, which is thought to be the reason that the absorption of PUFAs is much greater from animal rather than plant sources.

Nomenclature

Several different systems of nomenclature are used for fatty acids.[22] A more meaningful systematic nomenclature defined by standard International Union of Pure and Applied Chemistry (IUPAC) terminology are encouraged by naming fatty acid after its parent hydrocarbon (Table 1-2).[23]

Table 1-2 Terms and symbols designating major fatty acids

Trivial name	Chain length	Double bonds	symbol	Systematic name
Myristic	14	0	14:0	n-Tetradecanoi acid
Palmitic	16	0	16:0	n-Hexadecanoic acid
Palmitoleic	16	1	16:1 *n-7*	*cis*-9-Hexadecanoic acid
Stearic	18	0	18:0	n-Octadecanoic acid
Oleic	18	1	18:1 *n-9*	*cis*-9-Octadecanoic acid
Linoleic	18	2	18:2 *n-6*	*cis-cis*-9,12-Octadecatrienoic acid
α-linolenic	18	3	18:3 *n-3*	All *cis*-9,12, 15- Octadecatrienoic acid
β-linolenic	18	3	18:3 *n-6*	All *cis*-6, 9, 12- Octadecatrienoic acid
Gadoleic	20	1	20:1 *n-9*	*cis*-9-Eicosenoic acid
Arachidonic	20	4	20:4 *n-6*	All *cis*-5,8,11,14-Eicosenoic acid
EPA	20	5	20:5 n-3	cis-5,8,11,14,17-Eicosateraenoic acid
Cetoleic	22	1	22:1 *n-11*	*cis*-11-Docosaenoic acid
DHA	22	6	22:6 *n-3*	*cis*-4,7,10,13,16,19-Docosahexaenoic acid

In this thesis, the symbols are used. Unbranched fatty acids are described by the number of carbons followed by the number of double bonds. Thus, the saturated fatty acid (SFA), palmitic acid (Table 1-2) for instance, can be denoted as 16:0. Double bond positions may be described from either end of molecule. Double bond positions given from the methyl end of the carbon chain are commonly referred to by "*n*-". Monounsaturated fatty acid (MUFA), gadoleic acid (Table 1-2) for instance, may be denoted as 20:1 *n-9*. Double bonds in polyunsaturated fatty acid (PUFA) are typically separated by a single methylene unit. In this case, the complete molecular

structure can be described by specifying the number of carbons, the number of double bonds and the position of the double bone system. For instance, EPA (Table 1-2) can be donated as 20:5 *n-3*. If the double bond systems that do not have the regular methylene interrupted patters, the distance of all double bonds from the carbonyl group should be specified. Besides, the carbon chain may contain triple bonds, branches, as well as saturated and unsaturated carbon rings.[24,25] These complex fatty acids are out of our discussion and not presented in Table 1-2.

1.6 Lipids in Cod Liver

In cod the fat is mainly stored in the liver as TAG as it is reported by Meier *et al.*[8]. The lipid content in cod liver is as high as 70% relative to wet weight. The distribution of fatty acids in different lipid class for neutral lipid (NL, typically TAG), free fatty acid (FFA) and polar lipids (PL, typically phospholipids) are around 95%, 4% and 1%, respectively. The phospholipids account for a small percentage of lipids, but it is the building blocks for all biological cells membranes. The portion of phospholipids is relatively stable while the storage lipids are dependent on the energy condition of fish. The fatty acid composition may affect mobilization[26] and gonad maturation[27]. They are likely to be related to microsomal membrane, electron transport system[28], buoyancy mechanism[29] and fluidity of membranes[30].

There are several ways to affect the lipid compositions in cod liver, such as diet[31], water temperature[32] and water component[29,33]. The dietary is most likely to affect the fatty acid composition of neutral lipids.[31] Morais *et al.* studies the effect of protein/lipid ratios in extruded diet on liver and muscle composition. An interesting observation is that liver compositions (dominated by TAG) were more affected by diet, with muscle (containing more phospholipids) presenting a much more homogenous FA profile.[31] This research supported that phospholipids are more independent from dietary. However the phospholipids could be affected by other factors. A laboratory study show that AP released in produced water alters fatty acid profile in the polar lipid from the liver to contain more SFA and less PUFAs.[9] Similarly, Dey *et al.* reported less PUFAs were observed after exposure to petroleum in phospholipids. Besides, the amount of FFA increased, which indicates that rapid mobilization occurred due to oil exposure.[33] Hereby, our group is going to investigate whether the high intensive offshore oil activities affect the fatty composition in Atlantic cod liver. Both neutral lipids and phospholipids will be studies.

1.7 Lipid Analysis

Lipid research has recently gained prominence with the emergence of lipidomic, although it has been an intensive area of research already in the 1950s. There are several reviews[34-36] focus on lipidomic research and compared the last methods employed for lipid analysis recently. The chromatographic techniques[37], mass spectrometry[38], nuclear magnetic resonance[39] and biochemistry techniques[40] are all adapted to analyze lipids in complex matrices with high sensitivity. Thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC) are main techniques used in lipid research. The conditions have been well adapted for the analysis of various classes of lipid compounds. GC is a routine method for fatty-acid analysis after derivatization of target samples. The fatty acid composition of lipid samples is determined by assessing the corresponding fatty acid methyl esters (FAME) via GC. For GC analysis, the extraction and purification approaches, the initial lipid extraction by solvent and followed by their transmethylation are critical for the chromatographic separation. However, in case where the total fatty acid composition of the sample is sought, the fatty acids can be extracted and methylated with one-step procedures wherein methylation reagent is added directly to the samples without previous extraction.

Extraction Approaches

Folch method[41] and Bligh and Dyer method[42] based on chloroform/methanol solvent systems have been invariably used as standard methods. However, other solvents such as dichloromethane/methanol[43], diethyl ether[44] and hexane/isopropanol[45] have also been employed, but there are no established criteria for choosing the most appropriate one. The accuracy of different lipid extraction methods depends on the solubility of their constituent lipid classes in the solvents employed and the nature of sample matrix as both could influence the extent of lipid extraction. There are many literatures compared the extraction methods for different nature samples.[46-48] The results show that Folch method has better efficiency and yield to extract lipid from most nature samples when one want to analyze polar lipids.

Atlantic cod liver contains over 98% neutral lipids and 1% phospholipids. Majority of published methods frequently used in fractionating lipid mixtures offer limited possibilities for quantitative isolation of phospholipids occurring in neutral-lipids-rich animal tissues. To separate small

amount (43 mg) of phospholipids from a cream lipid fraction containing 20 g of TAG, Frankel *et al.*[49] used 4,650 ml of organic solvents to elute a 100 g silicic acid column. Further, an improved procedure was developed to isolate phospholipids from lipids mixture with ethanol, ether, and chloroform, which was extracted from buttermilk by using methanol/chloroform,.[50] A simpler extraction solvent system was introduced by Galanos *et al.*[51] later. They used petroleum ether/ethanol/water binary system to isolate phospholipids from triglyceride mixtures successfully. A similar extraction system is proposed and validated by our group. Hexane/methanol will extract the liver sample following by chloroform/methanol (2:1, v/v). This two-step method can extract most neutral lipid into hexane and isolate phospholipids into chloroform/methanol extract.

Directly Methylation

Considering the disadvantage of extractions (time consuming, health risk, solvent demand), one-step procedures have been devised for determination of FA in a large range of biological tissues.[52,53] Meier et al[53] optimized a one-step extraction/methylation method for FA determination in marine tissues using 2^3 full factorial design and studied the effect of reaction time, temperature, and presence of nonpolar solvent on FA recoveries, and further compared them with the Folch method. This study clearly emphasized that the two methods showed similar FA compositions when the values were expressed on wt/wt%, and the one-step method gave higher recoveries than the traditional Folch method. However, one-step extraction/methylation is not suitable for full lipid classes' research of complex matrices.

Purification approaches

For semi-preparative isolation of lipid classes, solid phase extraction (SPE) is a rapid and simple alternative to TLC[54]. Single aminopropyl bonded column has been employed for the separation of a broad variety of lipid mixtures of different origins. Fungal lipid mixtures[55] and lipid extracts from mixed microbial cultures[56] have been separated into three fraction comprising: neutral lipids, free fatty acid and phospholipids. Perez-Palacios *et al.*[57] improved the SPE introduced by Kaluzny, *et al.*[58] for fractionation of liver phospholipids into PC, PE, PS, PI. However, Russell and Werne [59] reminded us the use of SPE columns with tubes composed of high density polyethylene (HDPE), HDPE coated with a fluorinated polymer similar to Teflon and glass

released short chain fatty acids (significantly 16:0 and 18:0). Our research group also met the blank problem when we were doing previous study. The glass SPE column was adapted to conquer the problem. Our study showed that the glass SPE columns only released trace amount (less than 1.0 μg) of fatty acid while that of plastic columns were up to 25 μg for 16:0 (unpublished data). The amount of phospholipids extracted by traditional method is around 1 % and the SPE load limitation is around 10 mg. Therefore, the PS and PI are extremely low level in SPE eluates. The contaminant of short chain fatty acid affect the quantification of PS and PI dramatically. The extraction method we proposed intends to concentrate the phospholipids into Chloroform/method extract while the neutral lipids are in another phase. In this case, the separated fractions of phospholipids are of much higher concentration after purified by SPE. . Meanwhile glass SPE columns were used to avoid the problems related to blank sample.

Normally, to get pure phospholipid classes, large amount of solvents were used. Our research group decreased the volumes of eluates as long as reasonable resolutions are obtained. PE is co-eluted with PC, while PS/PI are not eluted because the high interaction with stationary phase. Instead of using huge amount of solvent to separate PS and PI, direct methylation will be conducted to stationary phase of SPE column.

1.8 Conclusion

Offshore oil exploration has been carried out in the North Sea for more than 30 years. As the oil fields are becoming "old", the discharges of produced water have increased. Therefore, there are big concerns about how this may affects the marine environment. Balk *et al.*(2011)[2] reported that the offshore oil production could alter fatty acid composition and metabolism of Atlantic cod in North Sea, when other factors, such as temperature and dietary, are not the sources of lipid composition changing. Instead of measuring chosen fatty acids (8 acids were measured by Balk *et al.*[2]), a full scan of fatty acid compositions in different lipid classes will be done to provide more evidence about effects of offshore oil production on lipid composition in Atlantic cod's liver.

However, the amount of phospholipids in cod liver is far less normal (1% of total lipid). Although the standard extraction method, Folch method, has good efficiency to extract polar lipid from animal tissue, it is difficult to analysis the small amount of phospholipids in SPE eluates when the sample load is limited by column ,. A new combined extracted method has been proposed and validated followed by using glass SPE column to separate lipid classes. The objectives of present work are to:

- develop and validate a combined extraction method to extract neutral lipids and polar lipids separately;
- compare the lipids profile with that of standard method (Folch method);
- analysis the fatty acid composition of livers of wild Atlantic cod fish (*Gadus morhua*) from the North Sea using combined extraction method;
- explore the potential effects of offshore oil activity in lipid composition of liver membranes.

2. Method Development

2.1 Experiment

2.1.1 Sample Collection

The Cod (*Gadus morhua*) was raised at the Institute of Marine Research, Bergen, Norway. The liver was dissected by scalpel and pieces were put in cryotubes. All the samples were immediately frozen in liquid nitrogen and later stored at -80 ℃ until further preparation and/or analysis.

