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Extraction and Analysis of Marine Lipids with Emphasis on Phospholipids- Evaluation and Improvement of Methods

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Summary

The unique and health- benefiting omega-3 long chain polyunsaturated fatty acids (LCPUFA) are present in high amounts in marine lipids and phospholipids (PL). These can give social, economic and environmental benefits. In addition, phospholipids are important natural examples of emulsion stabilizers, which are commercially produced at levels in excess of 250,000 tones per year. Krill and roe are good sources of PL and omega-3 LCPUFA. Krill products are becoming more popular in the marked, for example krill oil supplements.

Methods for extraction and characterization of marine lipids and PL are important for nutritional labeling of foods, in science as well as in industry. Today, numerous methods for the characterization of lipids exist. At SINTEF "Fiskeri og havbruk" research to evaluate efficient extraction methods in order to recover high lipid and PL yield were an effort.

This study conducted five aims of marine raw materials. The fist aim was to evaluate the significance of the mono and biphasic proportions during lipid extraction by Bligh and Dyer (B&D). The second aim was to study the capacity of different solvent systems in ASE to extract lipids and PL concentrations. The third aim was to investigate the performance of three extraction steps on the recovery of total lipid and PL amounts. The fourth aim was to evaluate the efficiency for complete lipid recovery of multiple extraction steps by B&D using washout curves. The fifth aim was to improve a method of analyzing PL-classes by HPLC-CAD.

Evaluation of the significance of solvent proportions in B&D extracts was conducted by comparing the modified B&D at SINTEF (in short SINTEF B&D) and the original approach. The two B&D approaches represent two different solvent proportions used in extractions. Total lipids and PL extracted by three extraction steps of herring roe were found. A theoretical argument was used to choose a reference method in further experiments. In this study, it was decided to use the original B&D method. Further, it was decided to employ the standard parameters for ASE to determine the extraction capacity from the basis of the solvents. ASE with chloroform and ethanol were tested. Both moist and dry samples with different lipid composition were applied. The results were compared to the B&D results. The sample compositions were determined from lipid-class analysis by Iatroscan. On the basis of the results and discussions it was decided to employ ASE ethanol for further research. Three extractions were performed on two different krill meals (*E. Superba*) and herring roe (*Clupea harengus*). Based on earlier results, a hypothesis was tested. It asked whether ASE chloroform: methanol 2:1, as well as crushing the roe and krill meal, would yield similar or more PL and lipids as the B&D method. In a washout curve, the PL and lipid concentrations remaining in the samples after extractions were plotted logarithmic as a function of extraction steps. The curve was relatively well fitted. Extrapolation from linear regression gave total lipid concentration of the sample. The extraction numbers needed to yield a complete recovery of lipids and PL were calculated from the linear regression. Mass balance between extrapolation values of similar samples obtained by different methods was decided. Several calibration steps in HPLC-CAD were performed. Afterword, the PL-classes as

well as the total PL concentration of all the extracts were determined. The PL values obtained from analysis by Iatroscan TLC-FID and HPLC-CAD was compared to find if the methods gave similar results.

The conclusions related to the aims of this work are summarized in the following paragraphs. By one extraction step, a modified method with higher water content within its solvent proportions achieved higher lipid and PL recovery for herring roe. Unfortunately, the theory states that the solvent proportions of the method applied may result in impurities of proteins within the extracts. The experiments indicate that the significance of solvent proportions had no impact on the lipid and PL recovery when three extractions were performed.

Observations of solvent systems capacity, using ethanol and chloroform, found that ASE ethanol gave higher PL recovery of meal samples than the B&D method. Furthermore, B&D achieved superior PL amounts of roe with one extraction step. As expected, the optimal solvent system for lipid recovery is dependent on the lipid composition of the sample. Samples high in TG favored non-polar solvent systems, like chloroform. In contrast, samples high in PL favored more polar solvents, like ethanol.

Experiments indicate that for samples high in PL and lipids, such as krill meal, yield sufficient amount of lipids and PL after three extractions. ASE ethanol, ASE chloroform: methanol and B&D are methods that can be used for several extractions with this objective. On the other hand, increasing extraction steps may be costly and more time consuming. The use of higher amounts of solvents may also be an issue.

Extrapolations of the washout curve of B&D extracts may give the total concentration of a component within a sample. Estimations of efficient lipid extraction steps can be obtained from this curve by linear regression. In this study, mass balance was obtained for krill meal 1, but not for herring roe. A suitable PL-class HPLC - CAD has been improved by calibration.

List of Abbreviations

ALA	Alpha Linoleic Acid
ASE	Accelerated Solvent Extraction
B&D	Bligh and Dyer
CAD	Charged Aerosol Detector
CHOL	Cholesterol
Chloroform phase	The lower layer in the biphasic solvent system of B&D
CN-analysis	Carbon and Nitrogen analysis
CV	Coefficient of Variation
CVD	Cardio Vascular Disease
DE	Diatomaous Earth
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty Acids
FFA	Free Fatty acids
FID	Flame Ionization Detector
HPLC	High Performance Liquid Chromatography
LCPUFA	Long Chain Poly Unsaturated Fatty Acids
LCUFA	Long Chain Unsaturated Fatty Acids
LN	The Natural Logarithm
LPC	Lyzophosphatidylcholine
LPE	Lyzophosphatidyletahnolamine
PA	Phosphatic Acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylionsytol
PL	Phospholipids
PUFA	Poly Unsaturated Fatty Acids
SEM	Standard Error of Mean
STD	Standard Deviation
TG	Triglycerides
TLC	Thin Layer Chromatography
Water phase	Water/methanol phase- the upper layer in the biphasic solvent system of B&D

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Chapter 1

Introduction

1.1 Background

For almost nine decades ago it was documented that the Canadian Inuit people, despite having a diet rich in fat (c. 39% of their calories), were free of cardiovascular disease (CVD), hyperglycemia, diabetes and cancer. In comparison, the Danish people, who consumed similar amount of fat (42%) experienced ten times higher death rate from CVD. The attributed contrast was the Intuits high consumption of omega-3 fatty acids and the Danes high consumption of saturated fat and trans fat[21]. Today, evidence suggests that omega-3 PUFA exert a protective effect in biological tissues [77]. Positive impacts of omega-3 LCPUFA are protection against heart attack, lowering high blood pressure and making the blood less likely to clot. A new result from treatment of cancer cachexia with salmon oil showed an increase in EPA and DHA in plasma phospholipids. Researchers think that EPA may prevent constant breakdown of phospholipids (PL) in tumor cells[98]. Knowledge of and interest in the heath benefits of marine lipids and phospholipids are growing in today's society, industry and research. A fast increasing amount of the world's population is suffering either from lifestyle diseases or from malnutrition. Many people are getting more aware of the health benefits provided from marine lipids, and some wish to consume healthier food. WHO recommends for the general population a regular consumption of Omega-3 PUFA (EPA and DHA) of 200-500mg twice a week, which approximate 1-2 servings of fish a week. This will be protective against CVD and stroke[55].

Fish oils and marine products are the major source of omega-3 PUFA[26]. Some seafood species that feed on for example microalgae contain high amounts of the omega-3 types EPA and DHA that is insufficient in other foods[108].

Fish roe products, such as caviars, are expanding on the international marked. In many roe products, for example from herring and cod, the dominant lipid component are PL[7]. Krill oil is a food source that is high in omega-3 PUFA and PL . In the future, opportunities to increase seafood harvest from traditional sources are limited and alternative sources such as krill may be required as sustainable supplies to achieve benefits of health, economic, social and environmental issues[70].

Marine lipids and phospholipids are used in foods, pharmaceutical, nutritional and cosmetic industry. PL is used to stabilize emulsions, and are important components in admixtures that are produced commercially at levels in excess of 250,000 tons per year[37]. Products with fortification and supplements that contain high quality marine lipids are getting more popular on the market; an example are all the omega-3 and krill supplements competing on the market and liver pâtè fortified with omega-3.

For food labeling requirements, health concerns or commercial reasons there is need to measure total lipids and to characterize processed and unprocessed food products[72]. Also, estimation of lipids in an aquatic animal can provide insight into growth strategies, health and condition, and survival potential[79].

In analysis of foods and their lipid extracts it is usually necessary to extract the lipids prior to analysis. The Bligh and Dyer (B&D) method is an established extraction method. However, it uses relatively high amounts of heavily volatile solvents, relies on manual work and can be time consuming. This can harm the laboratory workers as well as being inefficient. Accelerated Solvent Extraction (ASE) is an extraction method that relies on a mechanically driven machine. This method may have the potential to replace some of the older methods, and therefore achieve economical and health benefits for producers and their workers. Earlier research from project of Kalstad[49] developed a method using ASE300 for lipid extractions in general. Later, research from Master Thesis of Madsen[58] found that the lipid extracts contained close to only non-polar lipids, and that the total lipid yield was lower for ASE than for B&D. It has been discovered that the effectiveness of the lipid extraction will to a large extent depend on the solvent system employed[10].

Unfortunately, an abundance of phospholipids (PL) may remain in the sample after extraction[93]. Phospholipids interact with proteins and other polar components. For this reason, it can be difficult to extract PL without having any proteins joining the solution. Another issue is that it is important to exploit the raw material to a maximal extent. This requires efficient extraction methods to yield high concentrations of lipids and phospholipids. Higher yields of PL can occur if more than one extraction is carried out. However, it is uncertain if several extractions are efficient for all products. In, addition this may be costly and the high amounts of toxic waste that is produced from the extraction may be difficult to get rid of[92].

At SINTEF "Fiskeri og havbruk" a need for a simple and efficient method for separation and detection of PL-classes has been emphasized. High Performance Liquid Chromatography (HPLC) is a method that can be used for PL-class or lipid class analysis[72].

Research at SINTEF "Fiskeri og havbruk" was conducted to study the ability of different methods to extract lipids and PLs. The emphasis was to use marine samples to yield high quantity of PL and lipids. From the basis of this effort, five aims were constructed:

- to evaluate the significance of the mono and biphase solvent proportions during lipid extraction by Bligh and Dyer (B&D).
- to study the capacity of different solvent systems in Accelerated Solvent Extractor

(ASE) to extract marine lipids and phospholipids (PLs).

- to investigate the effects of three extraction steps on the recover of total lipid and PL amount.
- to evaluate the efficiency of extraction steps by B&D with washout curves for complete lipid recovery.
- to study the possibilities for improving a method analyzing phospholipids classes of marine lipids by HPLC-CAD.

1.2 Lipids

[18] describes the term lipid as:

> Lipids are a group of substances that generally speaking denotes a heterogeneous group of substances associated with living systems, which have the common property of insolubility in water but solubility in non-polar solvents.

Lipids are often referred to as fats and oils, but the lipid family also includes triglycerides, phospholipids and sterols. Fats give food a creamy mouth feel, and provide flavor and texture to foods. Most of the food we eat contains some fat. Fat serves as one of the main energy sources, an important component in hormone production and it supplies and act as fat-soluble vitamins (A, D, E and K). Protection of the organs and the body (some act as pigments) is another important purpose of fats. Although some fats are essential for good health, it has a bad reputation and its importance is often misunderstood[13].

1.2.1 Fatty Acids

Fatty acids (FA) are an essential part of most lipids and they provide a fundamental understanding of lipid structure and properties. Several different fatty acids exist and these can be described from their trivial names or their systematic names. The trivial name of a fatty acid is often linked to the source of the FA (for example palmitic which comes from palm oil) or to the scientist who first described it. Internationally accepted rules agreed upon by organic chemists and biochemists are the basis for systematic names. In this report the fatty acids are described as numbers, example 18:2(9,12) describes a fatty acid having 18 carbon atoms (in a straight chain) with two double bonds at position nine and twelve. When counting, the COOH=1 and the end of the molecule (the last methyl group, CH3, of the chain) is called omega (ω). An omega-6 or n-6 indicates that the first double bond occurs on carbon 6 counting from the methyl group[36].

The length of fatty acids is usually between 4 and 24 carbons. The fatty acid chains can be saturated, mono or polyunsaturated. A saturated fatty acid contains only single bonds between its carbon atoms. Two carbon atoms that is missing a hydrogen atom

each and form a double bond, is a monounsaturated fatty acid chain. A polyunsaturated fatty acid has two or more double bonds in its carbon chain. The shape of the unsaturated fatty acids can make a bend in the chain or it can be strait, zig-zagged. The double bonds that form a strait chain is called trans and the chains with a bend consist of a cis double bond[18]. The term long chain polyunsaturated fatty acids (LC-PUFA) is referring to chains having more than 18 carbon atoms[11]. Considering a nutritional significance the most important polyunsaturated fatty acids are the Omega-3 (α-linoleic acid) and the Omega-6 (linoleic acid) (see Figure 1.1)[13].

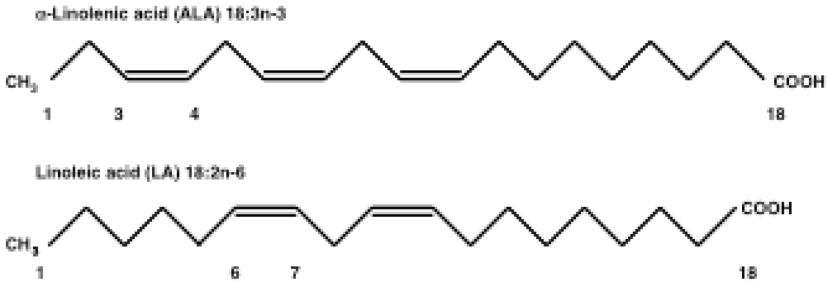


Figure 1.1: Chemical structure of representative polyunsaturated FA (Cis configuration), n-3 (α-linoelic acid) and n-6 (linoleic acid)[77].