2.1.2 Lipid Extraction and Gravimetric Analysis

Procedure 1: Folch extraction (FE)

Total lipid was extracted by a modified Folch method[41] with chloroform/methanol (2:1. v/v). The extraction of lipid was carried out in 25 ml glass tubes with Teflon lined screw caps. Approximate 0.5 g samples were defrosted and homogenized by on a SENTRY^{TM} microprocessor (Oslo, Norway) in 18 ml of chloroform/methanol (2:1. v/v) solvent. Afterwards, the mixture filtered through a glass filter funnel to remove undissolved tissues. Non-lipid material was removed by washing the extract with 0.88% KCl (aq). Subsequently, the extract was dried with $MgSO_4$(s) and filtered. The lipid content was determined from this extract by evaporating the solvent until constant weight. Methanol (HPLC-grade) and chloroform (HPLC-grade) were purchased from Merk (Oslo, Norway).

Procedure 2A: two-step extraction validation

Samples of 0.5 g were homogenized by microprocessor in a 6 ml of methanol/hexane solvent (1:3, v/v). The knife was washed by 4.5 ml of hexane and 9 ml of chloroform respectively. The extracted mixture was centrifuged and hexane phase was transfer to pear-shaped funnel after combined the hexane used to wash knife. The hexane extract in pear-shaped funnel was washed by 3 ml methanol four times and then evaporated separately by nitrogen gas until constant weight. The first methanol wash went to combine with methanol phase of extracted mixture and merged together with the chloroform used to wash knife. Finally the extract was in chloroform/methanol (2:1) one-phase solvent. After that, the extract was removed the non-lipid material and dried as described in ***procedure 1*** and evaporated until to constant weight by

nitrogen stream. The other three times' methanol washes were evaporated separately by nitrogen gas until constant weight. Quick freeze by dry ice was conducted to each methanol wash before transfer to break the micelle of liposome. The hexane phase appeared after freeze was transferred back to hexane extract. This procedure got five parts of extracts, hexane extract (HE), Chloroform/methanol extract (CME), 3rd methanol wash (3MW), 4th methanol wash (4MW) and 5th methanol wash (5MW). Hexane (GC-grade) was obtained from Merk (Oslo, Norway).

Procedure 2B: two-step extraction

Samples were prepared as described in *Procedure 2A*. Instead of washing hexane extract four times, methanol washed thrice and the entire methanols went to chloroform/methanol (2:1) extract and were evaporated together until constant weight. This procedure obtained two parts of extracts, hexane extract (HE) and Chloroform/methanol extract (CME).

2.1.3 Solid Phase Extraction (SPE) Procedure

The SPE procedure was adapted from the research result of Perez-Palacios *et al.*[57] using aminopropyl bonded phase columns to separate lipid mixtures into individual classes. All the extracts obtained in *section 2.1.2* went through column. Briefly, 0.5 ml of each extract (approximately 8 mg lipid) was loaded in a 500 mg aminopropyl modified silica minicolumn (MACHEREY-NAGEL GMBH&Co. Germany), which had been previously activated with 4 ml of hexane and 4 ml chloroform. Neural lipid (NL), free fatty acid (FFA), and phosphatidylcholine/phosphatidylethanolamine (PC/PE) were sequentially eluted with 7 ml of chloroform/isopropanol (2:1, v/v), 5 ml of 2% acetic acid in diethyl ether, and 10 ml of methanol. The eluates were saved in 15 ml thick-walled glass tubes with Teflon lined screw caps, which contained nonadecanoic acid (19:0) as internal standard. The internal standard was dissolved in chloroform and added to the reaction vials with a 100 μl Hamilton syringe. We collected the eluates drop by drop by controlling the vacuum. The vacuum of apparatus for collection was released immediately after the solvent wash to prevent the columns from becoming completely dry. The phosphatidylserine/phosphatidylinositol (PS/PI) fraction was collected by methylating the stationary phase of column directly. Blank column eluates were collected periodically without loading samples. All the eluates were evaporated to dry by nitrogen gas and stand by for the thin-layer chromatography (TLC) and gas chromatography (GC) analysis.

2.1.4 TLC analysis

TLC as described by Olsen[60] was performed. Briefly, lipid fractions obtained in *Section 2.1.3* and dissolved in 50 µl chloroform were separated by spotting on a silica gel 60 plate 10 ×10 cm (Merck, Darmstadt, Germany). The samples and standards were loaded and first developed to a distance of 4.5 cm form the origin using methyl acetate: isopropanol: chloroform: methanol: 0.25% KCl in water (25:25:25:10:9, by volume) as solvent system. After evaporation and dried in vacuum dessicator, the plate was then developed in hexane: diethyl ether: acetic acid (80:20:2, by volume) to ≈8.8 cm from the origin. Separated lipid classes were visualized by spraying the plate with 3% cupric acetate in 8% phosphoric acid followed by charring at 160 ℃ for 20 min. Tentative identification of the lipid classes was performed using standards that were spotted next to the samples.

2.1.5 Methylation

Dry HCl in methanol (2.5M) was used as the methylation reagent. The reagent was prepared by dissolving HCl gas in dry methanol as described by Meier et *al.*[50]. The tubes obtained from *Section 2.1.3*, as well as 0.3 ml of total lipids (TL) from hexane extract and chloroform/methanol extract, which also included internal standard (19:0), were added 1 ml of methylation reagent and reacted in the oven (100 ℃) for 2 h.

2.1.6 GC analysis

About half of the methanol aliquot from the methylation was evaporated under a stream of nitrogen gas and 0.5 ml distilled water was added. The corresponding fatty acid methyl ester (FAME) was extracted by 2 ml hexane two times. The extracted hexane was diluted or concentrated to obtain a suitable chromatographic response. One microliter was injected splitless (the split was open after 2 min) in a HP-7890A gas chromatograph (Agilent, Palo Alto, CA, USA) with a flame ionization detector (GC-FID), the column was a 25 m × 0.25 mm fused silica capillary, coated with polyethylene-glycol of 0.25 µm film thickness, CP-Wax 52 CB (Varian-Chrompack, Middelburg, The Netherlands). Helium (99.9999%) was used as mobile phase at 1 ml/min to 45 min followed by 3 ml/min for 23 min. The injector temperature was 280 C and the detector temperature 300 ℃. The oven was programmed as follow: 90 ℃ for 2 min, 15 ℃/min to 150 ℃, then 2.5 ℃/min to 240 ℃ where the temperature was held for 28 min, t.

total analysis time was 68 min. the last major fatty acid (24:1 n-9) eluted at approximately 35 min. the chromatographic peaks were identified by comparing retention times with a FAME standard (GLC-68A from Nu-Chek Prep. Elysian, MN, USA).

To monitor the performance of the GC, a standard mixture, with known concentrations of the FAME (GLC-463, Nu-Chek Prep. Elysian, MN, USA) was injected for each 10^{th} sample. Peak areas were correct by the use of empirical response factors relative to 18:0. The response factors for FAME not present in the standard mixture were estimated according to the identity and the retention time relative to the standard FAME.

2.2 Results and Discussion

2.2.1 Quality Control

SPE Control

SPE is widely used technique to separate lipid class[59, 61-62], even to be a popular student laboratory topic[62]. Russell and Werne reported that the SPE columns with tubes composed of high density polyethylene (HDPE), HDPE coated with a fluorinated polymer similar to Teflon and glass released short chain fatty acids (significantly 16:0 and 18:0).[59] However, the glass column yielded relatively less contamination. In our study, the blank samples are important quality control sample. The release of fatty acids from columns is presented in table 2-1. Majorities of FAMEs are negligible in blank sample, whereas 16:0 and 18:0 show detectable amount, which is consistent with previous study[59].

The SPE column has sample load limitations (less than 10 mg lipid mixtures). When the analyst contains very high level of specific lipid class, other lipid classes are of quite small amount. Consequently some analytical problems may occur due to the paucity. In our liver samples, 99% of total lipids are neutral lipids. The extract from Folch extraction only included 1.52 μg (Table 2-1) of 16:0 in the SPE eluate of PS/PI, which is 7 times of that released by blank samples. Thus, the quantification of 16:0 from PS/PI eluate could be interfered by columns while FFA and PC/PE fraction are also sensitive to blank control due to low amount of 18:0. The new method we used show obviously less neutral lipids and more phospholipids in one extract, which was eluted into four lipid classes, and all the lipid classes are much higher relative to blank control. Therefore, blank interference is avoided by using the new method.

Table 2-1 fatty acid methyl esters (FAMEs) in blank sample and extracts (unit: μg, Mean ± standard deviation)

	Blank sample (n=6)		FE (n=3)		CME (n=5)	
	16:0	18:0	16:0	18:0	16:0	18:0
NL	0.2±0.1	0.1±0.1	1928.8±476.6	572.8±140.1	603.7±107.6	163.7±31.7
FFA	0.4±0.4	0.3±0.2	2.8±0.4	1.2±0.1	148.7±29.3	13.1±1.6
PC/PE	0.3±0.3	0.2±0.2	35.7±2.6	5.2±0.8	148.7±29.3	16.4±5.0
PS/PI	0.2±0.2	0.1±0.1	1.5±0.2	2.6±0.0	11.0±1.5	7.7±2.3

(Abbreviations: NL, neutral lipids; FFA, free fatty acid; PC/PE, phosphatidylcholine/phosphatidylethanolamine; PS/PI phosphatidylserine/phosphatidylinositol; FE, Folch extraction; CME, chloroform/methanol extract)

GC Analysis Control

The GC performance was monitored by testing a standard mixture with known concentrations of the FAMEs. They were injected for each 10th sample. The empirical response factor, as well as control chart, was calculated from chromatograms of standard mixture. In present study, the control limits of response factor is 1.00±0.15. As the results presented in the Figure 2-1, all the peaks are in the control interval. However, the peak of 22:6 *n-3* is close to lower control limit during the whole analysis, which is agree with other represented results[63, 64]. The fatty acid of 22:6 *n-3* is the most likely loss sample in the liner or column in GC when traceable amount of nonvolatile compounds were left in instrument. In our study, 22:6 *n-3* is the key control point. The equipment maintains, such as cutting column, was done several times when the empirical response factor was out of control limit. The average response factor was used to adjust the peak area and quantification.

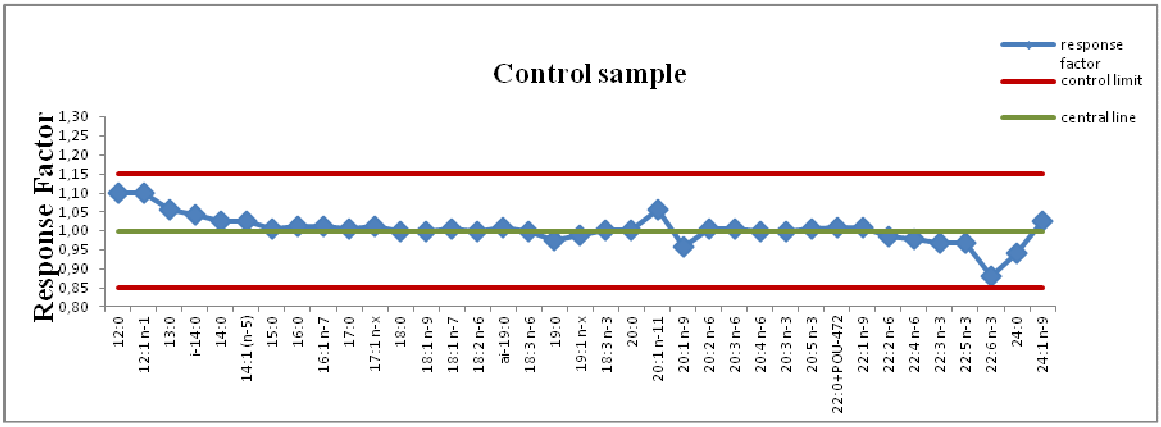

Figure 2-1 the mean value of response factors obtained from NU-chek standard mixture

2.2.2 Lipid Extraction Procedure

Isolate small amount of polar lipids from neutral-lipid-rich animal tissues has been discussed since more than 50 years ago. Some efforts have been done. Smith and Jack[50] improved procedure isolate phospholipids from lipid mixtures with ethanol, ether, and chloroform, which were extracted from buttermilk by using methanol/chloroform before.[50] Galanos *et al.*[51] used petroleum ether-ethanol-water binary system to isolate phospholipids from triglyceride mixtures successfully. Our group proposed and validated the Hexane/methanol (3:1, v/v) solvent system to extract neutral lipids following by chloroform/methanol (2:1, v/v) to extract phospholipids in the cod liver samples.

Effect of methanol wash

Compared to the classic method FE, this two-step method introduces methanol/hexane extraction system and the hexane phase was washed several times by methanol. The TLC analysis plate was presented in Figure 2-2. Only neutral lipids (typically TAG) were observed in hexane extraction. All the lipid classes can be found in the CME and 3MW. The spots related to polar lipids weaken as the increase of methanol wash. Besides the standards showed in plate, some other lipids can also be identified by reference literature[60].

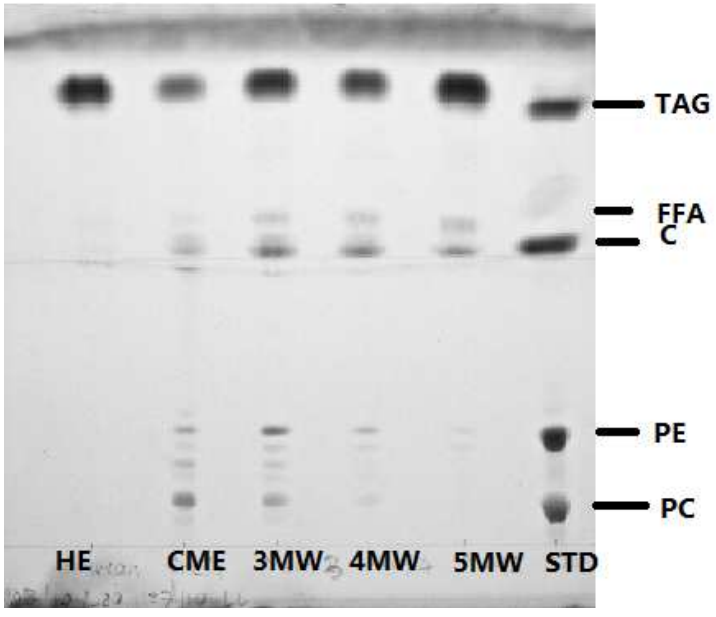

Figure 2-2 TLC analysis of two-step extraction validation (TAG, triacylglycerol; FFA, free fatty acid; C, cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine. HE, hexane extract; CME, chloroform/methanol extract; 3MW, 3rd methanol wash; 4MW, 4th methanol wash; 5MW, 5th methanol wash; STD, standard)

Sphingolipids (SL) was observed above PE; cardiolipin (CL) was located beneath PE followed by PI and PS. Sphingomyelin (SM), which was located between origin and PC, occurred in very small amount. All these extracts went through SPE procedure and were quantified by GC (Table 2-2). More than 90% neutral lipids stayed in HE while only 5.83% phospholipids were remained. The content of NL decreases as the increase of methanol wash. Nevertheless, the 5th methanol wash has higher amount of NL than the fourth, which might be interpreted as over methanol wash could have adverse effect to concentrate neutral lipid into hexane extract. The reason for increasing amount of NL could be the generation of micelle.

Table 2-2 lipid classes distribution in extraction fractions. (unit: %)

Fraction	NL	FFA	PC/PE
HE	91.92	28.95	5.83
CME	2.68	38.34	76.64
3MW	1.76	16.92	15.50
4MW	1.18	8.48	1.87
5MW	2.45	7.31	0.16

(Abbreviation as in legends to Figure 2-2)

This micelle generation is also proved by TLC analysis. As for the polar lipid, the percentage of 76.64 PC/PE was extracted by chloroform/methanol. Using methanol to wash hexane extract can obtain more PC/PE. The 5MW only had 0.16% PC/PE left. Thus, the 5th methanol wash is not used in the two-step extraction. The free fatty acid was with small amount in our samples since the samples were collected and stored in very low temperature. FFA is sample quality control parameter. They were not detailed as long as they were maintained with a reasonable amount.

Effect of micelle

Micelles form when the concentration of surfactant is greater than the critical micelle concentration, and the temperature of the system is greater than the critical micelle temperature. Micelle formation is essential for the absorption of fat-soluble vitamins and complicated lipids within the human. However, in our case, the formation of micelle has adverse effect for extraction. During the extraction procedure, the concentration of lipid in methanol/hexane solvent system was high to a point, which was reached at that the unfavorable entropy consideration, derived from the hydrophobic end of the lipid molecule, become dominant. At this point, the hydrocarbon chains must be sequestered away from the methanol.

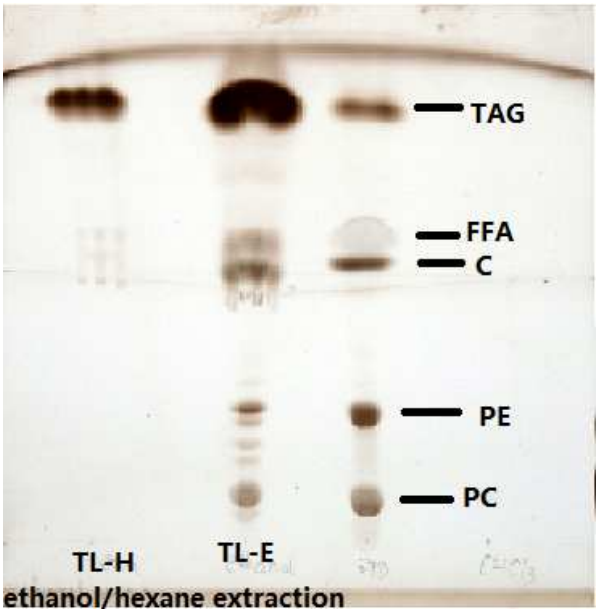

Figure 2-3 The lipid classes extracted by ethanol/hexane system (abbreviation as in legends to Figure 2-2. TL-H, total lipids in hexane extract; TL-E, total lipids in ethanol extract).

Besides, the lipid micelles possibly packed some hexane molecules inside. It was clear that the volume of hexane phase decrease after extraction. To destruct the micelle structure, quick freeze by dry ice was conducted before phase separation. Otherwise, some hexane solvent would be drag into methanol in the form of micelle, further affect the ratio of chloroform/methanol solvent system used in the second step extraction. Our exploratory experiment showed that more neutral lipids remained in polar phase when micelles were not broken (Figure 2-3). The TL in ethanol is the same as that extracted by methanol. There was big area corresponding neutral lipid located in the line extracted by ethanol. However, this area decreased dramatically after quick freeze action conducted (Figure2-2).

2.2.3 Lipid Classes Separation

Lipids are important constituents of all marine organisms and as such frequently have to be analyzed and quantitated in the study of marine ecosystems. Marine and freshwater lipids are frequently separated into lipid classes using TLC or SPE. Latter technique does not offer the same resolution as TLC and is usually preferred when a large quantity of sample is being separated. In present study, SPE was used to separated lipid extracted before; meanwhile TLC is used for verification of fractions.

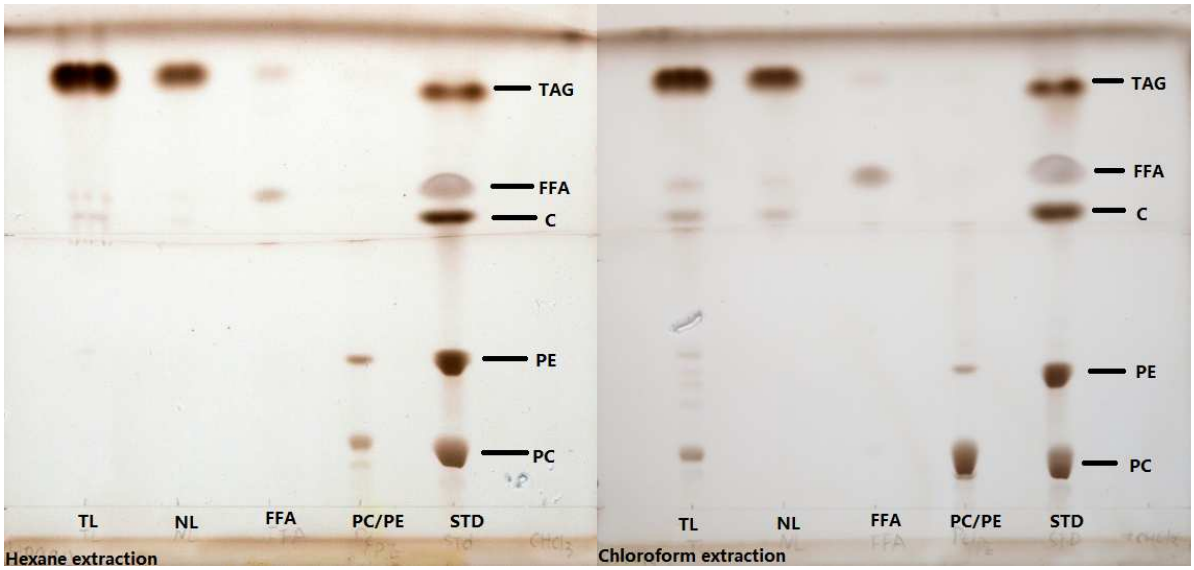

Figure 2-4 Separation by SPE of lipids extracted from cod liver. (Abbreviation as in legends to Figure 2-2. TL: total lipids; NL: neutral lipids)

The TLC solvent system used in present work was applied and verified by Olsen *et al.*[60] who separated total lipid extracted from different aquatic species. The resolution of TLC is good enough to verify the SPE eluted fractions. The verifications are presented in Figure 2-4. The SPEs separated lipid classes effectively according the TLC results. To decrease the use of solvent, PC and PE were co-eluted together, while the PS/PI were obtained by methylating the stationary phase of SPE column directly. Since we will discuss the phospholipids together, this separation is effective enough. The lines for NL and PC/PE were quite clean. However, a weak spot appears above the target FFA in hexane. These results indicate that NL, PC/PE eluted fractions were with high purity while the FFA fraction had some NL co-eluted.

There are no obvious points corresponding to polar lipids showed in TL of hexane extraction, which suggests the amount of polar lipids were with very small amount in hexane extraction. The chromatographic results (Table 2-3) show that more than 95% neutral lipids were extracted into hexane phase while the PC/PE left in hexane extract were less than 5 %. On the contrary, the areas related to PC/PE were clearly observed in both TL and PC/PE fractions in chloroform/methanol extraction. Compared to hexane extraction, the lipids extracted by chloroform/methanol were with higher amount of polar lipid, which were (95.1±2.0) % for PC/PE and (87.6±4.6) % of PS/PI. This selective distribution suggests that the extraction method we proposed concentrates neutral lipids into hexane extraction while the polar lipids prefer to go

to chloroform/methanol extraction. It can be used to remove neutral lipids when the targets are polar lipids. Moreover, by removing neutral lipids, the SPE eluates of extract from CME were of higher concentration of phospholipids. Compared to PC/PE and PS/PI eluate from Folch extraction, higher concentration of PC/PE and PS/PI were obtained after lipid classes' separation. Multiple tests are possible for higher concentration samples. Since the extraction and purification procedure are time-consuming, handy sample with befitting concentration is of great importance.