1.2.2 Omega-3 Fatty Acids

Humans are unable to desaturate omega-3 bonds and therefore, the omega-3 fatty acids are considered essential fatty acids. In addition, the human body cannot convert short chain to long chain omega-3. When insufficient amounts of omega-3 are consumed, growth can be impaired and vulnerability to a number of diseases will probably increase. It is shown that LC omega-3 protect against heart attacks, lowering high blood pressure, making blood less likely to clot and influence the narrowing of arteries. Therefore it is available as treatment for patient suffering from these problems[70]. The most common dietary omega-3 fatty acids are alpha α-linoleic acid (ALA; 18:3n-3), docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3). In many cases a high concentration of ALA is found in vegetables, nuts and seeds[21]. In contrast, EPA and DHA are almost exclusively found in certain types of fish, which consume algaes and one-celled microorganisms that are able to synthesize these fatty acids (containing EPA biosynthesis genes)[108]. Examples of these types of fish are Kippered herring (17% EPA +DHA of total fat) and Atlantic wild Salmon (23% DHA+EPA of total fat)[21]. Unfortunately, PUFA easily undergoes oxidation and undesirable changes. Therefore proper processing, handling and storage are crucial. An example of changes are cis to trans isomers during deodorization (removal of fishy smell) of fish oils with EPA and DHA[26]. To prevent oxidation a reduction of air access and addition of antioxidants is desirable[32].

The important functions of omega-3 are to serve as major component of biological membrane, to alter gene expression and for eicosanoid production[11]. ALA is a precursor for synthesis of EPA and DHA, which further is synthesized to eicosanoids (see Figure 1.2). Hence, EPA and DHA undergo minimal processing before integration into membranes[76]. Furthermore, ALA (omega-3) and LA (omega-6) competes for the active site of the enzyme δ6-desaturase (the enzyme crucial for a conversion in the first step of synthesis), which means that a diet high in omega-6 restrict ALA conversion[23]. It has been suggested that men convert 8% of ALA to EPA and less than 0.05-4% to DHA. While women convert 21% of ALA to EPA and 9% to DHA (see Figure 1.2, step 1). Therefore separate recommendations for intake different types of omega-3 are often given[11]. Another large intervariability concerning the omega-3 metabolism considers the age, genetic determinants and concurrent associated diseases. The WHO gives a daily-recommended intake (DRI) of average c. 50g/d. Today the global population average n-6/n-3 ratio is 20/1 meanwhile the recommended ratio is 4/1[64].

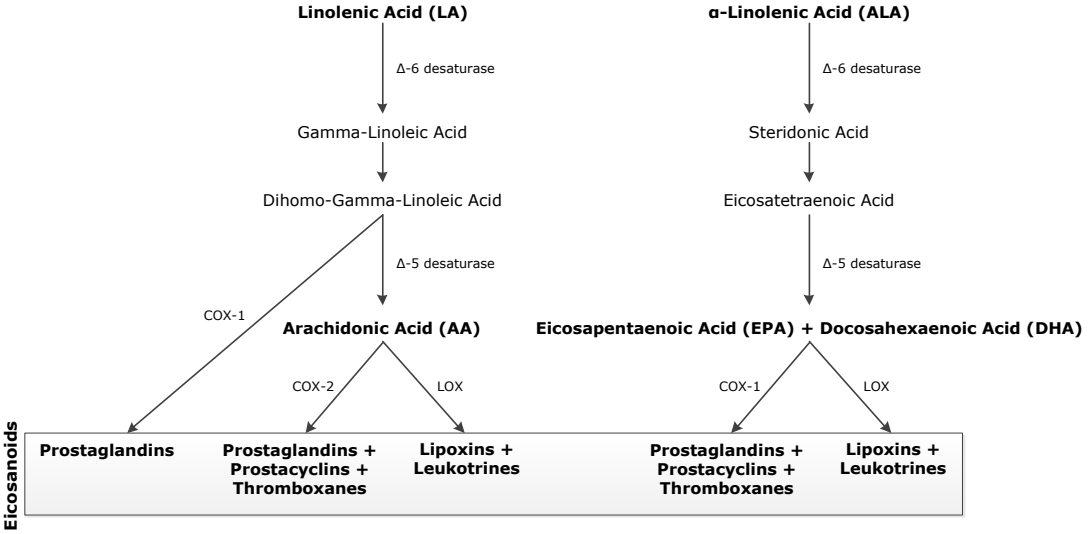


Figure 1.2: Dietary fatty acid metabolism and synthesis of eicosanoids from omega-6 and omega-3 fatty acids. The metabolic conversion of ALA (α-linoleic acid) to EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), and of LA (Linoleic acid) to AA (arachidonic acid) occurs in the ER (endoplasmatic reticulum, an organelle). The enzymes δ5-and δ6-desaturases are associated with the membrane. From this step the metabolism of EPA, DHA and AA to eicosanoids occurs. COX (Cyclooxygenase) and LOX (Lipoxygenase) enzymes metabolize EPA, DHA and AA to produce eicosanoids (thromboxanes, leukotrienes, prostagladines, lipoxins and prostacyclins). Eicosanoids play a critical role in cell homeostasis at low concentrations. At high concentrations they are involved in inflammation, cell proliferation and tumor genesis[77].

1.3 Lipid Classes

Lipid classes can be categorized into neutral, non-polar, and polar lipids. Neutral lipids are triglycerides, some ether lipids and sterol and wax esters. The polar lipids are phosphoglycerolipids (phospholipids are derivatives of it), sphingolipids and glycosyldiacylglycerols[36].

1.3.1 Triglycerides

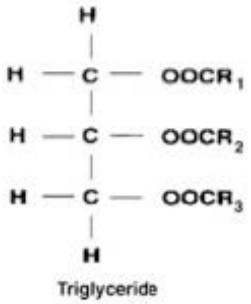


Figure 1.3: Structure of a Triglyceride[91]. R1, R2 and R3 represents fatty acids with variable chain length and unsaturation.

Triglycerides are built from a glycerol backbone and on each of glycerol's 3-hydroxyl group there is a fatty acid attached. These compounds are made from an esterification process. The fatty acids are attached to the glycerol from its carboxyl end and have different length of carbon and hydrogen linked together with a methyl group at the end (see Figure 1.3). The triglyceride composition can have very different saturation within its fatty acids. Fats and oils contain mixtures of different triglycerides[18].

1.3.2 Phospholipids

Phospholipids (PL) are important components of the membranes in the cell. Including both the membrane surrounding the cell, the plasma membrane, and the membranes that comprise the organelles inside the cell (such as the mitochondria). The PLs occur in small amounts of oils and crude fats and are a major constituent in egg yolk. Phospholipids are amphiphilic molecules. This means that a part of the molecule, which compose triglycerides, are non-polar/hydrophobic and another area of the molecule, which compose phosphate (PO_4), is polar/hydrophilic. These structural elements make molecules with large hydrophobic heads and long hydrophilic tails (see Figure 1.4). Because of the amphiphilic character PL tend to form bilayers, micelles or liposomes in water. Due to the polar and nonpolar regions of the PL oriented at the interphase between two immiscible phases PLs act as emulsion stabilizer (example in mayonnaise)[18]. Lysophospholipids occur in small amounts in cell membranes and they play a wide role

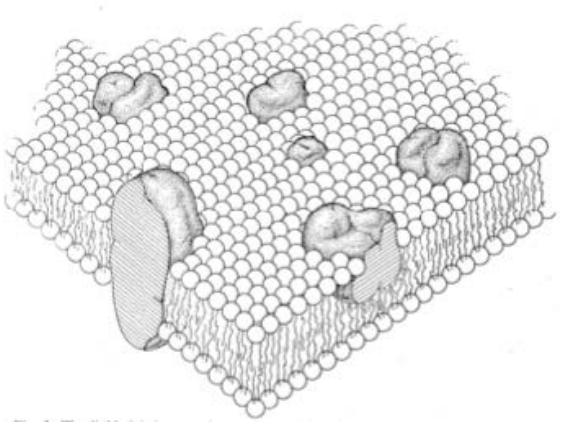


Figure 1.4: Fluid mosaic model of a cell membrane cross-section. The large globular proteins are integrated in a cell membrane composed of mainly phospholipids[45].

in cellular processes. Example LPC (see Figure 1.5) plays a role in signal transduction of protein kinase C, gating several membranes and lipid pore formation in bilayers[63]. New research suggests that LPA plays a part in regulating the integral and functional homeostasis of gastrointestinal mucosa[99].

Two basic classes of PL are glycerophospholipids and sphingolipids, The glyserophospholipids are built from FA, glycerol, phosphoric acid and a second hydroxyl, which can be choline, ethanolamine, serine, inositol and glycerol (see Figure 1.5)[37]. Lecithin from soya bean oil is called commercial PL and it contains a mixture of glycerolipids, typically around 15% PC, 12%PE and 10% PA and the rest is mostly TG[18].

The head group of PLs can be electrically neutral. In a neutral solution it has a zwitterionic character (both negative and positive charge) or is negatively charged, depending on the type of PL. Most PLs are soluble in chloroform, dichlormethane, hexane, methanol, ethanol, isopropanol and in mixtures of some of these solvents. The most common mix for dissolution and elution of PL are a mixture of hexane, isopropaniol and water. This mixture of solvents is preferred because of toxicological and environmental perspectives. Water is a poor solvent for PL, example PC with 16-18carbon atoms has a solubility of 10^{-10}M in room temperature[84]. The charge of the PL is dependent on the PH of the solution. The pka differs from the PL class, and reveals when the PL is deprotonated or protonated. A great change in PH over the pka values affect the PL properties and change its conformational structure and overall polarity. A great impact on the physical properties of PLs in water is the degree of unsaturation, which is normally 1-3 double bond in the lipid. If the PL has a negative charge the colloidal stability of dispersed systems increases, example emulsions and liposomes. If a salt is present the repulsive character diminishes and eventually leads to phase separation. A zwitterionic character of the PL possesses excellent dermatological properties, since these are unable to interact electrostatically in vivo[37]. Challenges in PL technology and extraction are

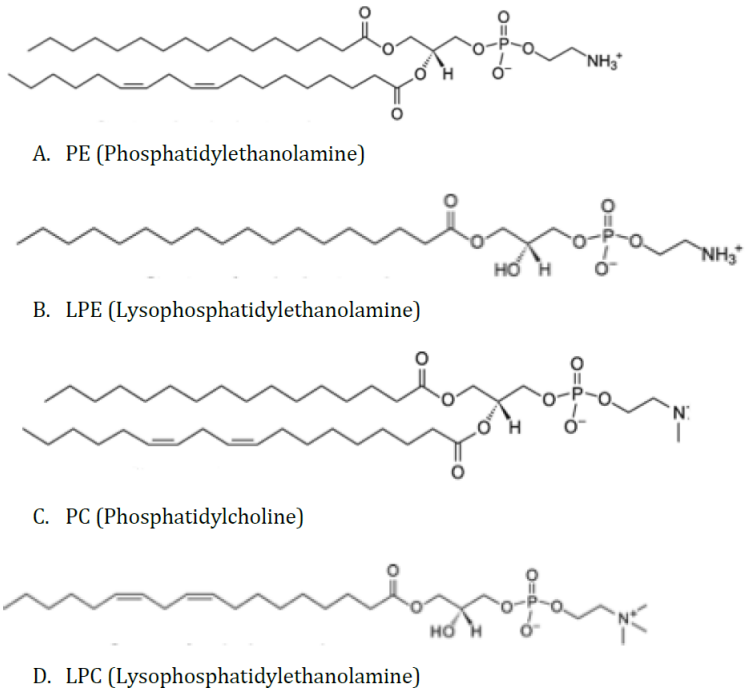


Figure 1.5: Structure of the major PL classes (predominant species) in living organisms[37].

that PL often is bound to proteins and separation could be difficult[3].

Modified lecithin is produced for increased stability and resistance of PLs to meet specific functional requirements; an example is more resistance to oxidation and therefore it has no taste or odor. Natural PL differs in both composition of class present and proportion of saturation in their FA chains, which is important for obtaining specific functions of biological systems. Also, all natural material contains small amounts of lysophospholipids. Only natural PL is allowed for food applications in Europe. However it is used in other products, example leather products, cosmetics, soaps, inks, paints, antioxidants etc. In the US it is permitted in all applications[46].

1.4 Marine Raw Material Rich in Phospholipids and PUFA

1.4.1 Roe

Roe is a term that describes fish eggs (ovocytes) gathered in skeins. Fish roe production is expanding on the international market, and Caviar is an example of a product made from roe. Extreme variability in the composition of fish roe has been detected. An example of such variation are lipid content measured from 8-32%[1]. Differences in species, environment, season and diets are some factors which explains the compositional

variability[7][2]. An extracellular envelope made of three layers of high- density proteins surrounds the plasma membrane of a fish egg. This envelope serves as a protection system and counteracts excessive mechanical pressure, like an eggshell[54]. Roe lipids contain high amounts of phospholipids, LCUFA and PUFA[43]. Herring roe lipid is the type of fish roe lipid that contains the highest amount of phospholipids[48]. Studies found that herring and salmon roe lipids had higher oxidative stability than fish oils from tuna and sardine, and that this is probably due to the higher presence of PL. Further research on rats fed with salmon and herring roe lipids found little increase in total cholesterol level of the rat plasma lipids, despite the fact that these fish roe lipids composed c. 6-10% cholesterol[67]. Studies indicating health benefits in addition to nutritional and sensorial values utilize fish roes as food materials[1][43].

1.4.2 Krill

Figure 1.6: Photograph of krill, Courtesy of Leif Grimsmo. Krill range in size from 0.01-2g wet weight and 0.8-6cm length[100].