Table 2-3 lipid classes distribution in two extracts (%, mean ± standard deviation, n=5)

	TL	NL	FFA	PC/PE	PS/PI
HE	95.6±0.9	95.0±2.5	57.9±32.1	4.9±1.9	12.4±4.6
CME	4.4±0.8	5.0±2.5	42.1±32.1	95.1±1.9	87.6±4.6

(Abbreviation as in legends to Figure 2-2)

Besides, the lipid class separation procedure has good recoveries. The recoveries for hexane and chloroform/methanol extracts were (98.5±2.9) % and (98.6±7.5) %, respectively. All the separations showed acceptable recoveries (90 % -110 %),

2.2.4 Comparison with Folch Extraction

In table 2-4, the total lipids determined by new two-step extraction and their gravimetric result are compared with that of Folch extraction. The total FAs determined by GC are significantly less than gravimetric results for both methods. Since the cod liver lipids nearly totally contain of triacylglycerids (>99%), the theoretic FA/lipid ratio should be 0.95. However, our results were around 0.75 and 0.68 for two-step extraction and Folch extraction. One explanation may be a systematic error in the concentration of the internal standard 19:0 used in the analysis. If the amount of 19:0 has been higher than expected in the calculation would one get a underestimation of the quantification of fatty acids in the samples. Another explanation for the low FA/lipid ratio could be that gravimetric lipids include non lipid material and that the lipid % relative to wet weight was too high. However there were very good agreement between the two different extraction methods, and the "dry" lipids after the evaporation of the solvent had a clear oily condition and there were no visible signs of particular or other non-lipid materials. None of the two explanations seems likely, so at the present moment I do not have any final explanation for this.

The lipid distribution obtained by these two methods did not show significant difference. Nevertheless, the two-step extraction got (0.26 ± 0.07) mg/100mg PC/PE while the Folch

extraction had (0.39±0.02) mg/100mg. It is most likely that two-step extraction has lower efficiency to extract PC/PE (t-test, p=0.051). In addition, the standard deviations of two-step methods are clearly higher than that of Folch extraction. This uncertainty is involved in multiple transfer as well as stability of instruments.

Table 2-4 Total lipid and lipid classes determined by two-step extraction and Folch extraction. Values are mg/100 mg wet weight of tissue (mean ± standard deviation).

	two step extraction n=5	Folch extraction n=2
Gravimetric total lipid	57.68±1.23	56.66±0.04
Total FA	43.01±3.17	38.64±2.90
NL	41.92±2.95	40.68±0.11
FFA	0.10±0.04	0.05±0.00
PC/PE	0.26±0.07	0.39±0.02
PS/PI	0,04±0.01	0.04±0.00
SPE recovery	98.5±2.9	106.6±7.6

(Abbreviation as in legends to Figure 2-2)

In cod liver, more than 98% of the total lipids (Table 2-5) were NLs. The traditional method only extracted approximate 0.96 % of PC/PE and even less (0.10 %) PS/PI relative to total lipids. In this case, SPE eluates corresponding to FFA, PC/PE and PS/PI were of very low concentrations. Subsequently, inaccurate quantification or interference from blank samples might occur. In our two-step extraction, majority (95.5 %, Table 2-3) of neutral lipids were extracted into hexane extract. Thus, the percentages of FFA, PC/PE and PS/PI have increased around ten times in CME, to be 2.50%, 12.27 % and 1.84 %, respectively.

Since 99.8% of lipids in hexane were neutral lipids; majority of PC/PE (95.1, Table 2-3) and PS/PI (87.6, Table 2-3) were contained in CME; FFAs were minority in samples, two ways to calculate the lipid distribution were discussed. One was using the sums of each specific lipid class in both HE and CME to be divided by the total fatty acids in two extracts, while another took the total lipids in HE and neutral lipids fraction in CME as total neutral lipids and the other three fractions only took the corresponding parts in CME into account. These two calculations showed coincident results except less FFA was calculated in calculation 2. However, the FAA is out of main concern. The similarity of two calculations suggests that the separation of HE is not necessary. The HE can be considered as pure neutral lipids. Therefore, only CME was separated by SPE and calculation 2 was used when analyzing wild Atlantic cod liver.

Table 2-5 lipid classes relative to total lipids (%, mean ± standard deviation)

	HE (n=5)	CME (n=5)	Two step method calculation 1 (n=5)	Two step method calculation 2 (n=5)	Folch extraction (n=3)
NL	99.83±0.14	83.39±1.08	99.09±0.30	99.3±0.13	98.80±0. 05
FFA	0.13±0.12	2.50±0.15	0.24±0.13	0.11±0.02	0.13±0.01
PC/PE	0.03±0.02	12.27±1.22	0.58±0.16	0.53±0.11	0.96±0.55
PS/PI	0.01±0.00	1.84±0.09	0.10±0.02	0.08±0.01	0.10±0.00

(Abbreviation as in legends to Figure 2-2)

Compared with the results of Folch extraction, significantly less PC/PE (t-test, $p<0.05$) was obtained by two-step method calculation 2, which coincides with the extraction efficiency aforementioned. The other three fractions showed coincident results between two-step extraction and Folch extraction.

Table 2-6 details the fatty acid composition in neutral lipids. The fatty acid profiles for neutral lipids are quite similar for these two methods. 16:0 was the main saturated fatty acid, while 18:1 n-9 was the main MUFA. The sum of SFA and MUFA were 25 % and 40 %, respectively. The dominants PUFAs were *n-3* series fatty acid, being 30% of neutral fatty acids, which is consistent with marine fish's characterization. However, the method we proposed extracted less (t-test, $p<0.05$) PUFAs, typically 20:5 *n-3* and 22:6 *n-3*. Thus the ratio of *n-3*/*n-6* was relative lower.

Most of profiles for PC/PE in both chloroform/methanol extract and Folch extract are comparative (Table 2-7). However, different extractions do occur in two methods. New method extracted significantly less (t-test, $p < 0.05$) monoenoic fatty acids. Besides, more polyunsaturated fatty acids appeared in CME, especially 22:6 *n-3*. The fatty acids of *n-3* series were significantly higher than that of Folch extraction. Meanwhile, the *n-6* series fatty acids were less extracted. Subsequently the discriminated extraction of *n-3* and *n-6* series leads to a higher ratio of *n-3*/*n-6*, which is an important biomarker for marine fish.

For PS/PI profile comparison, there were more differences. The profiles for saturated fatty acids, 16:0 and 18:0 showed significant differences. More 16:0 and less 18:0 were extracted by CME. Eventually, the saturated fatty acids extracted by CME were less than that of FE. Some significant differences also existed in MUFA extractions. The tendency to extract PUFAs is similar as that happened in PC/PE fraction with a larger difference.

Table 2-6 Fatty acid composition (wt. % of total fatty acids) of the neutral lipids of cod liver (mean values ± standard derivation)

	NL-HE (n=5)	NL-FE (n=3)
14:0	3.59 ± 0.11	3.70 ± 0.02
15:0	0.39 ± 0.01	0.36 ± 0.00
16:0	15.71 ± 0.32	15.42 ± 0.29
i-17:0	0.32 ± 0.04	0.29 ± 0.01
17:0	0.31 ± 0.00	0.31 ± 0.01
ai-18:0	0.41 ± 0.01	0.37 ± 0.00
18:0	4.45 ± 0.07	4.58 ± 0.01
∑SFA	25.87 ± 0.49	25.47 ± 0.31
16:1 n-9	0.39 ± 0.00	0.37 ± 0.03
16:1 n-7	6.26 ± 0.13	6.16 ± 0.08
17:1 n-x	0.39 ± 0.02	0.36 ± 0.01
18:1 n-11	1.10 ± 0.13	0.93 ± 0.08
18:1 n-9	17.82 ± 0.19	18.15 ± 0.08
18:1 n-7	4.22 ± 0.02	4.42 ± 0.04
20:1 n-11	0.98 ± 0.02	0.99 ± 0.00
20:1 n-9	4.40 ± 0.04	4.41 ± 0.03
22:1 n-11	**3.01 ± 0.04**	**3.20 ± 0.08**
22:1 n-9	0.33 ± 0.01	0.27 ± 0.08
∑MUFA	**39.80 ± 0.27**	**40.33 ± 0.20**
16:2 n-4	0.58 ± 0.01	0.56 ± 0.01
18:2 n-4	0.38 ± 0.01	0.38 ± 0.00
18:2 n-6	**5.03 ± 0.03**	**5.13 ± 0.06**
20:2 n-6	0.30 ± 0.00	0.31 ± 0.00
20:4 n-6	**0.63 ± 0.01**	**0.58 ± 0.03**
22:4 n-6	0.25 ± 0.01	0.26 ± 0.00
22:5 n-6	0.21 ± 0.00	0.22 ± 0.00
18:3 n-3	1.21 ± 0.01	1.23 ± 0.01
18:4 n-3	1.71 ± 0.03	1.73 ± 0.01
20:4 n-3	0.78 ± 0.02	0.80 ± 0.05
20:5 n-3	**9.59 ± 0.24**	**11.48 ± 0.20**
21:5 n-3	**0.47 ± 0.02**	**0.52 ± 0.01**
22:5 n-3	**1.90 ± 0.07**	**2.20 ± 0.00**
22:6 n-3	**10.43 ± 0.36**	**11.20 ± 0.06**
∑PUFA	**34.33 ± 0.72**	**37.26 ± 0.29**
∑ (n-6)	6.75 ± 0.09	6.82 ± 0.06
∑ (n-3)	**26.19 ± 0.66**	**29.32 ± 0.25**
(n-3)/(n-6)	**3.88 ± 0.07**	**4.30 ± 0.02**

(Numbers marked by boldface are significant different in pair (t-test, $p < 0.05$)

Table 2-7 Fatty acid composition (wt. % of total fatty acids) of the phospholipids of cod liver (mean values ± standard derivation)

	PC/PE-CME (n=5)	PC/PE-FE (n=3)	PS/PI-CME (n=5)	PS/PI-FE (n=3)
14:0	1.52 ± 0.11	1.79 ± 0.22	1.32 ± 0.14	1.11 ± 0.01
15:0	0.37 ± 0.02	0.33 ± 0.03	0.25 ± 0.04	0.24 ± 0.02
16:0	25.66 ± 1.09	25.04 ± 0.92	**12.86 ± 0.78**	**10.54 ± 0.11**
i-17:0	0.31 ± 0.01	0.27 ± 0.01	0.19 ± 0.02	0.17 ± 0.04
17:0	0.31 ± 0.02	0.34 ± 0.01	**0.35 ± 0.03**	**0.73 ± 0.03**
ai-18:0	0.02 ± 0.02	0.03 ± 0.01	0.05 ± 0.02	0.03 ± 0.02
18:0	**2.77 ± 0.35**	**3.59 ± 0.23**	**8.88 ± 1.47**	**17.48 ± 0.39**
∑SFA	31.35 ± 0.81	31.68 ± 1.16	**24.39 ± 0.61**	**30.90 ± 0.22**
16:1 n-9	0.24 ± 0.01	0.34 ± 0.01	0.43 ± 0.07	0.57 ± 0.07
16:1 n-7	**1.00 ± 0.07**	**1.25 ± 0.08**	**1.72 ± 0.19**	**1.16 ± 0.02**
17:1 n-x	0.21 ± 0.01	0.21 ± 0.02	0.21 ± 0.01	0.21 ± 0.01
18:1 n-11	0.41 ± 0.02	0.46 ± 0.03	0.66 ± 0.13	0.66 ± 0.05
18:1 n-9	12.97 ± 1.13	14.66 ± 0.81	7.42 ± 0.23	7.60 ± 0.63
18:1 n-7	**2.41 ± 0.27**	**2.88 ± 0.06**	4.53 ± 0.06	4.32 ± 0.33
20:1 n-11	0.35 ± 0.02	0.38 ± 0.04	0.36 ± 0.04	0.31 ± 0.08
20:1 n-9	1.11 ± 0.19	1.20 ± 0.06	**1.68 ± 0.19**	**2.39 ± 0.23**
22:1 n-11	**0.29 ± 0.05**	**0.42 ± 0.05**	0.33 ± 0.05	0.38 ± 0.13
22:1 n-9	**0.10 ± 0.02**	**0.17 ± 0.06**	0.04 ± 0.00	0.05 ± 0.02
∑MUFA	**20.55 ± 1.61**	**25.07 ± 1.06**	18.96 ± 0.47	21.64 ± 4.59
16:2 n-4	0.05 ± 0.00	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
18:2 n-4	0.24 ± 0.01	0.23 ± 0.01	**0.34 ± 0.01**	**0.24 ± 0.05**
18:2 n-6	**1.91 ± 0.08**	**2.45 ± 0.28**	3.64 ± 0.28	3.46 ± 0.79
20:2 n-6	0.19 ± 0.01	0.19 ± 0.01	0.50 ± 0.01	0.47 ± 0.01
20:4 n-6	1.50 ± 0.03	1.44 ± 0.04	**5.83 ± 0.84**	**9.30 ± 0.58**
22:4 n-6	0.07 ± 0.01	0.07 ± 0.00	**0.11 ± 0.01**	**0.08 ± 0.00**
22:5 n-6	0.39 ± 0.06	0.39 ± 0.05	**1.43 ± 0.11**	**1.78 ± 0.24**
18:3 n-3	0.29 ± 0.02	0.31 ± 0.02	**0.49 ± 0.03**	**0.36 ± 0.04**
18:4 n-3	0.30 ± 0.03	0.31 ± 0.02	**0.28 ± 0.01**	**0.16 ± 0.03**
20:4 n-3	0.39 ± 0.03	0.41 ± 0.03	0.53 ± 0.03	0.44 ± 0.04
20:5 n-3	**13.69 ± 0.61**	**15.02 ± 0.17**	8.43 ± 0.98	8.10 ± 0.08
21:5 n-3	0.22 ± 0.02	0.24 ± 0.01	0.14 ± 0.01	0.12 ± 0.02
22:5 n-3	1.84 ± 0.12	1.88 ± 0.04	**2.33 ± 0.11**	**1.87 ± 0.30**
22:6 n-3	**26.59 ± 1.84**	**21.71 ± 2.18**	**31.91 ± 1.79**	**23.28 ± 0.68**
∑PUFA	**47.93 ± 1.36**	**44.98 ± 1.99**	**56.22 ± 0.78)**	**48.28 ± 3.54**
∑ (n-6)	**4.27 ± 0.10**	**4.70 ± 0.28**	**11.87 ± 0.58**	**14.55 ± 1.82**
∑ (n-3)	**43.10 ± 1.43**	**39.95 ± 2.11**	**43.79 ± 1.26**	**33.43 ± 1.78**
(n-3)/(n-6)	**10.17 ± 0.53**	**8.53 ± 0.82**	**3.74 ± 0.29**	**2.31 ± 0.18**

(Numbers marked by boldface are significant different in pair (t-test, $p < 0.05$)

Although the extraction showed different profile with traditional methods, the repeatability of methods is stable. The standard deviations of main compounds are presented in Table 2-6 and Table 2-7. The fatty acid of 18:0 and 22:5 *n-3* showed relative larger variation in chloroform/methanol extract. The largest relative standard deviation (6.31%) occurs to 22:5 *n-3* in the fraction of neutral lipids in chloroform/methanol extract.

2.3 Conclusion

A combined two-step extraction was proposed. Four times of methanol washes were done for hexane extract to ensure the maximum extraction of polar lipids in the second step. The quick freeze by dry ice was adapted to break micelle formed during extraction procedures. The use of glass SPE column decreased the interference from blank samples. The results show that hexane/methanol extracted almost all the neutral lipids (95% of total neutral lipids) and concentrated 95% of PC/PE and 88% of PS/PI into chloroform/methanol extract. The SPE separated lipid classes effectively with very good recoveries. The profiles of neutral lipids are quiet similar with Folch extraction with a lower ratio of *n-3/n-6*. There are more differences of phospholipids profiles using two-step extraction compared with traditional Folch extraction. In PC/PE fraction, less MUFAs appeared. With a higher amount of n-3 series and lower amount of n-6 series PUFAs, the total PUFAs were significantly higher than that obtained by Folch extraction. In contrary to neutral lipids extraction, a higher ratio of *n-3/n-6* was observed. As for PS/PI fraction, a semblable discrimination of *n-3* and *n-6* series fatty acids was noted as PC/PE.
Although the extraction is different from traditional method, the new method is reproducible. Moreover, the method separated neutral lipids and phospholipids into two extracts effectively. Higher concentration of phospholipids in chloroform/methanol extract makes it possible to multiple tests of membrane lipids in one sample. It is important to have enough chromatographic samples to test more than one time when analyzing wild samples which were collected difficultly. Moreover, adequate lipid distribution between two extract avoid potential interference from blank sample caused by SPE column load limitation. Considering the effectivity and stability of the two-step extraction, it is fit for analyzing lipid compositions in livers of wild Atlantic cods collected from Tampen area and at the Egersund bank, where are with high offshore oil activity and no oil production, respectively.

3. Lipid Composition Analysis

3.1 Experiment

3.1.1 Sample Collection

Atlantic cod (*Gadus Morhua*) was collected in the Tampen area and at the Egersund bank in the summer 2010 (Figure 3-1). The daily discharge of produced water in Tampen is 278,000 m^3 as documented by the oil industry in 2002, whereas there is no oil or gas production at Egersund bank, used as control. The liver was dissected and pieces were put in cryotubes. All the samples were immediately frozen in liquid nitrogen and later stored at -80 ℃ until further preparation and/or analysis. The details of samples are included in Table 3-1.

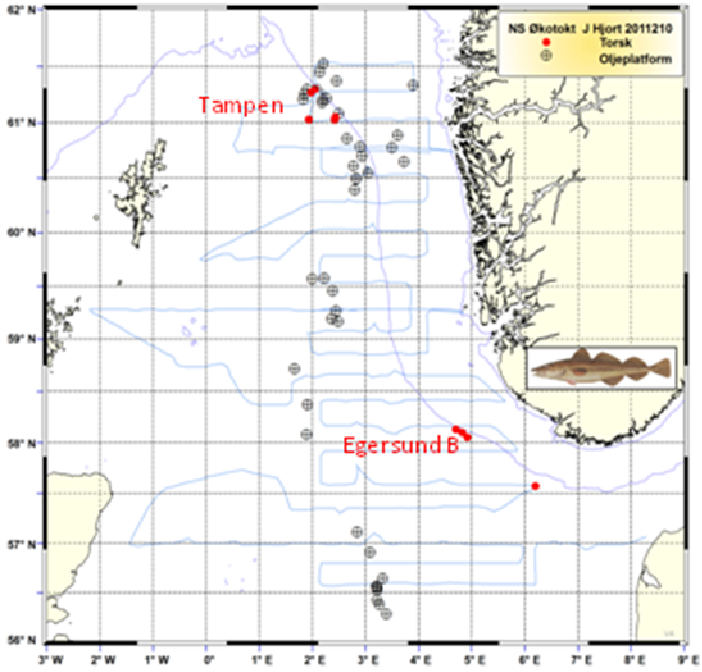

Figure 3-1. Station of fish sampling July 2011. The red marks shows the sampling position of fish, the black marks shown oil installation in the Norwegian sector of the North Sea.

The liver index (hepatosomatic index) was calculated as

$$\text{LSI (\%)} = \frac{LW}{L} \times 100$$

where LW is the liver weight (g) and W is the wet weight of the fish (g).

Fulton's condition factor:

$$\text{Fulton's C} = \frac{W}{L^3} \times 100$$

where W is the wet weight of fish (g) and L is the length of the fish (cm)

3.2 Result and Discussion

3.2.1 Sample Conditions

43 samples from two areas (Tampen and Egersund bank) were analysis (Table 3-1). There was no discriminate of weight and length between these two places and genders statistically due to very large biological variances. However, it seems that males are larger than female, males was weighted (850~2870) g compared to female (750~2040) g at Tampen and male (385~3380) g relative to female (485~2620) g at Egersund. The males have some very fat fish in our analytical group. There was no significant difference found between these four groups for liver size, which is characterized by LSI. As for Fulton condition, it is influenced by age of fish, sex, season, stage of maturation, fullness of gut, type of food consumed, amount of fat reserve and degree of muscular development. The Fulton conditions of cods from Tampen were a little bit higher than cod samples from Egersund (t-test, p = 0.051). Nevertheless, the difference is not significant, being 6% higher. Compared the total lipids contained in fishes from two different areas, no statistic difference is observed.

Table 3-1 Biological information of samples

		n	weight (g)	length (cm)	Liver weight (g)	gonad weight (g)	LSI (%)	Fulton C	Lipid (%)
Tampen	female	12	1105±398	47±6	22±12	2.7±1.1	1.9±0.6	1.04±0.08	29.6±15.0
	male	10	1357±633	50±6	35±42	-	2.3±1.5	1.04±0.08	32.5±21.4
Egersund	female	11	910±711	43±10	30±36	2.9±2.9	3.0±1.4	0.98±0.09	42.0±15.0
	male	10	1323±980	49±13	21±14	-	1.7±0.8	0.98±0.09	29.0±16.6

The correlation between LSI and lipids contents is obviously observed. There is a tendency that larger livers have higher amount of total lipids (Figure 3-2). This tendency is well fit for all four groups. Since liver is used to storage energy in cod fishes and storage lipids are dominate in total lipids, it is expectable this tendency from biology viewpoint. As we can see in Figure 3-2, the content of neutral lipids is nature logarithm of total lipids content. The regression constants are 0.799 and 0.827 respectively.