Krill is a term originally applied to "fish fry", and generally the term involves various crustaceans that whales eat. In appearance, krill looks like shrimp (see Figure 1.6), possessing a chitinous exoskeleton, but differing from other crustaceans in many other biological properties, example external gills and luminous organs etc. Krill are one of the most populous animals species, and are found in oceans worldwide[100][24]. There are many existing krill species, but till now only Euphasia superba (the most abundant Antarctic Krill) and Euphausia pacifica (Pacific Krill) are harvested as a source for human supplements and food[9][100]. From a nutrient perspective, Krill is a source of high- quality protein, ranging from 32-50% dry weight in species M. Norvegica and Thysanoessa. In addition, krill possesses the advantage of low fat concentration, which varies from 2.7-6% wet weight of Euphausiva superba species dependent on season[85]. Lipid content and composition also depend on species, age and time between capture

and freezing[33]. The lipids compose high amounts of PLs, detected c. 44-47% of total lipids of Euphausiva superba, and long chain omega-3 fatty acids[28]. An overwintering strategy of Euphasia Superba involves lipid storage of both TG and PC, suggesting that PC not only serve as an essential component in membranes, but although unusual like and as a reserve lipid for this krill species[38]. Research found that most of the omega-3 fatty acids were bound to triglycerides in cod oils and to PL in krill oils. It is suggested that this difference causes the krill omega-3 to be more easily incorporated into membranes[106]. Benefits of less oxidative damage is probably higher in krill than in fish because of higher antioxidant levels in krill[100].

Comparison studies show that krill oil (2g/day, providing 390mg/day EPA:DHA 2:1), but not olive oil or menhaden oil (2g/day, providing 390mg/day EPA:DHA 1:1), could significantly decrease plasma 2-arachidonoylglycerol (2-AG) levels in obese subjects. It is suggested that 2-AG levels are influenced by the balance between n-6 and n-3 LCPUFA, which is present in optimal proportions in Krill oil[5]. Studies of phoshhatidylserine (PS) isolated from krill found that it gave a protective role, which might be useful for slowing the progression of age related degeneration of brain neurons. This may indicate that it can function as therapy for the improvement of diminished memory function in elderly people[53].

The Krill shell composes a high concentration of fluoride, and a survival decline of catfish having diets supplemented with high fluoride krill meal has been observed. This may limit krill meal as a fish feed resource[39]. Observations of shrinking populations of penguins in Antarctic are probably due to disruptions in food supply, which means that there is too few krill to eat. Warming Climate may cause the krill supply to decrease[96].

1.5 Extraction of Lipids

The total lipid content of biological samples is an important quantity used in many biochemical, physiological, and nutritional studies. Many different methods for determining total lipid content have been developed, example Folch et al, Bligh and Dyer and Soxhlet. Many of these extraction methods are time consuming and result in much manual work. Demonstrations found that the different methods receive different lipid recovery[59]. Soxhlet is probably the most common technique used for fat extraction from foods[15][104]. The effectiveness of a lipid extraction method depends on the chemical nature of the lipids as well as the type of association in which they occur in the cell. Membrane associated lipids, such as PL, require polar solvents to disrupt the hydrogen and electrostatic bonding with proteins. Lipids with a hydrophobic nature are extracted with non-polar solvents. Anyway, covalent bonding is not disrupted by any solvents, and can be a challenge in lipid extraction procedures[10].

1.5.1 Bligh and Dyer

Bligh and Dyer is a rapid and simple method developed for extraction and purification of lipids from biological material[8]. The original report described a method to determine

the total lipid content in fish muscle. However, the Bligh and Dyer method is widely used to measuring the total lipid content of whole fish and other tissues[44].

The Bligh and Dyer method was developed from examinations of the water-methanol-chloroform phase diagram (Figure 1.7). The sample is mixed with water methanol and chloroform in specific proportions, and homogenized to create a monophasic solution. Dilution with chloroform and water produce a biphasic system. The upper layer is made from methanol and water and the lower layer is chloroform. The water-methanol layer constitutes the polar components of the solution. Proteins, carbohydrates, free fatty acids and phospholipids are examples of cellular components that are dissolved in the water-methanol layer. The chloroform layer contains the non-polar components, like most lipids. Polar lipids, like PL, can also dissolve in this layer. The chloroform layer is isolated from the biphasic solution and the lipid content is determined when the lipids are purified[8].

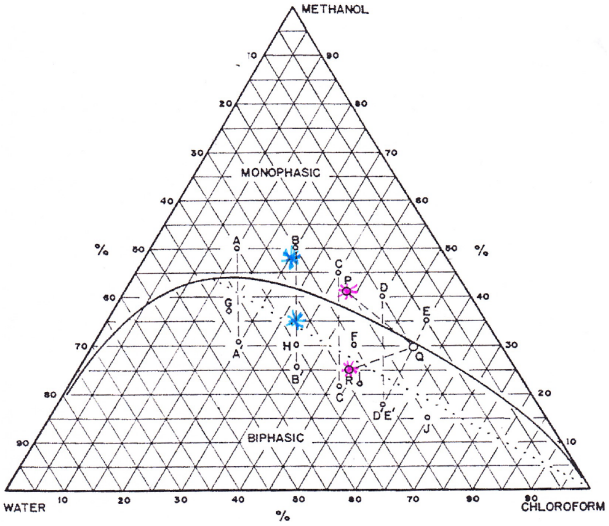


Figure 1.7: Chloroform-methanol-water phase diagram. The solvents are presented as %-proportion in a solvent system at 20°C[8]. Point R represents the biphasic proportions and point P represents the monophasic proportions of the original B&D method. The blue stars represent the proportions of modified B&D approach. Notice that the biphasic point lies above the chloroform tie line. This is commented in Section 3.5.2.

The chloroform-methanol-water phase diagram is shown in Figure 1.7. It describes a triangle solvent system of the solvents employed in B&D. Dependent on the solvent proportions the system can be biphasic or monophasic. The tie line that is drawn in the biphasic area is called the chloroform tie line. A biphasic system having compositions below or on the chloroform tie line results in a chloroform layer that is contaminated by water and methanol. Folch et al showed that this chloroform layer is contaminated with methanol and water, by determining chloroform insoluble material in the chloroform layer[25][8]. The point P represents the ideal solvent proportions of the monophasic

system, that is 1:2:0.8 chloroform: methanol: water. This point was chosen of two reasons; First, its solvent proportion yields the highest amounts of lipids. Second, it uses less chloroform for extraction and the chloroform layer in diluted state has no detected impurities. These solvent proportions can remove most of the lipids from the tissue. B&D found that 6% of the total lipids were left in the tissue of the cod muscle tissue. Analysis determined that these lipids represent membrane bound lipids and more polar lipids, like phospholipids and free fatty acids. The point Q is the composition of the solution after dilution with chloroform. Point R represents the ideal composition after dilution with water, which is 2:2:1.8 chloroform: methanol: water. Adding the solutes in this order is crucial. The chloroform has to be added before the water, and each state followed by mixing, to result in a rapid separation of layers and to obtain a high yield[8]. The Bligh and Dyer method is in fact thought to yield the recovery of more than 94% of total lipids[44].

Depending of the solvent composition there is a possibility that proteins are denaturized during the process. Protein inclusion with lipids may occur and affect the kinetics in the extraction. GC analyses of the chloroform phase reviled that the chloroform phase consists of 10% methanol in the Q mixture. Polar lipid components show a higher solubility in chloroform-methanol solution than in pure methanol and non-polar lipids are insoluble in methanol and water[93][8]. Demonstrations of similar applications of Bligh and Dyer methods performed in different labs show results that are comparable[80].

The primary advantage of the Bligh and Dyer method is a reduction in the solvent and sample ratio to (3+1): 1[44] , as compared to 20:1 solvent: sample ratio of Folch[25].Comparison of the Folch and the Bligh and Dyer methods found that samples containing less than 2% lipids gave similar yield, but that the B&D method underestimated the lipid content in samples with a high lipid content. An example is Herring samples, which resulted in about 45% lower extraction using the Bligh and Dyer method[44].

Discrimination of acidic phospholipids, which will partly appear in the aqueous phase, is an effect when a biphasic solvent system is exhibited. In comparison to the single-phase method of Christiansen, the Bligh and Dyer achieved lower than 10-35% yield of phospholipids[51]. Analysis of lipid extracts from cod flesh determined that some of the remaining lipids are inositides, many of the phospholipids was undefined because they are only partially separable[31][8].

Negative aspects of the B&D extraction method are that it uses lots of solvents and that this can be a waste disposal problem. The method can be slow and exhausting for the workers, because it relies on manual work. Also, for complete extraction no emulsions must occur between the two phases[92].

1.5.2 Liquid-Liquid Phase Extraction Washout Curve for B&D

Liquid-liquid extraction from a two-phase (B&D) system uses two immiscible solvents and is an equilibrium phenomenon that is governed by distribution laws. The extracted solute (Lipids or PL) distributes itself between both phases. One of the solvent systems, for example chloroform, dissolves the solute better than the other, for example

water/methanol. The concentration of the solute is therefore higher in this solvent, chloroform, than in the other phase, water/methanol. The relationship existing between these two-phase concentrations is in equilibrium.

$$[solute]_{water/methanol} \Longleftrightarrow [solute]_{chloroform} \tag{1.1}$$

Ideally the stabilized equilibrium between these concentrations is constant and not affected by temperature or concentration of the solute. The dissociation constant, K, is the equilibrium constant, and reveals the molar concentrations of the solute in each of the phases.

$$K = \frac{[solute]_{chloroform}}{[solute]_{water/methanol}} \tag{1.2}$$

The distribution constant can be used to calculate the concentration of solute remaining in a solution after certain number of extractions[10].

1.5.3 ASE

Accelerated Solvent Extractor (ASE) is an automated system that employs a machine to perform extractions (see Figure 1.8). The extraction procedure of ASE uses organic solvents at elevated temperatures and applies pressure to maintain the solvent at liquid state. A solid or semisolid sample is enclosed in a stainless steel sample cell. The sample cell is heated in an oven maintaining high temperatures and pressures. The cell is filled with extraction fluid and static extraction is carried out for short time periods. After heating and experiencing static extraction the sample extract is purged from the cell into a collection bottle by compressed gas[107].

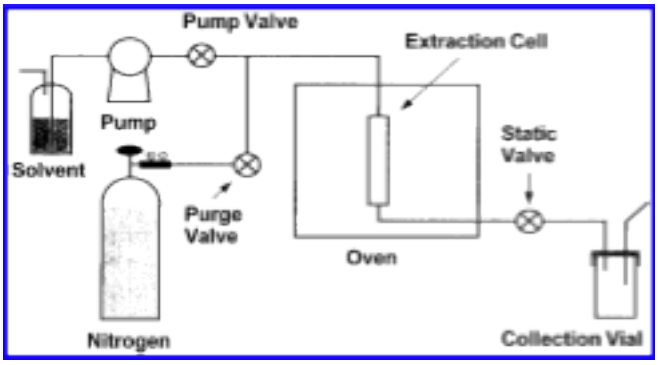


Figure 1.8: Overview of the Accelerated Solvent Extraction (ASE) system[82].

Enhanced performance by ASE lies within elevated temperatures and pressure. The increasing temperature results in faster diffusion rates, increasing the solvents to dissolve analytes and improved mass transfer and extraction rates. Moreover, disruption of equilibrium between bound molecules in matrix may occur and the thermal energy

can decrease the activation energy required for the desorption process. Higher temperatures increase the viscosity of the solvents, making it easier to penetrate the matrix. Increased pressure makes it possible to maintain solvents as liquids when the temperature is above boiling point. High pressure may increase the contact area by forcing solvent into "sealed" matrix areas or making the solvent rapidly coming in contact with the surface area of the entire sample. Studies of ASE extraction found no evidence of thermal degradation during the extraction of temperature sensitive compounds[82].

The high extraction efficiency of ASE elicit that less amount of sample is required than what is routinely used. Large quantities of solvent can be both harmful and expensive. In classical lipid extraction substantial consumption of sample (at least 1g) is often needed and the majority of the methods, including many modifications, are time consuming and cumbersome. Also contamination, loss and oxidation of sample is a high possibility for classical methods because extensive sample handling is required[22].

Employment of high pressures and temperatures reduce the solvent quantity needed for extraction and allow for faster analysis relative to other liquid-liquid extraction based techniques[82]. Unfortunately, high temperatures can lead to oxidation of the extracted lipids[95]. In comparison to Soxhlet, ASE has the advantage of being rapid, c.20 minutes instead of 8-18 hours, and requiring less solvent, c. 90ml instead of c. 200[17]. Also extraction by ASE is simple and operates in a single run, eliminating possible systemic errors and dependence of highly experienced workers[102].

ASE can be applied to extract organic compounds from a variety of samples. Example determination of PCB in Cod, lipid biomarkers from environmental samples and organic compounds from soils, sludges and waste and much more[17][57][78]. It has been suggested that because ASE is a mild analytical method and is suited for extraction to prevent degradation and formation of instable cholesterol[95].

Some boundaries using ASE is that solvnets with an auto ignition point of 40 to 300°C is not to be used, example diethylether and carbon disulfide, acid or bases. Also samples containing salts is usually avoided because the salt may accumulate in the machine[107]. Concentrated acid and base reagents can cause corrosion and interfere with reliable operations in the ASE instrument[102]. Evaporation of volatile solvents may occur during analysis, causing underestimation of the extract volume[58].

1.6 Analytical Methods for Qualitative Measurements of Lipid Classes

In the previous chapter some extraction methods were introduced. Overview of the lipid class composition in the extracted lipid can be established by analysis with Iatroscan TLC-FID. Detailed quantitative measurements are obtained by HPLC, where both the fat composition and its concentrations are revealed.

1.6.1 Iatroscan-TLC-FID

Thin layer chromatography (TLC) is a separation method, composing a liquid mobile phase and a stationary phase of a thin solid layer, which lies upon a sheet made of glass, plastic or metal. Traditional TLC was first introduced in 1958, but this method has been developed, altered and refined several times till present. Analysis of several samples in a single run is compatible on TLC. HPLC and TLC share similar theoretical and chemical concepts, for details see the HPLC section. Analysis process of TLC is described in general terms as: First, soluted sample stains are fixed to a distinctive area on the TLC plate. The sample solvent is evaporated before placed in a chamber employing the mobile phase solvent. Capillary forces cause the mobile phase to migrate on the stationary phase, transporting the sample components along with variable velocity. Attractive forces between the mobile phase and the components, dependent of the type and its functional groups, determine the transport velocity. When optimal separation of the components has occurred, the TLC plate is removed form the mobile phase chamber and the components are detected visually or by the employment of an instrument[35].