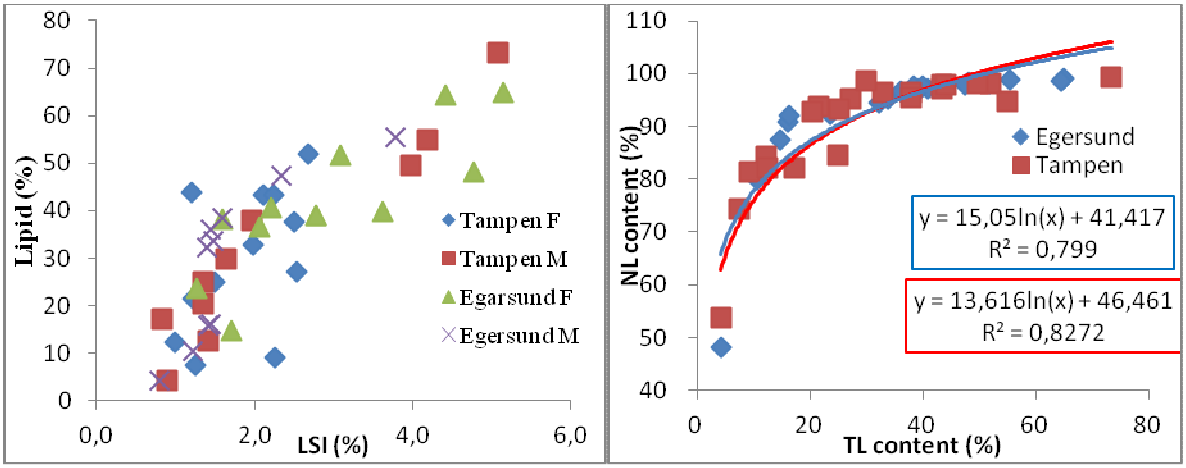

Figure 3-2 The correlation between LSI and total lipids content and neutral lipids content

3.2.2 Lipid Classes Distribution

The lipid classes' distributions are detailed in the Table 3-2. The samples were classified as lean fish (lipid content less than 20% in liver) and fat fish (lipid content more than 20%). Compared to report from Meier *et al.*[9] and the result of sample used for method development (raised fish), wild samples show much higher amount of PL. Usually, only 1 % of total lipids could be ascribed to phospholipids in raised fish samples, while the wild sample show the PC/PE were response for more than 10% of total lipids in lean fish and around 3% in fat fish. Meier *et al.*[9] suggested that the exposure of APs could alter the distribution of fatty acids between lipid classes in the liver. As exposed to APs, significant higher amount of NLs and lower amount of phospholipids were observed. However, our works show opposite results and the distribution altered similarly as the effect of brain samples. The area of Tampen is with high oil activity and discharge of huge volume of produced water. 323 tons of APs were discharge to North Sea from Norwegian shore in 2009.[5] whereas, there is no oil production documented at Egersund bank. Our results reveal that more phospholipids and less neutral lipids existed in the samples living in high offshore oil activity field. These observations are limited in lean fish comparison, and the observed differences are not significant because of larger biological variance. As we mentioned, multiple temporal and spatial variables make deciphering the effects extremely difficult. We are not sure about how long the samples stayed at the selected areas; we are lack of information about marine environment changing; and we have no thorough understanding of the inter-effect between lipid content and fatty acid composition. However, some deduction can be done based

on present information. The observations strongly indicate rapid mobilization and utilization of stored pool of lipids occurred in lean fish. The reduce of levels of neutral lipids may be due to either a reduction of the rat of accumulation of neutral lipid from food or an increase in rat of mobilization of the lipid reserves. Balk et al.[2] suggested that extreme diets, which can result changes of lipid composition, are not to be expected in the natural diet of Atlantic cod in the Tampen and Egersund. Therefore, the most possible reason for neutral lipids decrease is mobilization of reserved lipids. The elevated levels of phospholipids are likely to be related to microsomal membranes and electro transport system which have been demonstrated by others to be induced under exposure of petroleum condition .[65,66]

Table 3-2 lipid classes distributed in sample (wt % of total lipids, mean values ± standard derivation)

Lean fish Lipid content <20%		Tampen (n=6)	Egersund (n=4)
	Lipid content (% wt)	13.9±6.3	20.9±14.9
	NL	81.5±3.7	85.2±21.2
	FFA	1.5±0.3	2.0±2.3
	PC/PE	15.5±3.6	11.4±17.1
	PS/PI	1.4±0.4	1.4±2.2
fat fish Lipid content >20%		Tampen (n=16)	Egersund female (n=16)
	Lipid content (% wt)	39.5±14.4	42.89±11.1
	NL	96.4±2.1	95.3±4.8
	FFA	0.3±0.2	0.5±0.7
	PC/PE	3.0±1.8	3.8±3.6
	PS/PI	0.3±0.2	0.5±0.6

3.2.3 Fatty Acid Composition

Fatty Acid Composition in Neutral Lipids

Table 3-3 details the fatty acid composition of the neutral lipids of the different tissues. This class of fatty acids is variable and labile and is utilized principally as a source of fatty acids destined for oxidation to produce adenosine triphosphate (ATP).

The fatty acid of 16:0 was the main saturated fatty acid, while SFA contributed around 20 % of neutral lipids. There was no statistic difference of SFA between two areas. However, the amount of SFA is lower than report[9]. More than 32 % of total lipids were SFAs and the number increased

as exposed to APs. In our study, around 22% of total lipids were corresponding saturated fatty acid. The monounsaturated fatty acids were dominated, being more than 50 % of total neutral fatty acids, which is much higher than Meier's report[9] and the cod sample raised by IMR (Table 2-6). the main monoenoic fatty acids were 18:1 *n-9*, 20:1 *n-9* and 22:1 *n-11*, accounted for 7.79~9.99 %, 8.64~12.08 % and 10.34 ~ 11.58 % respectively. The amount of 18:1 *n-9* is consistent with previous study[9] and the validation sample raised by IMR (Table 2-6). It is interesting to find high amount of long chain MUFAs (20:1 *n-9* and 22:1 *n-11*) in wild fish. Since the liver is the lipid energy store of the cod and does mainly contain neutral lipids, the fatty acid profile of NL is strongly influenced by the profile of the diet.[67] Lie et al. has proved that fatty acid composition in the liver was clearly modified when the fish were fed by peanut oil, cod liver oil or Greenland halibut oil. Thus, the dietary is the main effect of fatty acids profile of neutral lipids. The 22:1 *n-11* has proved to be an important fatty acid when using adipose tissue fatty acid composition to study diet in marine organism.[68] Although theoretically vertebrates can synthesize 22:1 *n-11*, this FA primarily originates from the fatty alcohols (wax esters) of certain copepod species.[69] The concentration of this FA also varies widely among different fish and invertebrate species, making it a good indicator of diet.[68] However, it more likely to be chain shorten by peroxisomal β-oxidation. Cooper *et al.* [70] found that the main product of the chain shortening of 22:1 n-11 was 18:1 PUFAs. The similar chain shortening could occur in cod. The differences of 22:1 *n-9* (p = 0.188) and 18:1 *n-9* (p = 0.285) are not significant in statistic due to limited sample size and big biological variance. Only the less 20:1 *n-9* was detected by ANOVA at the field of Tampen.

As could expected, the dominate PUFAs are n-3 family polyenes. The most important two PUFAs are 20:5 *n-3* and 22:6 *n-3*, accounting to 20 % of total lipids. The main *n-6* series PUFAs are 18:2 *n-6* and 22:4 *n-6*. The ratio of n-3/n-6 is between 7.43 ~ 11.33, which agrees previous study.[9]

Fatty Acid Composition in Phospholipids

The composition of phospholipids is more independent from the diet. Fish have high flexibility in remodeling the lipid composition of cell membranes[71] and optimize the membrane fluidity to maintain normal physiological functions. The mechanisms responsible for the perception of changes in membrane fluidity have not been fully characterized, but include changes in fatty acids unsaturation, changes in the proportion of phospholipids classes or cholesterol and changes in the lipid-protein ratio[72]. Lie et al.[73] reported the major phospholipids in the liver are dominated by PC (54%), PE (28%), PI (11%), and PS (7%). In our study, PC and PE were eluted together and PS stuck together with PI. The PC/PE fraction contributed 90% of phospholipids while others are PS/PI.

Table 3-4 and Table 3-5 present fatty acid profiles information for PC/PE and PS/PI fractions. The fatty acid profiles found in both fractions show a typical picture of phospholipids from cod with their very high level of *n-3* PUFAs. Saturated fatty acid accounted for 25% of total fatty acids. A characteristic high content of 16:0, 18.78~19.69 % of total lipids was noted, which was almost four times higher than that in PS/PI. Lie suggested that the high amount of 16:0 is assigned to PE.[71] The main SFA in PS/PI was 18:0 and it is from PI[73]. The monoenes accounted for around 25% of the fatty acids in both phospholipid fractions. The major monoenes was 18:1-*n-9* in PC/PE, followed by two *n-7* MUFAs (16:1 *n-7* and 18:1 *n-7*) and two *n-9* series long chain fatty acids (20:1 *n-9* and 24:1 *n-9*). Unlike what was noted in storage lipids profile, only trace amount of 22:1 *n-11* was observed in membranes lipids. In the PS/PI fraction, *n-9* and *n-7* family monoenes also dominated the MUFAs.

The major PUFAs were 22:6 *n-3* and 20:5 *n-3*. They are of particular importance of the regulation of localized membrane structure and functionality in actively metabolizing tissues. 20:4 *n-6* was also prominent in PS/PI, being 10.40 ~ 12.30 % of total lipids, which is characteristic composition of PI[72]. However, 18:2 *n-6*, mention by Lie *et al.*[73], was only trace amount in PC/PE. They also stated that the higher level of 18:2 *n-6* is pointing to the influence of the diet rather than the environment effects we are discussing. Because of contribution of 20:4-*n-6* from PI, the *n-3*/*n-6* of PS/PI fraction showed the lowest among three fractions we discussed.

Table 3-3 Fatty acid composition (wt. % of total fatty acids) of the neutral lipids of cod liver (mean values ±standard derivation)

	Tampen		Egersund,	
	Female (n=12)	Male (n=10)	Female (n=11)	male (n=10)
14:0	5.07 ± 1.11	4.76 ± 1.19	5.14 ± 0.76	5.32 ± 0.93
15:0	0.44 ± 0.13	0.42 ± 0.12	0.34 ± 0.06	0.43 ± 0.08
16:0	12.55 ± 1.65	12.47 ± 1.32	11.66 ± 0.74	11.47 ± 1.69
i-17:0	0.39 ± 0.14	0.39 ± 0.14	0.27 ± 0.05	0.40 ± 0.13
17:0	0.35 ± 0.19	0.34 ± 0.12	0.22 ± 0.05	0.30 ± 0.17
ai-18:0	0.34 ± 0.18[b]	0.33 ± 0.144[b]	0.54 ± 0.11[a]	0.35 ± 0.20[b]
18:0	2.79 ± 0.95	3.01 ± 0.66	2.32 ± 0.37	2.22 ± 0.67
ΣSFA	22.15 ± 1.99	21.94 ± 2.12	20.67 ± 1.16	20.75 ± 1.92
16:1 n-9	0.30 ± 0.13	0.32 ± 0.08	0.25 ± 0.08	0.31 ± 0.08
16:1 n-7	6.02 ± 0.64	5.73 ± 1.31	6.56 ± 0.34	5.97 ± 1.47
17:1 n-x	0.39 ± 0.14	0.40 ± 0.13	0.29 ± 0.07	0.32 ± 0.07
18:1 n-11	2.38 ± 0.51	2.15 ± 0.88	2.70 ± 0.48	2.43 ± 0.66
18:1 n-9	9.99 ± 3.32	9.12 ± 3.03	8.70 ± 2.04	7.79 ± 1.74
18:1 n-7	3.35 ± 1.10	3.36 ± 0.68	2.97 ± 0.24	2.84 ± 0.59
20:1 n-11	2.69 ± 0.44	2.69 ± 0.83	2.28 ± 0.35	2.47 ± 0.67
20:1 n-9	9.04 ± 2.60[b]	8.64 ± 2.23[b]	12.08 ± 1.15[a]	10.68 ± 1.43[ab]
22:1 n-11	11.16 ± 3.73	10.34 ± 2.13	10.42 ± 1.02	11.58 ± 3.66
22:1 n-9	0.66 ± 0.18	0.62 ± 0.11	0.71 ± 0.06	0.75 ± 0.13
24:1 n-9	0.74 ± 0.24[ab]	0.89 ± 0.54[a]	0.44 ± 0.07[b]	0.80 ± 0.45[ab]
ΣMUFA	51.53 ± 10.66	50.62 ± 13.70	52.41 ± 10.38	51.33 ± 10.26
16:2 n-4	0.51 ± 0.23	0.51 ± 0.19	0.69 ± 0.10	0.54 ± 0.14
18:2 n-6	1.15 ± 0.11	1.21 ± 0.16	1.15 ± 0.14	1.34 ± 0.43
20:2 n-6	0.40 ± 0.19	0.40 ± 0.16	0.28 ± 0.04	0.33 ± 0.09
20:4 n-6	0.77 ± 0.51	0.95 ± 0.95	0.44 ± 0.08	0.55 ± 0.26
22:4 n-6	1.03 ± 0.98	1.71 ± 2.22	0.52 ± 0.38	2.29 ± 2.50
22:5 n-6	0.19 ± 0.04[a]	0.21 ± 0.06[a]	0.12 ± 0.02[b]	0.19 ± 0.06[a]
18:3 n-3	0.68 ± 0.10	0.67 ± 0.17	0.66 ± 0.13	0.75 ± 0.31
18:4 n-3	1.72 ± 0.71	1.74 ± 0.80	2.18 ± 0.28	1.68 ± 0.76
20:4 n-3	0.62 ± 0.14	0.66 ± 0.14	0.66 ± 0.10	0.80 ± 0.55
20:5 n-3	8.36 ± 2.29	8.76 ± 1.58	9.73 ± 1.27	7.64 ± 3.12
21:5 n-3	0.45 ± 0.16	0.47 ± 0.17	0.62 ± 0.10	0.44 ± 0.21
22:5 n-3	1.74 ± 0.33	1.88 ± 0.27	1.93 ± 0.56	1.92 ± 0.52
22:6 n-3	12.25 ± 1.57	13.33 ± 4.14	11.50 ± 1.48	13.46 ± 2.14
ΣPUFA	30.42 ± 3.33	33.06 ± 6.20	31.11 ± 1.48	32.47 ± 1.33
ΣPUFA (n-6)	3.54 ± 1.26	4.47 ± 3.22	2.50 ± 0.43	4.71 ± 2.56
ΣPUFA (n-3)	26.03 ± 3.29	27.71 ± 4.46	27.45 ± 1.62	26.87 ± 2.81
n-3/n-6	8.12 ± 2.61[ab]	8.02 ± 3.74[ab]	11.33 ± 2.27[a]	7.43 ± 4.09[b]