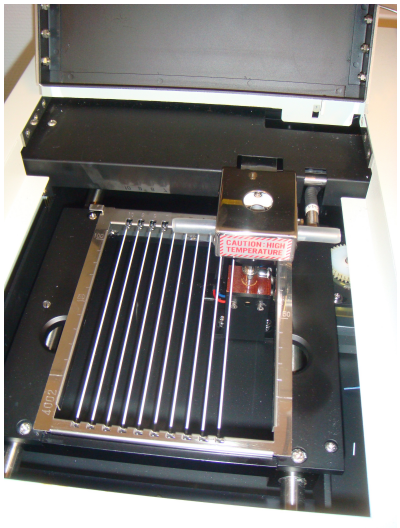


Figure 1.9: Iatroscan chromarods -III.

Iatroscan (Iatroscan TLC-FID) is an instrument that utilizes the resolution of thin-layer chromatography and the quantification of flame ionization detection, by combining them[19]. The Chromarods of the system constitute the TLC component (see Figure 1.9). They are quartz rods coated with a thin layer of soft glass powder and absorbent (Silica gel in this thesis) with specific particle size, referring to the nature of the separated sample mixture. The FID scanner consists of a hydrogen flame jet and an ion collector. The sample, which are eluted on the chromarods, are burnt during analysis. The ions produced from the sample burning are collected by collector electrode and the signal is amplified[88]. The sensitivity of the FID is dependent of many factors, e.g. hydrogen

flow rate, sample load, scan speed and the nature of the sample[42].

The Iatroscan approach can measure lipid classes, e.g. triglycerides, sterol esters, sterols, free fatty acids and phospholipids. It can also measure total lipids of a sample[56]. Unfortunately, the instrument may lack quantitative accuracy and reproducibility. Early studies of TLC reported relative standard deviations of 6-83% for this method using low sample loads[27]. Since then, more sensitive detectors, uniform chromarods and better application techniques have been developed. Newer research found that some of the lipid classes gave a curve linear relationship between the sample loads of 0.5-10μg detected on chromarods-III, indicating a drastically improvement in FID response[42]. However, due to nonlinear responses in the detector of Iatroscan TLC-FID underestimations of total lipid content may occur[75][56]. Other challenges concerning Iatroscan are that manual sample applications give variable results and are dependent on the workers and routines[88][89].

In comparison to conventional liquid chromatography the Iatroscan TLC-FID obtains data from a large sample rapidly, are more simple to operate and can be less time consuming[6]. Comparable results of Iatroscan TLC-FID and HPLC-ELSD has been detected from quantitative measurements of fish oil lipids[42].

1.6.2 HPLC with CAD

High Performance Liquid Chromatography (HPLC) is an analyzing method, which separate sample components in a liquid phase. The components in a mixture, that are intentionally separated, are distributed differently between a stationary phase and a mobile phase. The mobile phase is the liquid moving trough the instrument, carrying the components, and the stationary phase is the column material. In general the HPLC instrument is composed of a reservoir, a mobile phase, a pump, an injector, a column, a detector and a computer (see Figure 1.10).

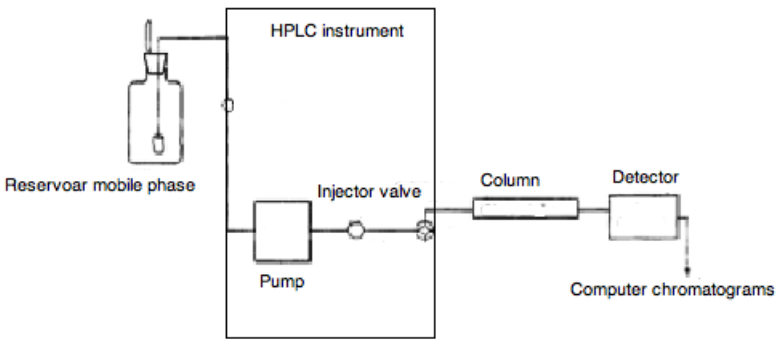


Figure 1.10: Overview of the HPLC instrument set-up.

A known sample amount is injected into the instrument. Pressure from a pump causes the mobile phase to carry the sample trough the column. In the column the components

are separated and they are carried further to the detector. Detected results are sent to a computer, which compute a chromatogram showing the quantity and quality of each separated component. Different methods, depending on the physical and chemical properties of the analytes to be separated, are employed for optimal separation. Some of the most important parameters to consider are sample injection, temperature in the column and in the sample vials, pressure in the system, solvent flow, mobile phase composition and changes during analysis. The column attached together with the mobile phase composition and the temperature applied, reveals the main separation principles of the method. The detector attached gives the detector principles[35].

Normal phase-column with silica gel is today the most commonly used stationary phase for the separation and determination of phospholipids. Silica columns are relatively robust and inexpensive[69]. Examples of other separation mechanisms include columns such as reverse phase for separation of hydrophobic groups like non-polar lipids[90]. A challenge with silica gel columns is that silanols are more acidic and has a high affinity to water. Molecules having groups engaging in proton donor or acceptor, dipole-dipole or dipole induced and dispersion forces interactions affect the elution and decrease the overall adsorption[81].

It is crucial that the sample is dissolved in the solvent of the mobile phase. Therefore the mobile phase should match the nature of the analytes as well as the sorbent layer being used. Example a polar solvent is required to cause migration for polar substances[29]. Solvent strength is a description of the mobile phase effect on the retention of sample components. Increasing solvent strength will increase the elution time and decrease retention. Depending on the retention mechanism, the solvent strength has various descriptions. The solvent strength in normal phase chromatography is regarded as polarity. Polarity is the ability to form hydrogen bonds[81]. A small increase of a polar solvent that is present in small concentrations produce a large increase in solvent strength[83].

The mechanism of the CAD detector is that the analyte forms particles that are dried, charged and detected. Prior to detection the HPLC effluent is nebulized with nitrogen gas and droplets are generated. The small droplets evaporate, leaving analyte particles that are sent to a mixing chamber to be mixed with ionized nitrogen gas. A diffusion charge is transferred from the gas to the analyte particles before they pass trough an ion trap, which quantifies the charge by an electronmeter. The greater the amount of particles, the greater the charge causing a higher integrated peak of the chromatogram[66]. Corona CAD is capable of detecting analytes ranging from low nanogram to high microgram and the detector response does not rely on the analytes optical properties, their ability to be ionized by gas phase nor their chromophore content[41]. A negative aspect with CAD detectors is that they do not detect volatile analytes, probably because they evaporate and do not form aerosol particles[65]. Response for the Corona CAD detector varies with the mobile phase composition and the analyte concentration[41].

Calibration is important to verify the response of the detector to a given analyte concentration. A straightforward method for quantitative chromatographic analysis involves preparation of standard solutions with approximate compositions. The standards

are run and a chromatogram is obtained. The peak area of each standard is plotted as a function of the amount standard in the analyzed concentration. A plot of the data often yields a straight line that passes trough origo. Quantitative analysis is based on this plot. Qualitative determination of component is fond from the retention time of its peak being similar to a known standard[92].

1.7 CN-Analysis

Quantitative determination of organic carbon and nitrogen (CN-analysis) can be applied for the detection of carbon and nitrogen cycling within marine environments or mapping of organic carbon in soil for crops, as well as determination of the number of PL within an extract.

The instrument principle of this method is dry combustion analysis. The main instrument setup combines a combustion reactor, a gas chromatography column and a mass spectrometer. At the start Helium is the carrier gas but later it is switched to oxygen. Prior to the arrival of oxygen in the combustion reactor, the samples are dropped. Oxygen and samples react in a redox reaction, and combust at 1700-1800°C. The sample is broken down, oxidized, to its elemental components N_2 and CO_2. High performance copper wires absorb the excess oxygen gas. The evolved gasses, N_2 and CO_2, are separated in a gas chromatography column, and then detected by TCD. A computer identifies the detected peak signal by comparing it to a known standard calibration. The separated gasses are carried to the mass spectrometer and its quantity is determined[40].

Leaching of non-carbonate inorganic matter result in too high carbon concentration, because extra CO_2 combustion from the inorganic matter is composed and detected[71]. Comparison of ten different laboratory determinations of total carbon in marine particles found that the results were accurate and precise on a variety of instruments, giving a standard error of the mean was $\pm 7\%$ of the mean nitrogen. However, the analysis is open to technical errors and provides that appropriate standards are used, and that the subsamples analyzed are representative of the whole sample[50].

1.8 Performance and Aims

This work was part of a master thesis conducted at SINTEF "Fiskeri og havbruk". Marine products are the only raw material used. The limitations of this work were based on projects running at the same time. The main focuses were to evaluate the methods according to the quantity of PL and lipids. Fatty acid composition, saturation and oxidation was not considered or tested. The work were conducted in four stages, depending on the analysis performed:

- Extraction of samples by B&D and ASE were performed at the beginning. Total lipid concentration of each sample was decided. Based on the experimental results, further experiments were constructed.

- A more detailed study of the B&D extracts were conducted with a lipid class analysis by Iatroscan TLC-FID.
- It was decided to calibrate the HPLC-CAD method for PL-analysis due to the need for an analysis method to find total lipids within the extracts.
- PL-class analysis by HPLC-CAD of the different sample extracts.

Chapter 2

Materials and Methods

2.1 Overview of Experiments and Procedures

The analytical procedures for lipid assessment of the samples were carried out in three steps: extraction, purification and separation/quantification, see Figure 2.1. The lipid yield was calculated from extraction procedures of Bligh and Dyer and ASE. Purification of the lipid extract was performed by distillation by Rotavapor followed by evaporation of the solvent under a stream of nitrogen gas. Separation and quantification of lipid classes were accomplished by Iatroscan TLC analysis. HPLC analysis was employed for separation and quantification of the phospholipids classes of the purified lipid extract.

2.2 Materials

2.2.1 Raw Material

An overview of the samples processed for lipid extraction and analysis in this work, is displayed in Table 2.1. All samples were stored at -20°C. The extracted lipids from the samples were stored at -80°C after treated with N_2 gas. In prior to the experimentation the moisture of each sample was analyzed by drying c. 2g (0.000g accuracy) sample in a drying oven (TS8000, Termeks) at 100°C for 24 hours. Two different krill meals were analyzed, but not compared. Figure 2.2 shows the difference between the two krill meals used. The moisture profiles detected were in most respects similar to expected values of 78-80% in Cod roe, 8.91% in fish meal, 6.33 in krill meal 1. Expected value of Herring roe was 73-77% moisture and the detected value was 68.08%. The salt within the roe is probably the reason for the decreased moisture content detected[103][7]. Notice the comments in the table.

2.2.2 Standards for Calibration

The standards used in this study is presented in Table 2.2. Notice that none of the samples were of marine origin. The standards contained different fatty acid compositions.

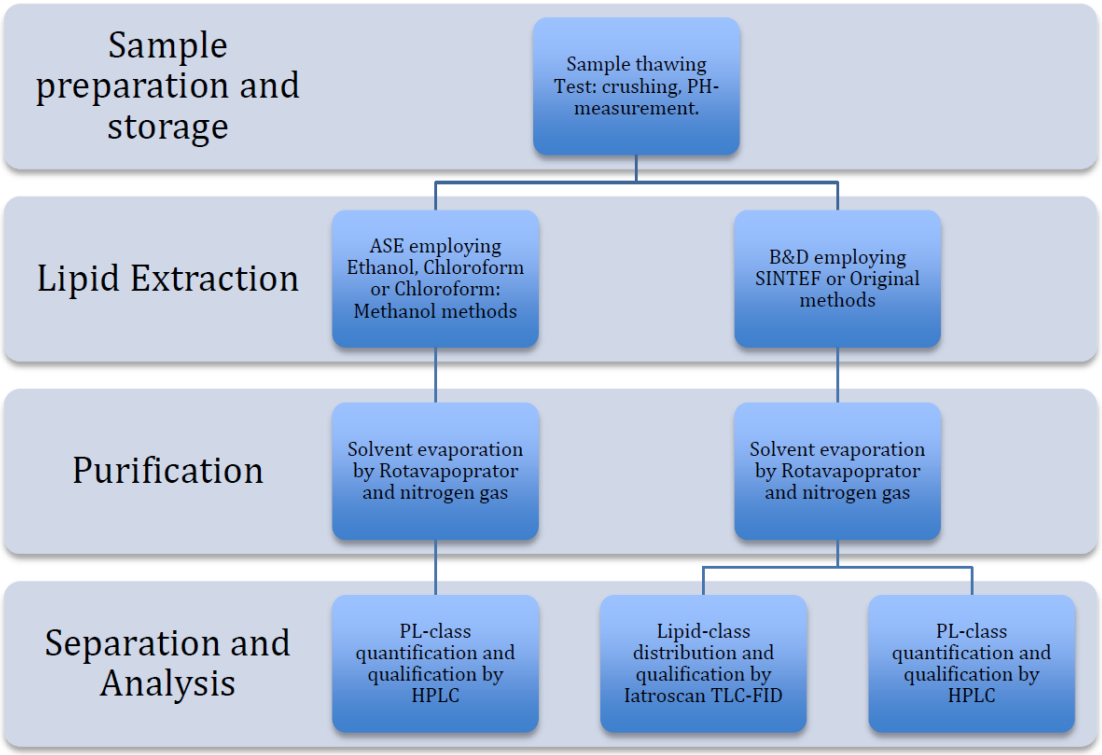


Figure 2.1: Scheme of the experiments of lipid extraction and analysis methods.

The dominant species were used for calculations of concentrations from results obtained by CN-analysis. It is assumed that all standard samples were pure.

2.2.3 Chemicals

The chemicals used in the research of this study are shown in Table 2.3. For all chemicals used in HPLC analysis, air bubbles were removed in ultrasound bath prior to analysis.

2.3 Lipid Yield

Lipid concentration from each extraction is given as g/100g lipids. The recovered lipid extracts was calculated from the formula:

$$\text{Lipid concentration} = \frac{(a \times b \times 100\%)}{c \times d} \tag{2.1}$$

Where a is the weight of the purified extracted lipids in grams, b is the volume of the solvent used for extracting the lipids in milliliter, c. is the weight of the sample in grams

Table 2.1: Description of the raw material (see Appendix A).