Table 3-4 Fatty acid composition (wt. % of total fatty acids) of the phosphatidylcholine/phosphatidylethanolamine of cod liver (mean values ±standard derivation)

	Tampen				Egersund			
	Female (n=12)		Male (n=10)		Female (n=11)		male (n=10)	
14:0	2.91 ± 0.53		2.85 ± 0.58		3.13 ± 0.53		3.07 ± 0.63	
15:0	0.52 ± 0.15		0.48 ± 0.10		0.44 ± 0.07		0.49 ± 0.07	
16:0	19.58 ± 1.50		19.11 ± 1.24		19.69 ± 1.26		18.78 ± 1.82	
i-17:0	0.44 ± 0.14		0.46 ± 0.12		0.39 ± 0.09		0.44 ± 0.10	
17:0	0.36 ± 0.17		0.35 ± 0.14		0.27 ± 0.09		0.30 ± 0.09	
18:0	2.14 ± 0.24		2.28 ± 0.28		2.04 ± 0.23		1.99 ± 0.30	
ΣSFA	26.18 ± 1.34		25.77 ± 1.29		26.15 ± 1.27		25.27 ± 1.75	
16:1 n-11	0.16 ± 0.05		0.16 ± 0.07		0.24 ± 0.09		0.23 ± 0.07	
16:1 n-9	0.42 ± 0.11		0.38 ± 0.07		0.38 ± 0.08		0.42 ± 0.10	
16:1 n-7	2.08 ± 0.18		2.03 ± 0.20		2.19 ± 0.13		2.16 ± 0.52	
16:1 n-5	0.26 ± 0.04[b]		0.24 ± 0.04[b]		0.28 ± 0.04[ab]		0.32 ± 0.06[a]	
17:1 n-x	0.29 ± 0.09		0.27 ± 0.10		0.23 ± 0.07		0.25 ± 0.05	
18:1 n-11	1.24 ± 0.30		1.20 ± 0.36		1.40 ± 0.30		1.33 ± 0.33	
18:1 n-9	6.85 ± 0.92[ab]		7.19 ± 0.95[a]		6.19 ± 0.59[b]		6.22 ± 0.34[b]	
18:1 n-7	2.62 ± 0.49		2.73 ± 0.62		2.36 ± 0.42		2.48 ± 0.43	
18:1 n-5	0.26 ± 0.04		0.27 ± 0.04		0.29 ± 0.04		0.29 ± 0.06	
20:1 n-11	0.67 ± 0.20		0.88 ± 0.68		0.64 ± 0.10		0.65 ± 0.20	
20:1 n-9	2.35 ± 0.96[b]		2.15 ± 0.74[b]		3.17 ± 0.43[a]		3.28 ± 0.64[a]	
22:1 n-11	0.80 ± 0.65		0.84 ± 0.44		0.79 ± 0.14		1.11 ± 0.65	
24:1 n-9	2.07 ± 0.36		2.18 ± 0.30		2.13 ± 0.31		2.19 ± 0.29	
ΣMUFA	24.90 ± 11.15		26.11 ± 11.07		25.34 ± 11.21		26.46 ± 11.46	
18:2 n-4	0.17 ± 0.13		0.15 ± 0.04		0.18 ± 0.03		0.14 ± 0.05	
18:2 n-6	0.60 ± 0.11[b]		0.62 ± 0.07[ab]		0.58 ± 0.05[b]		0.75 ± 0.21[a]	
20:2 n-6	0.32 ± 0.12		0.33 ± 0.15		0.26 ± 0.11		0.32 ± 0.09	
20:4 n-6	2.81 ± 0.79		2.77 ± 1.13		2.04 ± 0.85		2.16 ± 0.73	
22:4 n-6	0.31 ± 0.14		0.29 ± 0.13		0.25 ± 0.14		0.37 ± 0.25	
22:5 n-6	0.36 ± 0.04[a]		0.35 ± 0.04[a]		0.28 ± 0.06[b]		0.30 ± 0.06[ab]	
18:3 n-3	0.25 ± 0.07		0.24 ± 0.06		0.25 ± 0.04		0.32 ± 0.11	
18:4 n-3	0.48 ± 0.22		0.47 ± 0.16		0.60 ± 0.10		0.62 ± 0.22	
20:4 n-3	0.39 ± 0.10[b]		0.43 ± 0.07[ab]		0.44 ± 0.06[ab]		0.48 ± 0.06[a]	
20:5 n-3	16.06 ± 1.14		16.61 ± 1.13		17.02 ± 1.31		15.66 ± 1.32	
21:5 n-3	0.27 ± 0.12		0.25 ± 0.05		0.31 ± 0.05		0.26 ± 0.06	
22:5 n-3	1.54 ± 0.44		1.63 ± 0.30		1.60 ± 0.22		1.76 ± 0.47	
22:6 n-3	29.81 ± 1.22		29.14 ± 1.71		29.30 ± 2.17		30.18 ± 1.99	
ΣPUFA	53.62 ± 1.14		53.54 ± 1.49		53.40 ± 1.05		53.61 ± 1.81	
ΣPUFA (n-6)	4.39 ± 0.91		4.36 ± 1.38		3.41 ± 1.11		3.90 ± 0.99	
ΣPUFA (n-3)	48.92 ± 1.01		48.90 ± 1.51		49.63 ± 1.25		49.41 ± 1.64	
n-3/n-6	11.61 ± 2.59[b]		12.35 ± 4.14[ab]		15.63 ± 3.92[a]		13.45 ± 3.62[ab]	

Table 3-5 Fatty acid composition (wt. % of total fatty acids) of the phosphatidylserine/phosphatidylinositol of cod liver (mean values ±standard derivation)

	Tampen		Egersund	
	Female (n=12)	Male (n=10)	Female (n=11)	male (n=10)
14:0	1.15 ± 0.68	0.90 ± 0.44	0.88 ± 0.37	0.93 ± 0.25
15:0	0.15 ± 0.08	0.13 ± 0.04	0.13 ± 0.04	0.14 ± 0.04
16:0	5.70 ± 1.13	5.03 ± 1.09	5.98 ± 1.19	6.42 ± 3.38
i-17:0	0.23 ± 0.08	0.23 ± 0.07	0.18 ± 0.04	0.22 ± 0.05
17:0	1.05 ± 0.50	1.01 ± 0.68	0.80 ± 0.40	0.87 ± 0.76
18:0	11.66 ± 1.80	12.92 ± 1.74	13.16 ± 2.68	12.02 ± 1.74
ΣSFA	20.11 ± 1.66	20.36 ± 2.88	21.28 ± 2.91	20.75 ± 4.37
16:1 n-9	0.36 ± 0.16	0.37 ± 0.08	0.35 ± 0.16	0.41 ± 0.11
16:1 n-7	2.83 ± 0.58	2.63 ± 0.24	2.59 ± 0.32	2.58 ± 0.50
17:1 n-x	0.28 ± 0.17	0.25 ± 0.05	0.23 ± 0.09	0.22 ± 0.06
18:1 n-11	1.14 ± 0.25	1.10 ± 0.33	1.32 ± 0.32	1.30 ± 0.50
18:1 n-9	4.90 ± 0.71	4.70 ± 0.56	4.33 ± 0.56	4.17 ± 0.75
18:1 n-7	3.17 ± 0.44	3.08 ± 0.35	3.02 ± 0.31	3.12 ± 0.39
18:1 n-5	0.37 ± 0.35	0.26 ± 0.03	0.28 ± 0.04	0.30 ± 0.08
20:1 n-11	0.55 ± 0.15	0.50 ± 0.10	1.04 ± 1.85	0.44 ± 0.10
20:1 n-9	3.73 ± 1.39	3.54 ± 1.11	4.53 ± 1.88	4.60 ± 0.87
22:1 n-11	0.84 ± 0.32	0.78 ± 0.41	0.94 ± 0.50	0.79 ± 0.31
24:1 n-9	1.03 ± 0.58	1.48 ± 0.49	1.38 ± 0.57	1.21 ± 0.54
ΣMUFA	25.36 ± 14.27	24.46 ± 10.49	25.80 ± 13.03	26.21 ± 14.67
18:2 n-6	1.03 ± 0.15^{ab}	1.12 ± 0.11^{ab}	0.92 ± 0.16^{b}	1.14 ± 0.26^{a}
20:2 n-6	0.59 ± 0.10	0.58 ± 0.13	0.53 ± 0.08	0.61 ± 0.12
20:4 n-6	11.67 ± 2.08	12.30 ± 2.08	11.09 ± 2.88	10.40 ± 1.66
22:4 n-6	0.18 ± 0.10	0.15 ± 0.09	0.11 ± 0.08	0.13 ± 0.10
22:5 n-6	0.79 ± 0.13	0.80 ± 0.13	0.66 ± 0.14	0.68 ± 0.16
18:3 n-3	0.36 ± 0.06^{ab}	0.36 ± 0.06^{ab}	0.33 ± 0.06^{b}	0.43 ± 0.13^{a}
18:4 n-3	0.21 ± 0.09	0.21 ± 0.06	0.25 ± 0.06	0.24 ± 0.10
20:3 n-3	0.28 ± 0.08	0.28 ± 0.08	0.25 ± 0.04	0.30 ± 0.11
20:4 n-3	0.48 ± 0.08	0.52 ± 0.06	0.47 ± 0.05	0.48 ± 0.09
20:5 n-3	6.43 ± 1.13^{b}	6.38 ± 0.88^{b}	8.22 ± 1.92^{a}	7.64 ± 1.39^{ab}
22:5 n-3	1.90 ± 0.34	1.98 ± 0.32	2.10 ± 0.54	1.81 ± 0.33
22:6 n-3	35.96 ± 3.25	35.53 ± 3.82	32.83 ± 5.32	35.28 ± 6.07
ΣPUFA	60.32 ± 2.88	60.60 ± 4.58	58.29 ± 5.13	59.55 ± 6.90
ΣPUFA (n-6)	14.26 ± 2.07	14.96 ± 2.23	13.32 ± 2.90	12.96 ± 1.66
ΣPUFA (n-3)	45.75 ± 3.29	45.35 ± 3.68	44.61 ± 5.32	46.29 ± 6.23
n-3/n-6	3.29 ± 0.61	3.09 ± 0.51	3.51 ± 0.97	3.60 ± 0.55