Raw Material, Species	Producer/ Supplier	Bach nr, Production date	Water Content (g/100 g Sample)	Comment
Krill meal 1, *Euphasia Superba*	Emerald Fisheries A/S, Ålesund	Nr.11 in the SINTEF achive	6.72	Krill meal with salt and 100-200mg/kg astaxanthin esters.
Krill meal 2, *Euphasia Superba*	Olympic seafood, Emerald Fisheries A/S, Ålesund	280410, 28.04.2010	1.54	Salt free, Krill PL and peptide mix, 100-200mg/kg astaxanthin esters
Fish meal	Felleskjøpet A/S, Trondheim	13.10.10	7.93	Grain
Salmon meal	Natura, Felleskjøpet A/S, Trondheim	4045824190, 19.10.10	6.52	Grain
Herring meal	Vedde A/S, Langervåg	91003, 19.01.09	8.85	Grain
Herring roe, *Clupea harengus*	Grøntvedt Pelagic, Breksted	24.09.10	68.08	With salt, Fresh
Cod roe, *Gadius marhua*	Mills	15.03.08	77.63	Salt free, Frozen and separated

and d is the volume of the pipetted solvent extract prior to purification and evaporation, in this work it was 2ml.

2.4 Bligh and Dyer Method

A B&D procedure based on the original method and a modified SINTEF B&D procedure is described in this section.

2.4.1 Original B&D Procedure

First homogenization
The extraction of total lipids was performed according to the method of Bligh and Dyer[8]. 5.00-10.00g of a sample was weighted in a 250ml flask. X ml of water, followed by 40.00ml methanol and 20.00ml chloroform to the sample solution before and homogenized homogenizing (Ultra- Turrax T25, IKA) for 2 minutes by an Ultra-Turrax (Model T25, IKA).

Figure 2.2: Picture of krill meal 1 (right) and krill meal 2 (left). The picture demonstrates the difference between the two krill meals employed. The variable surface, size and texture of the meals can be observed from this photo, emphasizing why they are not comparable.

Xml of water was calculated from the equation :

$$16ml - \left(\left(\frac{\%\text{water content in sample}}{100}\right) \times 10 \text{ or } 5\text{g}\right) \tag{2.2}$$

When the principle is to keep water: methanol: chloroform proportions 0.8:2:1, including the presence that amount of water in the sample.

Second homogenization and centrifugation
Another 20ml chloroform was pipetted added to the homogenized solution by pipetting and mixed for 40 seconds by the Ultra-Turrax. Finally, 20 ml water was added to the sample by pipetting before pipetted and mixing for 40 seconds. The homogenized solution was centrifuged (KR22i, Jouan) at 5000rpm for 15 minutes (KR22i, Jouan).

After centrifugation the chloroform phase was transferred pipetted from the biphasic solution to a 100ml conical flask by a glass Pasteur pipette, leaving the remaining water-methanol layer and the tissue residue in the flask.

Determination of lipid content in the extracts
From determination of lipid content 2.00ml of chloroform phase was pipetted to a test tube of known weight. The chloroform was evaporated from the sample on a heating module (Reacti- Therm, Pierce) at 60°C under a stream of nitrogen gas until the sample remained dry. The test tube with the lipid sample was transferred and kept overnight in an exicator before weighing (0.000g accuracy). The lipid concentration obtained from the sample was calculated according to Equation 2.1.

The remaining extract was evaporated using a rotavapor (Laborota 4000, Heidolph) at 40°C in a water bath at 200 psi. The remaining extract was transferred to a 5ml collection bottle and remains of chloroform were evaporated under a stream of nitrogen

Table 2.2: The new standards employed for HPLC calibration. The standards are purchased at www.avantilipids.com. All standards were stored at -80°C. The molecular weights of each standard represent the predominant species given on the sample information.

	Phospholipid	Molecular weight (g/mole)	Molecular formula (Predominant species)	Origin	Catalog nr	Description
PE	L-α-Phosphatidylethanolamine	715.94	$C_{39}H_{74}NO_8P$	Soy	840024P	Powder (25mg)
LPE	L-α-Lysophosphatidylethanolamine	453.55	$C_{21}H_{44}NO_7P$	Egg, Chicken	860081P	Powder (25mg)
PC	L-α-Phosphatidylcholine	775.04	$C_{42}H_{80}NO_8P$	Soy	840054P	Powder (25mg)
LPC	L-α-Lysophosphatidylcholine	513.56	$C_{24}H_{50}NO_7P$	Soy	84007P	Powder (25mg)

gas. The lipid extracts were stored at -80°C prior to analysis.

Second and third extraction

Second and third isolation steps extraction were performed as describe above, only after removal of the chloroform water-methanol layer and the tissue residue remaining in the flask and 40 amount of chloroform was added. The mixture was homogenized for 2 min followed by centrifugation. For the third extraction this step was repeated.

Moisture was determined gravimetrically after drying at 104°C for 24 hours. Ash content was estimated by charring in a crucible at 600°C until the ash had a white appearance (AOAC, 1990), see Table 2.1.

2.4.2 Modified Bligh and Dyer - SINTEF method

First homogenization

The extraction of total lipids was performed according to the method of Bligh and Dyer[8]. 5.00-10.00g of a sample was weighted in a 250ml flask.16.00 ml of water, followed by 40.00ml methanol and 20.00ml chloroform to the sample solution before and homogenized homogenizing (Ultra- Turrax T25, IKA) for 2 minutes by an Ultra-Turrax (Model T25, IKA).

The rest of this procedure was similar to the original method. See Second homogenization and centrifugation of the original B&D method.

Note that the only difference between the two B&D methods are the amount of water added prior to the first mixing. The difference between the methods are therefore noly expressed in moist samples. The SINTEF method achives water: methanol: chloroform

Table 2.3: Description of the chemicals.

Chemical	Producer	Density (g/ml, 20°C)	Molarity (g/mole)	Purity (%)	Chemical formula	Analysis
Chloroform	Merck Licrosolv, Germany	1.480	119.38	99.0-99.4	$CHCl_3$	Extraction of lipids, B&D, ASE, Fat class analysis,Iatroscan
Diatomaceous Earth	Dionex Corporation, California	2.3	60.8	100	SiO_2	Extraction of lipids, ASE (absorpition)
Diethyl Ether	Merck Emsure, Germany	0.71	74.12	99.7	$C_4H_{10}O$	Lipid-class analysis, Iatroscan
Ethanol	NTNU lab, Trondheim	0.789	46.07	80-90	C_2H_6O	Extraction of lipids, ASE
Formic Acid	Merck Emsure, Germany	1.18	46.03	80	CH_2O_2	Lipid-class analysis, Iatroscan
Hexane, n-Hexane	Merck Licrosolv, Germany	0.660	86.18	98	$CH_3(CH_2)_4CH_3$	PL analysis, HPLC
Isopropanol, 2-propanol	Merck Licrosolv, Germany	0.786	60.10	99.9	$CH_3CH(OH)CH_3$	PL analysis, HPLC
Methanol	Merck Licrosolv, Germany	0.792	32.04	99.9	$CH_3(OH)$	Extraction of lipids, B&D+ PL analysis, HPLC
Water, Ion free	SINTEF lab, Trondheim	1.00	18.02	100	H_2O	PL-analysis, HPLC

proportions 1.2:2:1 for moist samples for the monophasic solution. Hence, 2.2:2:2 for the biphasic solution.

2.5 Accelerated Solvent Extraction (ASE) method

Accelerated Solvent Extraction (ASE300) was used for automatic extraction of lipids, see Figure 2.3.

2.5.1 Sample Preparation

The samples were stored at -20°C and thawed in room temperature before further processing subsamples of 10.00g - 15.00g. 20.00gDE was added to samples with high water content (20-99%), while 10.00-15.00g DE was added to the samples with low water content (2-20%). 10.00g DE was packed on the top of the sample mix to fill the gap in the ASE cell, when the rest of DE was mixed with the sample to a homogeneous mix and introduced into a 100mL Dionex ASE cell before l. A cellulose filter paper (47mm, 0.7μm pore size, Whatman) was placed at the outlet on both sides of the packed cell before it was closed. Three or more cells were prepared for each of the different samples analyzed.

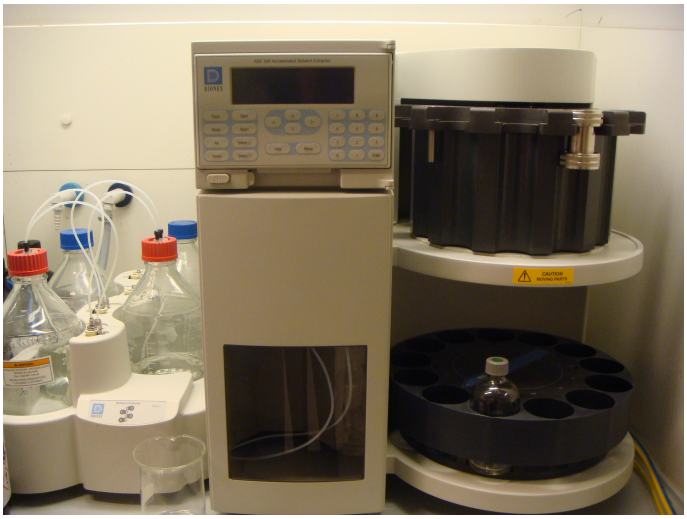


Figure 2.3: The ASE 300 machine employed for lipid extractions.

2.5.2 The Extraction Method by ASE

Prior to extraction the prepared cells were placed on the machine vials. All sample parameters of the ASE machine were held constant to establish an appropriate matching of the solvent and the sample. The extraction parameters of the method are shown in Table 2.4. The machine operates automatically, and after 20 minutes the receiving collection bottle is filled with the lipid extract in the solvent.

Three extractions of the same cell with the same analytical conditions were performed on selected samples.

2.5.3 Determination of Lipid Content and Purification of the Extracts

The volumes of the extracts in the collection bottles were determined by weight (0.000g accuracy). A 2.00 ml subsample of the extract was pipetted to a test tube with a known weight, and the solvent in the test tube was evaporated under a stream of nitrogen gas using a heating module (Reacti- Therm, Pierce) at the solvent boiling point until dryness. The tube containing the purified lipids was kept overnight in an exicator and weighed (0.000g accuracy) and the lipid content was calculated from Equation 2.1.

The machine was programmed to perform several extractions automatically by flushing the cells a second and third time. Each extract were collected in separate collection bottles.

The extract was concentrated using an evaporator (Laborota 4000, Heidolph) at a bath temperature of the solvent boiling point, until most solvent was evaporated. The concentrates were transferred to a clean 5mL collection bottle and the remaining solvent was evaporated under a stream of nitrogen gas. All samples were stored at -80°C prior to further procesing.

Table 2.4: ASE parameters used for lipid extraction (ASE300, Dionex)

Temperature (°C)	100
Pressure (Psi)	1500
Heat up time (min)[A]	5
Static time (min)[B]	5
Flush volume (%)[C]	60
Purge time (sec)	100
Number of static cycles	2
Solvent	Multiple[D]
Sorbent	DE[E]

[A]The sample cell was heated in the oven before extraction

[B]Extraction is performed by direct contact of solvent and heated sample

[C]The solvent flush volume is expressed by the % of cell volume (ml/100ml)

[D]A variety of solvents were employed, see Table X

[E]Diatomaceous earth

2.6 HPLC-CAD Calibration Method

Figure 2.4 shows a scheme of the experiments performed in order to receive a calibration curve that was acceptable.

2.6.1 Material accuracy, standards and solvents

The following standards were used: L-α-Phosphatidylcholine (Soy), L-α-Phosphatidylethanolamine (Soy), L-α-Lysophosphatidylcholine (Soy), L-α-Lysophosphatidylethanolamine (Egg, Chicken) (see Table 2.2). All standards were stored in a freezer at -80°C.

The solvents used were Isopropanol, Hexane, Water (ion-free), Methanol and Chloroform, (see Table 2.3).

The exact amount of solvents from equipment was decided by weighing (0.000g accuracy) the content of solution in an automatic pipette, a glass syringe and a glass pipette.

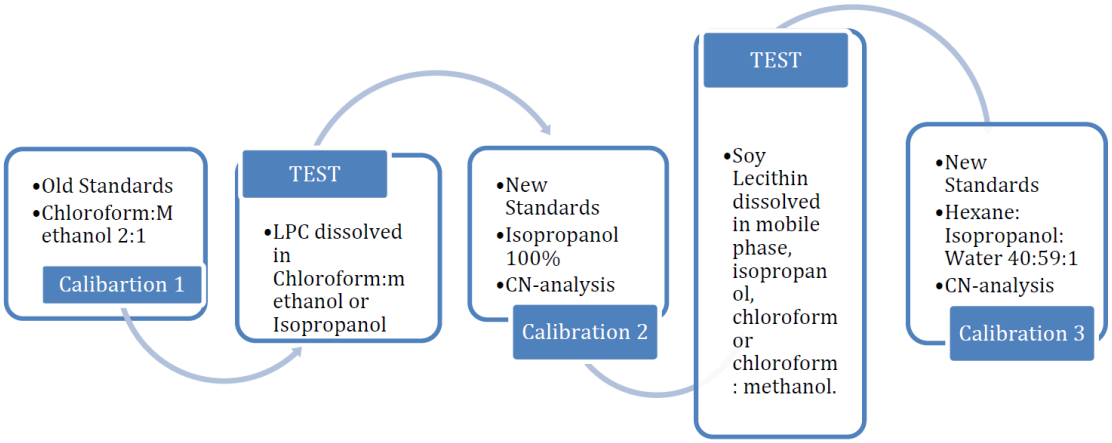


Figure 2.4: Overview of the HPLC calibration steps.

2.6.2 Calibration 1: Preparation of Standards

Standard samples of LPE, LPC (1mg/ml) and a mix of 0.437mg/ml PE and 0.413mg/ml PC were dissolved in chloroform: methanol (2:1). These were analyzed by HPLC-CAD. LPE and LPC were diluted to a concentration of 0.500mg/ml by transferring 250μl with an automatic pipette of each solution to 1.5 ml HPLC vials and adding 250μl of the solvent system to the solution. Then, sealing and mixing with a vortex was done followed by analysis by the HPLC-CAD system (see HPLC procedure).

2.6.2.1 Test: Deciding the Proper Solvent for LPC

Individual solutions of an old sample were made of LPC. Concentrations of 1mg/ml LPC in chloroform (by glass pipette) and 1mg/ml LPC in isopropanol (by automatic pipette) were made. The solutions were analyzed on the HPLC-CAD system.

2.6.3 Calibration 2: Preparation for Standards

New standards were ordered (see Table 2.2). The Stock solutions were prepared by weighing 25.000 mg standard samples on a micro weight. Both the glass pipe and the sample were transferred to a 100 ml glass bottle. The isopropanol was flushed with nitrogen gas 25.000ml isopropanol was weighed and transferred to the 100ml bottle and sealed. The stock solutions were mixed on Vortex and LPC and PC were heated at a 40°C water bath for complete dissolution. The solutions were stored in sealed HPLC vials at -80°C.

Two mixes were made. At first, by automatic pipette, 250μl of each standard stock solution were transferred to a 1.5 ml HPLC vial, and a mix of 1mg/ml was made. A mix of 0.600mg/ml was made from transferring 200μl of PE and PC and 100μl of LPE

and LPC. The samples were analyzed by the standard HPLC procedure (See the HPLC procedure). Samples of 1, 3, 5, 10 and 15μl were injected from each sample.

2.6.3.1 TEST: Deciding the Proper Solvent for all PLs

20.000mg Soy Lecithin was weighted on a micro weight, and the sample and glass pipe were transferred to a test tube. This was repeated four times, resulting in four sample test tubes. A final concentration of 1mg/ml was made by adding (with glass pipette) 20.00 ml of a different solvent or solvent mix to each of the four test tubes. The solvent and the mixes added was: a mix of hexane, isopropanol and water 40:59:1, 100% isopropanol, 100% chloroform and a mix of chloroform: methanol 2:1. The solutions were shaken on a vortex, transferred to 1.5 ml HPLC vials and analyzed by the HPLC-CAD (see HPLC procedure). Subsamples of 10μl of all samples were injected.

2.6.4 Calibration 3: Preparation of Standards

100ml solution of the mixed solvent containing hexane: isopropanol: water (ion free) 40:59:1 was made by mixing 26.4g hexane, 46.37g isopropanol and 1.00g water. From density calculations the exact weight was found for each solvent. The Stock solutions were prepared by weighing 10,000mg standard samples on a micro weight. Both the glass pipe and the sample were transferred to a 20.000 ml glass bottle. The isopropanol and hexane were flushed with nitrogen gas and 10.000ml of the solvent mix was weighed and transferred to the 100 ml glass bottle by a plastic cup. This was repeated four times to remain four stock solutions (1mg/ml) of the different standards PE, PC, LPE and LPC.

200μl PE, 200μl PC and 100μl LPC were transferred by a automatic pipette to a 1.5ml HPLC vial. The vial was sealed and analyzed by HPLC-CAD. LPE was heated at 40°C and mixed on vortex to ensure dissolution before the analysis. Subsamples of 1, 3, 7 and10μl was injected from each sample.

2.7 Analysis

Analyses of carbon and nitrogen concentrations of the second and third calibration standards were performed by CN-analyses. PL-class analyses of all the samples extracts were performed by HPLC-CAD. Lipid class analysis of the B&D extracts were performed by Iatroscan TLC-FID.

2.7.1 CN-analysis

Total nitrogen (N) and carbon (C) was determined by CHN-S/N elemental analyser 1106 (COSTCH instruments, ECS model 4010, Cernusco s/Nav. -MI (Italy)). These measurements were performed running five parallels. A nitrogen concentration of 1.6±0.2g/100g in phospholipid product was determined.

This method was performed by Marte Schei at SINTEF "Fiskeri og havbruk".

2.7.2 HPLC-CAD Method

All extracts obtained from ASE and B&D extractions was analyzed by a HPLC combined with a Corona CAD detector, see Figure 2.5. In prior to analysis the samples were stored in a freezer at -80°C.

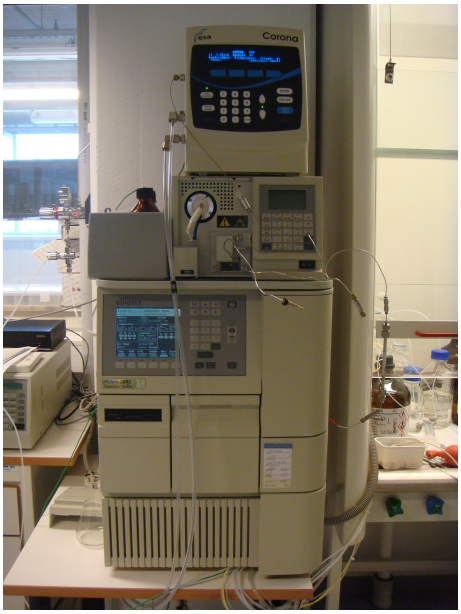


Figure 2.5: The HPLC and CAD instruments employed for PL-class analysis.

2.7.2.1 Sample Preparation

The extracted lipid samples contained small amounts of organic solvents, which were removed after flushing with nitrogen gas (N_2-gas). To prevent oxidation the samples were not heated while flushed. The purified sample was weighted by an accuracy of 0.000mg. The chromatographic mixture was made by dissolving the sample in a mixture of hexane: isopropanol: water (de-ionized) 40:59:1 (w:w:w) at an concentration of 1mg/ml. Samples containing less phospholipids were concentrated to 2mg/ml. The solvent was transferred to the sample bottle by a glass pipette (0.00g accuracy). The samples were stored in the freezer at -80°C.

2.7.2.2 Chromatographic Equipment and Analysis

The HPLC instrument (Waters, Manchester, England) is equipped with 515 and 510 pumps and a Water 717 auto sampler. A gradient system is employed to improve accurate and sensitive elution. The analytical column (150 mm × 4.6 mm I.D., 10 μm, Prep-SIL Scalar, Agilent) is a normal phase silica column. Chromatographic separation

was carried out with a flow rate of 1.25 mL/min via a gradient according to the following program: t0 min: 40% A, 59% B and 1% C, t3 min 40% A, 54% B and 6% C, t17 min 40% A, 48% B and 12% C and finally isocratic conditions (40% A, 59% B and 1% C) for 3 min. Eluent A was hexane, eluent B - isopropanol and eluent C - water.). The injected volume was 10μl for each sample. The instrument autosampler containing the sample vials was at cooled to 4°C.

Corona CAD detector (ESA Inc., Chelmsford, MA, USA) was used for detection, at a sensitivity of 200 pA and a N_2 flow of 35psi. The column temperature was at 20 °C. To prevent accumulation of contaminants in the column, a washing procedure was performed once each week.

Summing detected amounts and reporting them as percentage of total soluble materials in extracts calculated total phospholipids. The computer program EZchrom was used for calculating the integrated area of the peaks. The calibration curve was obtained for each of standards with a correlation coefficient of regression (R2) >0.95, and the sum of the concentrations of all PL-classes gave the total PL obtained.

2.7.3 Iatroscan TLC-FID Analysis

Individual lipid classes of the B&D extracts were quantified by TLC-FID analysis. The PL concentration was compared to the PL concentration from the HPLC analysis. All samples were run with four parallels.

Solutions of 1.00mg/ml lipids dissolved in chloroform: methanol (2:1) was made from the frozen and purified B&D extracts.

TLC-FID was carried out on an Iatroscan MK-6 (Iatron Labs, Japan). In prior to application chromarods (S-III, 10 rods) were scanned until all pollutants were removed. Each sample was applied onto the marked line of a chromarod with a 10μl Hamilton syringe, until the spots contained the same width and depth (c.2μl applied on the rod), followed by drying under a low stream of nitrogen. The dried chromarods were transferred to a constant humidity chamber (30%) saturated with aqueous $CaCl_2$ in 8 minutes.

2.7.3.1 TLC-Separation and Detection by FID

For 28 minutes the Chromarods were eluted in a closed glass chamber (15*17*3cm) containing a filter paper (cut 20*20cm, Camag) and a solution of hexane, ether and formic acid (85ml: 15ml: 40μl) at 22-25°C. Drying of the eluted chromarods was performed in a heating oven (Termaks) at 90°C for 5 minutes. Lipid class analyses of the eluted and dried chromarods were performed on the Iatroscan TLC-FID at a hydrogen flow rate of 160ml/min, air flow rate 2L/min, 2 bar hydrogen pressure and at scan speed 30s/rod. The instrument was connected to a computer running the software Spectra Physics (Mountain View, CA. USA), quantifying the peaks by automatic chromatogram integration.

This scheme allowed for the separation of six lipid peaks; Cholesterol esters, Triglycerides, Free fatty acids, Cholesterol, Mono and Di-glycerides and Phospholipids. The

chromatograms showed %-distribution of each lipid class in a sample.

A mixture of lipid standards from plants was run to qualitatively secure the sample analysis. Preparation of standards and analysis was performed similar to the samples.

2.8 Washout Curve

The two immiscible systems of the washout curve represent the biphasic solvent system of B&D. The upper phase is composed of methanol and water, and the lower phase is composed of chloroform. The solute is the lipids, PL or TG to be extracted, which is dissolved to a higher extent in the chloroform phase. From Equation 2.3 the equilibrium existing between the biphasic systems is:

$$[Lipids]_{Methanol:Water} \Longleftrightarrow [Lipids]_{Chloroform} \tag{2.3}$$

The stabilized, ideally constant, equilibrium relationship between the lipid concentrations in the biphasic system is written from Equation 2.4:

$$k = \frac{[Lipids]_{Chloroform}}{[Lipids]_{Methanol:Water}} \tag{2.4}$$

The phases are constant and independent of the concentrations in the systems. After one extraction, the chloroform phase and the dissolved lipids are removed. For a second extraction, pure chloroform is added to the tissue and water phase with some lipids. A new equilibrium is established between the pure chloroform and the water: methanol phase. More lipids are dissolved in the chloroform phase before the equilibrium is stabilized. The same principle occurs when the chloroform and its lipid concentration are removed and pure chloroform is added for a third extraction. For each extraction more lipids are removed from the water phase and sample, and more lipids are extracted in the chloroform phases removed.

The equation used for calculating the remaining x concentration in the water phase is:

$$\begin{aligned} k &= \tfrac{x_2}{x_4} \\ x_1 &= x_2 \times k \\ x_3 &= x_4 \times k \\ x_5 &= x_6 \times k \end{aligned} \tag{2.5}$$

Where X_2 is the lipid concentrations of the first extraction and X_4 is the lipid concentration of the second extraction. X_1, X_3 and x_5 represent the lipid concentrations of the water/methanol phase after the first, second and third extraction. X_6 is the lipid concentration of the third extraction. K is the equilibrium constant.

For detailed derivation of these equations, see Appendix I. The calculated x values are the remaining lipid concentration in the water phase after three extractions. The natural logarithm (LN) of the concentrations was plotted as a function of extraction

numbers. A linear regression of the LNx values was found and plotted as a function of number of extractions.

The curve presenting an overview of the concentration fraction in the water phase after each extraction occurs is called a washout curve. When extrapolating the linear regression line, made from the plot points, represent the total lipid extraction of the sample. The angle, α, of this linear relationship is the same value as K, the dissociation constant.

2.9 Statistical Analysis

In this thesis GraphPad Prism version 5 (GraphPad software Inc. 2007) was employed for all statistical analysis. Mean and Standard deviation (STD) is given in text and on graphs. In addition, the standard error of mean (SEM) is given in text and tables for most analysis. Differences were considered significant at $P<0.05$ for all tests.

All data were tested for outliers with Chauvenet's criterion (The probability of a measurement within t standard deviations on either side of the mean of the normal error integral) and data were rejected when the probability of the deviant measurement is less than 0.5. The outlier was decided from a data set, by formula:

$$\begin{aligned} &T_{sus} = \frac{X_{suspected} - X_{average}}{STD} \\ &Prob(outside(t_{sus} \times STD)) = 1 - Prob(within(t_{sus} \times STD)) \end{aligned} \tag{2.6}$$

The probability values are found from a normal integral table, of the probability percent as a function of t[97].

Lipid concentration obtained from different methods from each sample were compared in respect to the mean values with One-way-Anova followed by Tukey's multiple comparison test for unequal samples. Some selected samples experienced three numbers of extractions. A Two-Way-ANOVA was used to compare two different methods within each extraction number of similar samples. The extraction number and the method were evaluated as significant or not.

To find the relation between the extracted lipid concentration of selected samples and number of extractions performed by B&D, linear regression was employed. A washout curve was plotted where LN of the remaining lipid concentration was plotted as a function of extractions performed on a single sample. Extrapolation of the calculated straight line to X=0 found the expected lipid concentration of the sample and to Y=0 the extraction nr were we can expect close to zero lipid concentrations extracted (see Section 1.5.2).

Calculations of the phospholipids concentration in different samples were performed by multiplying the average value of phospholipids found by analysis with the average value of lipids obtained by extraction[97].

The uncertainty in the PL classes summed to total PL follows the equation:

$$\delta q = \sqrt{(\delta x_1)^2 + (\delta x_2)^2} \tag{2.7}$$

The uncertainty of the PL classes in an extract multiplied by total lipid extract in a sample is calculated from:

$$\frac{\Delta q}{\mid q \mid} = \sqrt{\left(\frac{\delta x_1}{x_1}\right)^2 + \left(\frac{\delta x_2}{x_2}\right)^2} \tag{2.8}$$

The variability ratio can be quantified as the coefficient of variation, CV, which is the SD divided by the mean. If CV equals 0.25, the SD is 25% of the mean[68]. In this thesis the CV is employed for comparison of scatter of the variable measured concentrations obtained by CN-analysis for Nitrogen in HPLC standards. The coefficient variation of the standards is an indication of how trustworthy the calculated concentrations are. If the CV is high, the average concentration value is ignored in this thesis and an indication that the solvent system does not dissolve the standards satisfactory to employ for the HPLC analysis is supported.