(Numbers with different letters are significantly different, $p < 0.05$)

Several statistic differences were observed in the aforesaid monoenes in two different areas. Higher amount of 18:1 *n-9* and lower amount of 20:1 *n-9* were detected in high offshore oil activity field. Both of these two *n-9* MUFAs are main fatty constituting phospholipids and bilayer of membranes. Besides, they all could be oxidized from 22:1 *n-11* which was of high amount in dietary. Even though there are some links between fatty acids of phospholipids, few if any detailed studies have been carried out on mechanisms of phospholipids in fish. My present understanding of this area in fish rests heavily on extrapolations from the situation in mammals, which is itself imperfectly understood. Some authors argued that 22:1 *n-11* is more likely oxidation to 18:1 *n-11*[70,74] in mink and gray seal. The differences of these two monoenes may support the hypothesis of altered fatty acid metabolism by offshore oil production proposed by Balk *et al.*[2]. Similar as the report of Balk *et al.*[2], the ratio of *n-3* to *n-6* fatty acids was reduced in Atlantic cod from Tampen compared with control area (Egersund). The ratio is both an indicator of the fatty acid composition and a measure of the nutritional value for human consumption. Some explanations were listed by Balk for the changes. First, petroleum hydrocarbons may accumulated in the membranes, thereby altering their properties, or interfere directly with metabolic reactions and/or molecular signaling regulating the fatty acid composition of membranes[2]. This propose was given by adapting the results from Meier et al.'s research. They found that reduced amount of *n-3* fatty acids were confirmed in Atlantic cod exposed in the laboratory to APs.[9] However, our study is pointing another possibility. It could be higher amount of n-6 fatty acids were biosynthesized in liver. The ANOVA showed a confidence level $p = 0.141$ to support the difference in statistic. This hypothesis of higher amount of n-6 fatty acids works in concert with another observation, the concentration of 20:4 *n-6* in the liver was elevated in Atlantic cod from Tampen. 20:4 *n-6* is an important constituent of biological membranes. The elevated amount of 20:4 n-6 also indicates rapid mobilization of reserved lipids could happened in liver of Atlantic cod in high offshore petroleum activity area, which is agree with the explanation of lipid distribution changes. Another explanation for low ratio of *n-3/n-6* is that oxidative stress alters the fatty acid composition of the membranes by lipid peroxidation. This explanation was proposed based on other environment effects investigation. Natural factors, like temperature and diet are less likely to be responsible for the fatty acid composition changing.

3.2.4 Principal Components Analysis (PCA)

PCA is a way of identifying patterns in high-dimensional dataset, and expressing the data in such as way as to highlight their similarities and differences. A comprehensive tutorial on PAC is given in by Wold *et al.*[75]. Generally, the original objects and variables can be investigated by score plots and loading plots. Some experience is needed to interpret PC-plots; I used PCA to explorative analysis the similarities and difference between the fatty acid compositions from two areas and interpret the plots based of some simple rules. The distance between the objects in the score plots is used to measure the similarity between objects and the direction between variables in loading plots explains the correlation between variables.

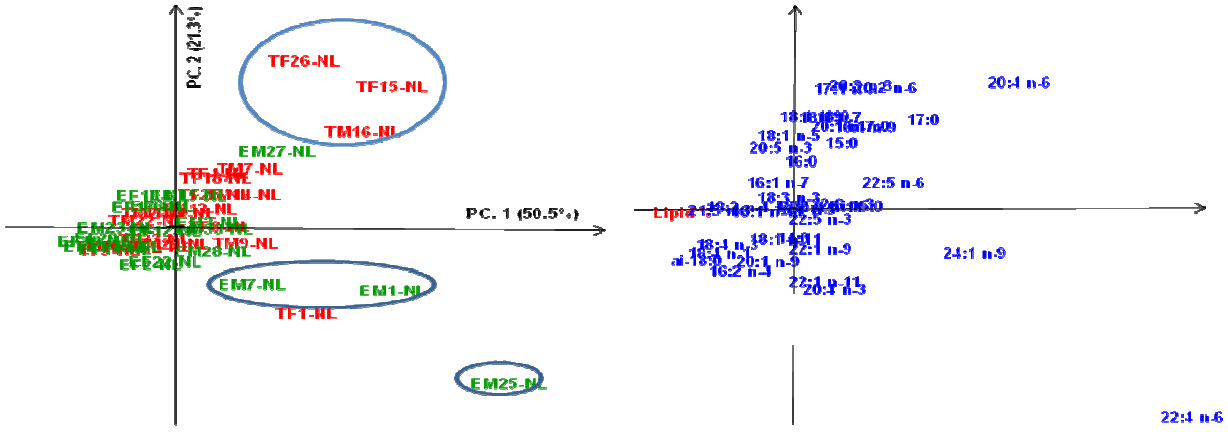

Figure 3-3 PCA plots of neutral lipids of cod liver. TF: Tampen Female; TM: Tampen Male; EF: Egersund Female; EM: Egersund male.

The PCA plots using samples as objects and fatty acid profiles as variables are present in Figure 3-3. One extremely lean sample was detected as outline, which shows very high level of phospholipids. The main variance in the dataset, 50.5% is explained by PC1, while 21.3% is caused by PC2. Major objects were located around origin. There are three Tampen samples are separated from majorities because of high level of 20:4 *n-6*. These samples are all have lower Fulton conditions relative other Atlantic cods from the same area. One Egersund sample was characterized by higher amount of 22:4 *n-6* while another two owned more 24:1 *n-9*. The total lipid content was also included in the PCA model. All the separated samples were of lower lipid content. The lipid content has adverse correlation with 20:4 *n-6* and 22:4 *n-6*.

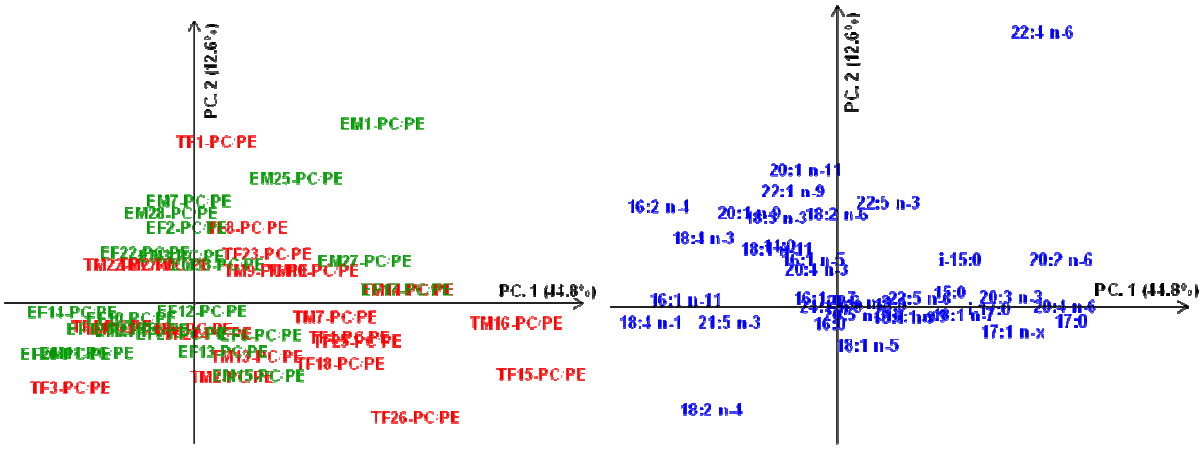

Figure 3-4 PCA plots of PC/PE of cod liver. (Abbreviation as in legends to Figure 3-3)

No interesting observation was found in phospholipids PCA analysis (Figure 3-4 and Figure 3-5). There is no obvious group of samples to reveal similarity. The objects from two areas are mixed. The PCA analysis suggests that no clearly differences of fatty acid composition are observed between the two areas.

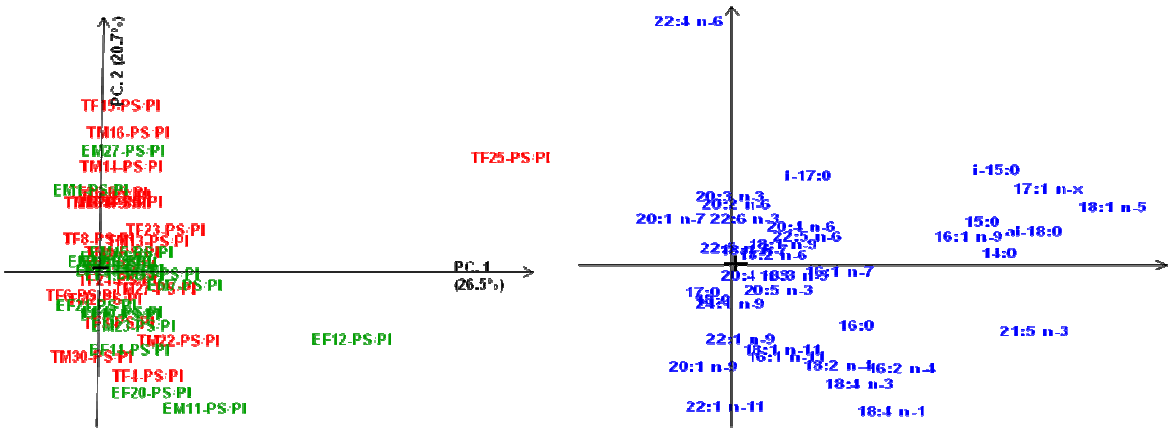

Figure 3-5 PCA plots of PS/PI of cod liver. (Abbreviation as in legends to Figure 3-3)

3.3 Conclusion

Forty-three of wild Atlantic cod fish (*Gadus morhua*) were analyzed by the two-step extraction we proposed. The lipid distribution and fatty acid composition of different lipid classes were studied. The total lipids content was correlated to liver size of samples. There was no significant difference of lipid distributions between two areas. More MUFAs were observed in neutral lipids of wild samples. High level of 22:1 *n-11* was detected in neutral lipids as an indicator of diet. Higher amount of 18:1 *n-9* and lower amount of 20:1 *n-9* were found in phospholipids at Tampen. Lower level of *n-3*/*n-6* was also observed, which could be resulted by increased amount of *n-6* series FAs. These observations support the hypothesis of altered fatty acid metabolism proposed by Balk *et al.* However, the evidences were limited by the small sample size and larger biological variance. The Principal Components Analysis (PCA) did not reveal clearly group of objects. No effect of offshore petroleum activity on fatty acid composition in liver of Atlantic cod (*Gadus morhua*) can be concluded based on present study.

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