3-12 samples were analyzed in HPLC, and the corresponding STD was calculated. It was discovered that most samples obtained similar STD for 6 and 12 samples analyzed. Therefore, it was decided to used 6 parallels for each HPLC analysis.

Chapter 3

Results and Discussion

In this work the results and discussion trying to answer the aims are organized in five main chapters:

1. Insecurities of the methods.
2. Lipid extraction by Accelerated solvent extraction (ASE) and Bligh and Dyer (B&D).
3. Improvement of the High performance liquid chromatography (HPLC)-CAD phospholipids (PL)-class analysis.
4. Lipid-class analysis of the B&D extracts by Iatroscan Thin layer chromatography (TLC)-FID.
5. PL-class analysis of the isolated lipids by HPLC-CAD.

In each chapter emphasis on explaining the reason for the experiments performed and the next step from the results obtained is presented. At last, a summary of the main results of the main objects is given. The importance of the results and probable areas of objects are stated.

3.1 Insecurities of the Methods

The analytical insecurities of the results obtained vary within each method employed as well as the natural variability of the sample population. The standard deviations of each method represent the insecurities of the method as a whole. The Standard deviation (STD) calculated for the extraction method is shown in Table 3.1. The samples employed gave high lipid yield and were relatively dry and is therefore not affected by the differences in the two B&D methods employed. For the ASE method a total of 26 parallels were analyzed. Small changes in STD from 6 to ten samples were observed from experiments by B&D. Hence, 12 samples were analyzed to decide the standard deviation of the method. The uncertainties of each method lie within the uncertainties

of the observed samples and it will enlarge the STD of the samples analyzed. Knowing the methods uncertainties contribute to narrow down and find a better estimate for the actual biological variability of the samples.

Table 3.1: Standard deviation (STD) calculated for the extraction methods; B&D and ASE. N is the number of samples analyzed, B&D is the Bligh and Dyer method, ASE is Accelerated solvent extraction (see Appendix C and Appendix B)

Method, Sample	Mean (g/100g sample)	STD	N
B&D, Herring meal	15.259	0.280	12
ASE, Krillmeal 2	22.678	1.055	26

The insecurities in for the analyses of the different lipid classes by Iatroscan are listed in Table 3.2. Figure 3.1 show the observed relative STDs of the HPLC analysis of Krill meal 2. From this graph it was decided that an injected sample of 10μl gave the most accurate analysis judged by the STD. Further, Krill meal 2 was analyzed and the STD for the different PL classes was calculated.

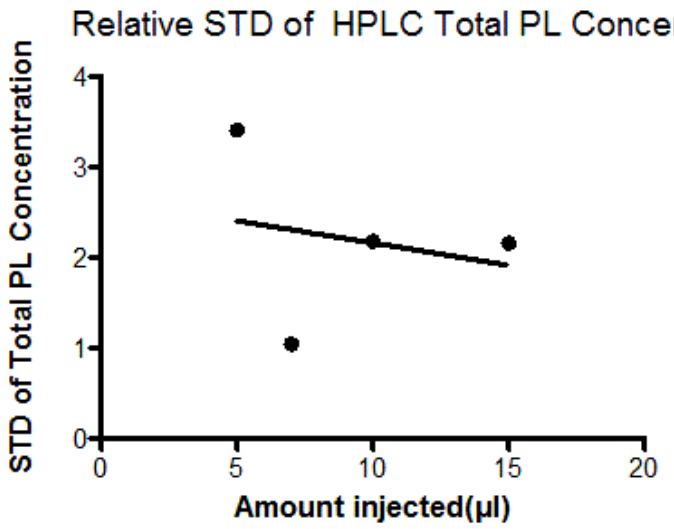
Table 3.2: Standard deviation (STD) calculated for the Iatroscan TLC-FID analysis of the lipid classes using standard samples of lipid classes from plants. N is the number of samples analyzed (see Appendix B).

Lipid class	Mean (g/100g lipid)	STD	N
Cholesterolester	26.102	0.647	10
Triglyserid	16.977	0.570	10
Free fatty acids	11.673	0.851	10
Cholesterol	38.908	0.969	10
Polar lipids	5.895	0.920	10

Unfortunately the insecurities of this thesis are highly dependent of the person performing the lab work. Appliance of the sample using a syringe may give unknown errors to the sample STD.

Table 3.3 shows the STD of the different PL-classes from HPLC analysis. PL extract concentrations were calculated from g/100g lipid to g/100g sample.

In general a possible source of errors for all extraction methods is that the sample may contain other components than lipids, for example proteins or coloring components. There is also a possibility that small part of solvents remain within the extract after nitrogen flushing and purification of extract. These errors result in an overestimation



	Total PL STD
Best-fit values	
Slope	-0.04934 ± 0.1528
Y-intercept when X=0.0	2.656 ± 1.526
X-intercept when Y=0.0	53.84

Figure 3.1: Graph of the relative STD for the HPLC analysis, showing the variation in the STD for the different injected amounts of PL from krill meal 1. The STD of 10μl lies closest to the linear regression line and therefore this injection volume was employed for all analysis (See Appendix B for detailed information on the regression line or the data table).

of the lipid concentration in the samples. Systemic errors are also a challenge when a single person performs the manual work. The pipetting may lead to constantly transfer of incorrect volume. Finally, if parts of the organic phase may be dissolved in the chloroform phase, the membrane may form bicelles and micelles. This may also contribute to an exaggeration of the lipid content in the sample[37].

A high STD may be a result of inconsistency of the analysis performed, or high biological variability[68]. Also, the lipids and other nutrients within the salmon meal, fish meal etc is not necessarily equally distributed in a meal package. The mixing is unknown and may contribute to large variation in lipid yields extracted from each method. 10-5g sample is used for each analysis. Mixing of the meal contents within the bags were not conducted, although this could be helpful to gain a more homogenized sample. Errors of the B&D method include adsorption of organic phase to the tissue. This contributes to incomplete extraction leading to underestimation of lipids concentration[93].

Chloroform is a highly volatile solvent and is extremely easily evaporated when

Table 3.3: Standard deviation (STD) calculated for HPLC with 10μl injections. The sample employed was krill meal 1 (see Appendix B.

PL class	Mean (g/100g lipid)	STD	N
PE	4.074	1.09	10
LPE	1.018	0.44	10
PC	27.498	1.47	10
LPC	3.985	1.09	10

heated. However, research found that the temperature of the organic solvent leaving a cell and oven at 100°C of the ASE machine was less than 35°C. This was due to long tubing (≈30cm) from cell to collection bottle and low heat capacity[82]. On the other hand solvent evaporation may occur from the extraction bottle, because the membrane on top of the bottle contain holes. Evaporation of solvent affect the volume extract measured after extraction. This will affect the calculated lipid concentration resulting in too low values. At last, ASE method cannot be completely automated. The possibilities of systemic errors should therefore be taken into account.

3.2 Extraction

In this chapter the modified SINTEF B&D method is compared to the original B&D method. Next, the most suited solvent system to yield high lipid concentration with ASE was discovered. Further the best solvent systems were used to perform three extractions of the same sample. A hypothesis was constructed and its results are compared to other extractions of similar samples. In the end, a washout curve was calculated from the sample extracts of three extractions obtained by B&D.

3.2.1 Bligh and Dyer Methods

A comparison of the B&D approaches was conducted. The aim was to find the optimal method for lipid extraction. Based on both research results and the theoretical aspects of this method, the different approaches were evaluated. Herring roe was employed because it contains around 70% water, which is crucial for distinguishing the SINTEF and original B&D approaches.

Figure 3.2 shows the results of the lipid extraction by SINTEF and Original B&D methods.

First extraction
The first extraction yields highest lipid concentration for the modified SINTEF approach, as was considered significant ($P<0.05$, $t=3.663$). One extraction of herring roe gives

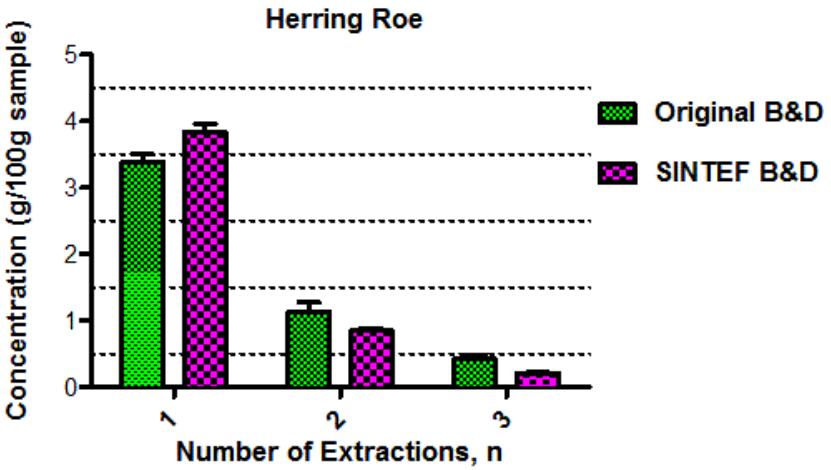


Figure 3.2: Lipid concentration in herring roe obtained from two different Bligh and Dyer (B&D) extraction methods, SINTEF and Original, with three extractions of each sample. The data bars are given in mean values (± SEM, n=6). See Appendix C for detailed raw data and result values.

higher yield by the SINTEF B&D in comparison to the original B&D. As stated in Figure 1.7, the two B&D approaches gave different solvent proportions. The original B&D approach has solvent proportions of 2:2:1.8 chloroform: methanol: water in the biphasic solvent system. This proportion lies below the chloroform tie line and is considered to have a 100% pure chloroform phase[8]. A combination of the water content of the roe with the solvent amount of SINTEF B&D reveal a solvent proportion of 2:2:2.2 chloroform: methanol: water. This solvent combination lies within the biphasic area of the phase diagram, above the chloroform tie line (see Figure 1.7). Its chloroform phase is considered contaminated by a small fraction of water and methanol. Therefore there is a possibility that more polar components, like proteins, are dissolved in the SINTEF chloroform phase. From these facts, it seems like the SINTEF approach may result in a lipid extract with protein impurities.

Second, third and total extraction

The second and third extractions yield a small increase of lipid concentration for the original approach, but the difference is not considered significant ($P<0.01$, second; $t=2.313$, third; $t=1.707$). The overall differences between the methods were considered not significant ($P=0.839$). When three extractions are performed, the methods give a similar yield, that is 4.9±0.2g/100g sample for Original B&D and 4.9 ±0.1 g/100g sample for SINTEF B&D (mean± SEM). This is observed from Figure 3.2.

Due to the possibility of getting impure extracts by the SINTEF approach, it was chosen to continue extraction by the original B&D method. Correct and pure extractions

are crucial to give true values of total lipids, especially when the B&D extractions are supposed to serve as a reference. It is assumed that the extractions obtained by B&D original method is 100% lipids. Therefore no analysis of the extractions from the method was conducted.

The Bligh and Dyer (B&D) were used as reference because it is one of the most recommended methods for determining total lipids in biological tissue. Also, it is a method developed for phospholipids and lipids extraction in fish[44]. In the rest of this study, the original B&D approach is presented as the B&D method.

3.2.2 The Effect of Extraction Method on Lipid Yield

The lipid extractions obtained by B&D and ASE with different solvents were compared. The goal was to investigate what solvent system, with the employment of ASE, which extracted most lipids. Four different samples were tested with three different extraction methods. Cod roe represents a moist and natural sample. The different meals represent relatively dry and processed samples.

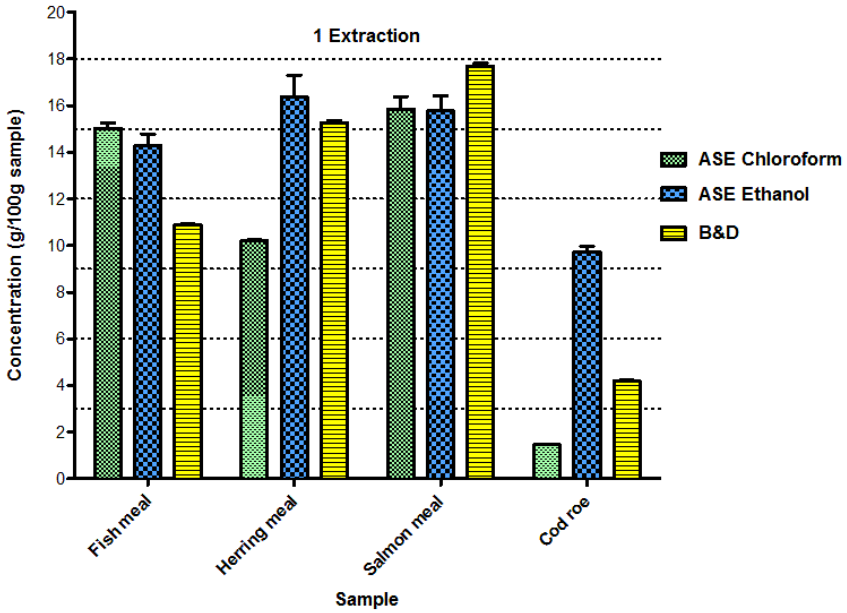


Figure 3.3: Lipid concentration of different samples obtained by three different extraction methods: ASE with chloroform, ASE with ethanol and Bligh and Dyer (B&D). The data bars are given in mean values (± SEM, n=6). See Appendix C and Appendix D for presentations of raw data.

Figure 3.3 presents the lipid concentration of salmon meal, herring meal, fish meal and cod roe obtained by different extraction methods.

Fish meal and salmon meal
The methods were significantly different for all samples (P>0.05). No significant difference in extracts from salmon meal (P>0.05,t=0.106) and fish meal (P>0.05,t=1.575) by ASE chloroform and ASE ethanol was observed. From extractions by Folch, a total lipid content of c.9 g/100g dry weight was found[103]. observation is comparable to the result obtained in this study. Indications of realistic and reliable results obtained for fish meal by B&D were achieved.

The salmon meal (P<0.05, t=3.297) yielded highest lipid concentrations by B&D than ASE ethanol. Different solvent properties in comparison to the lipid components within the sample may be due to this observation. The mix of different solvent systems employed in B&D may be favorable for dissolving different lipid compounds within salmon meal. See the lipid class analysis chapter for detailed discussion.

Herring meal
Significantly more lipids were extracted from herring meal (P<0.0001, t=10.58) and cod roe (P<0.0001, t=17.36) with ASE ethanol compared to ASE chloroform. Similar extractions by B&D and ASE ethanol were obtained in Herring meal (P>0.05, t=2.088). From the producers of the herring meal, VEDDE, it was informed that a former lipid extraction by the Soxhlet method received 12.5% yield (g/100g sample, ww)[101]. From this experiment, the lipid concentration of herring meal by ASE chloroform, is lower than this value. Both the B&D and ASE ethanol received a higher yield of herring meal than the Soxhlet method. Soxhlet uses only non-polar solvents, which does not dissolve polar lipids completely. Underestimation of lipid concentration could be expected, especially for the samples rich in PLs[95].

Cod roe
ASE ethanol was significantly greater than B&D in extractions of cod roe (P<0.0001, t=9.475) and fish meal (P<0.0001, t=7.110). Cod roe lipid content varies from 3% to 9%, depending on the development stage in the egg[7]. ASE ethanol and B&D yielded lipid concentrations within this range. This may indicate an underestimation from the ASE chloroform method. The extremely high value obtained from ASE ethanol in comparison to B&D may be due to proteins, that are bound to the polar lipids, are extracted with the solvent[72]. ASE employs high temperatures to increase the diffusion rates[82]. This may be another reason for high yields from ASE methods. Also, the polar character of the ethanol solvent dissolves more polar lipids, such as phospholipids[37]. This is discussed in more detail for herring roe extractions in the next section.

In the end, it was decided to employ ASE ethanol in further extractions. Due to its high lipid yield obtained in this experiment, and because it is often employed for industrial production of PL[37], it was interesting to explore this method.

3.2.3 The Effect of Three Extractions on Lipid Recovery

Three extractions steps were performed because three points were sustainable for the calculation of washout curve regression line. The intention was to explore if the lipid yield

of these samples would be much greater than with one extraction. Based on the further extractions, a hypothesis performed on ASE was proposed and formulated. Herring roe and Krill meal 1 were employed to represent samples with high and low moisture. Both contained high proportions of phospholipid concentration of the total lipid yield. Krill meal 2 see Table 2.1, and herring roe was used for experiments of the hypothesis test.

3.2.3.1 Herring Roe

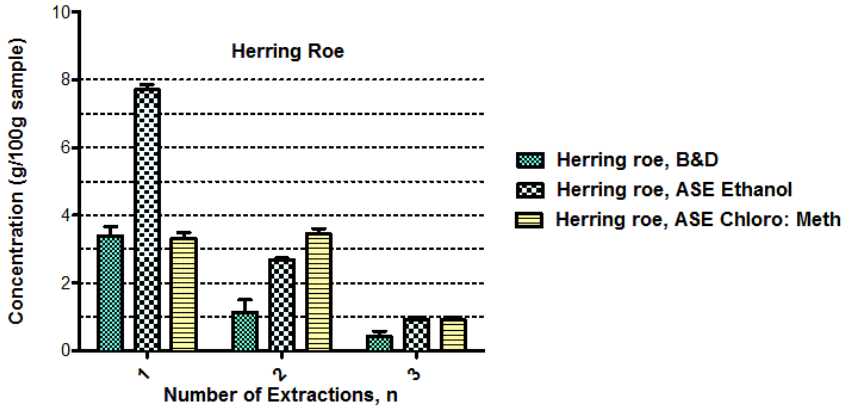


Figure 3.4: Lipid concentration of herring roe obtained from three extractions by three different methods. The data bars are given in mean values (± SEM, n=6). See Appendix C and Appendix D for presentations of raw data. The method ASE with chloroform: methanol 2:1 represents the hypothesis test of this research. Total sum of lipid yield from all extractions from each method is: 11.3±0.2 for ASE ethanol, 4.9±0.2 for B&D and 7.7± 1.5 for ASE chloroform: methanol (mean± SEM).

The results from three extractions of herring roe by three different methods are viewed in Figure 3.4.

First and Second Extraction
Lipid yield of the first extraction were significantly greater for the ASE ethanol method than B&D ($P<0.001$, $t=25.34$) and ASE chloroform: methanol ($P<0.0001$, $t=25.77$). The second extraction yielded highest lipid concentrations from ASE chloroform: methanol (B&D; $P<0.0001$, $t=13.71$). Herring roe compose many polar lipids, and these are dissolved easily in ethanol. Only a small proportion PL is dissolved in chloroform. The variable extraction efficiencies of the herring roe extractions may explain diatomous earth (DE) behavior, se arguments put together later in this section. Based on the DE properties the attraction of PL to the water and silica of DE is strong. The PL attraction to chloroform is relatively weak. Therefore many polar lipids may remain in the adsorbed area of DE. However, ethanol has stronger attractions to PL than water and

DE. Easily accessed polar lipids are probably extracted the first time. The PLs that are embedded in the membrane with strong attractions may be extracted in the second or third extraction.

Third Extraction and Total Extraction
There was no difference between the two ASE methods in the third extraction ($P>0.05$, $t=0.04$). Overall there were significant differences between the three methods ($P<0.05$). An error to this may be that the polar solvent extracts more polar components (Proteins and Carbohydrates) than just PLs. Hence, leaving a polluted extract. The ASE ethanol method gave total lipids c. 11 g/100g sample Lipids. Expected lipid concentration are 2-11g/100g sample[7]. In addition, if the washout curve gives true values of total lipids in the sample (see Section 3.2.4), indications of a polluted extract is strongly suggested from these results.

The Hypothesis, ASE Chloroform: Methanol
The B&D journal explains that the solvent proportion chloroform: methanol 2:1 denature the cell membrane. Therefore it is easier to extract the lipids and PL within the sample. In addition, mechanical forces from mixing with ultraturrax also contribute to break the cell membrane[8]. The hypothesis test regarded ASE using chloroform: methanol solvent. To break cell structures prior to extraction, crushing of the samples was conducted. By experiencing three numbers of extractions, the main achievement was to extract most lipids and polar lipids (PL). Also, it was desirable to yield similar or more lipids than B&D. The hypothesis test method, ASE chloroform: methanol, gave similar yield as the B&D method. This indicates that it is a good extraction method for herring roe lipids. The method received total 4.9g/100g sample of lipid concentration, which is quite similar to the extrapolated value of the Washout curve (see section X).

Unfortunately, the employment of Diatomous earth (DE) expels the possibility of adding water solvents. A test performed in the lab found that the DE employed in this thesis adsorbed 60ml water per 100ml cell full with DE. This hinders the employment of many solvent mixing systems that are perfect for PL dissolvent. An example is that hexane/isopropanol dissolves polar lipids poorly[22].

3.2.3.2 Krill Meal 1 and 2

Figure 3.5 shows the lipid concentration of krill meal 1 and krill meal 2 by ASE with different solvents and by B&D.

Krill Meal 1
Krill meal 1 extractions were significantly greater ($P<0.05$) by B&D method compared to ASE ethanol in all extractions (1;$t=3.271$, 2;$t=11.12$, 3;$t=11.82$). Krill meal 1 is a more ordinary type of meal, meaning dried krill processed from a specific method where the shell is removed. However, variable production methods employed for the production of different krill meals may yield different lipid compositions. Therefore, they are often not comparable. Discussions of the total lipid concentration of krill meal 1 are presented

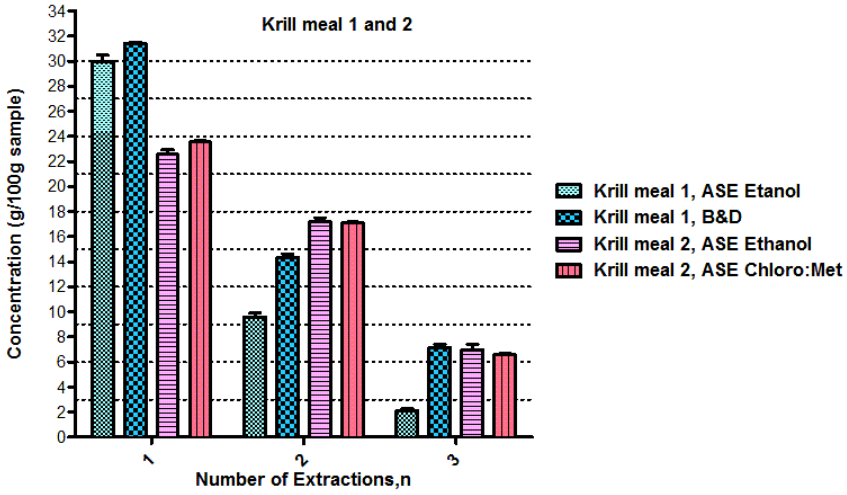


Figure 3.5: Lipid concentration of two different krill meals. Krillmeal 1 was analyzed by ASE with ethanol and Bligh and Dyer (B&D). Krillmeal 2 was analyzed by ASE ethanol and by ASE chloroform: methanol 2:1 (this method represents the hypothesis test performed). The data bars are given in mean values (± SEM, n=6). See Appendix C and Appendix D for presentations of raw data.

together with the washout curve calculation, in the next section. More lipids of krill meal 1 were extracted when B&D was applied. A probable explanation to this is the vigorous homogenization process used in B&D. This homogenization may result in more lipids being dissolved in the solvent of extraction. In addition, if the lipids of this krill meal compose high amounts of non-polar lipids. Non-polar lipids are more likely to dissolve in chloroform than in ethanol[10]. This is further discussed in the next chapter, where the lipid class composition of each sample is revealed. A mix of solvents with polar and non-polar properties may give high solubility of all lipid components. Also, the B&D uses proper solvent composition of the solvents to attain most lipids to dissolve in the organic phase[93].

Krill Meal 2

There was no significant difference (P=0.507) between the different extractions of krill meal 2 obtained by ASE ethanol and ASE chloroform: methanol from overall all extractions. The first extraction yielded higher lipid extraction by ASE chloroform: methanol ($P<0.05$ $t=2.536$). Krill meal 2 is a special meal containing mostly PL and peptides. Even if Ellingsen discussed the lipids of krill [85],[85], no reference was comparable to this raw material.

ASE Chloroform: Methanol
Earlier research of ASE (100°C) found that ASE chloroform: methanol gave high and efficient total lipid values. Although the ASE chloroform: methanol gave high yields, it was implied that the results were greater than expected. Nontrivial quantities of non-lipid materials are probably extracted with this method[22]. On the other hand, emulsions of micelles or bicelles within the broken sample structure are unstable in high temperatures[37]. In addition, increased diffusion rates between sample and solvent make them easier to extract. Also, the extraction of krill meal 1 gave similarity of results between ASE ethanol and B&D. It is therefore reasons to believe that these methods employed for ASE, yield PL concentrations that are compatible with other extraction methods. This was especially the case for krill meal 1 and 2 extractions.

Unfortunately, we did not have time to perform a B&D extraction of the krill meal 2 or an ASE chloroform: methanol extraction on krill meal 1. As a result, it is unknown if the extracts were contaminated with non-lipids.

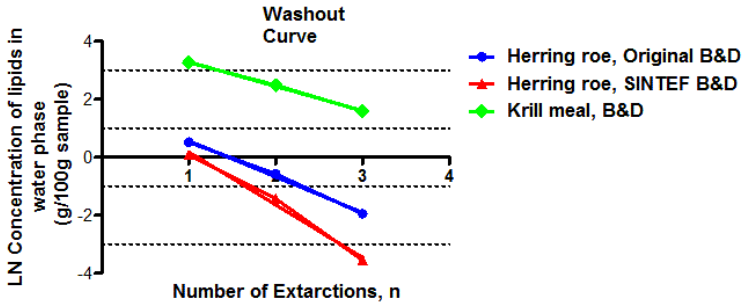
Diatomous Earth
Diatomous earth (DE) (sand in ASE sample cell) responds differently to the solvents employed in the different extractions by ASE. Based on the DE chemical properties, a conceivable explanation of the different lipid concentrations is stated. DE is a drying agent and is mixed with the samples in the extraction cell of ASE. It is considered to be a chemically inert matrix and has a low thermal conductivity[62]. Distinct equilibrium principles between the absorbed material within DE and the solvent depend on the solvent properties and the material adsorbed. The polar material, DE and water, may adsorb the PL so strongly that it will take two extractions to receive most of it in the extraction bottle[92]. This may explain why the experiment of ASE chloroform: methanol of herring roe, achieved similar lipid concentration as the first extraction. DE particles construct different diameter size, typically 10-200 microns across with variable shapes[62]. The distribution of the particles within the mix with sample is often uneven. Roe has a relatively large particle diameter compared to meal particles. This may contribute to unequal extraction and absorption of solvent.

General Comments
Newer research of total lipid recovery in soybean, calf brain and egg yolk found that a single extraction by PLE (pressurized liquid extraction) yield 94% of total lipids. The modified Folch method gave 77-83% lipids after one extraction. Four extractions were necessary to recover the total lipid content[110]. In general, the results obtained in this work suggest that by ASE extractions most marine lipid should be extracted by tree extractions. For both Herring roe and Krill meal 1 and 2 at least two extractions is necessary to yield high and efficient lipids concentrations of the samples. However, the B&D extractions of Herring roe yield low lipid concentrations after one extraction. The number of extractions needed for total lipid recovery is discussed in the next section.

Unfortunately, solvents are expensive and some are health hazardous. The employment of more extractions leads to increased solvent exposure and the need for more solvents may be costly. Also the ASE instrument is fairly expensive.

3.2.4 Washout Curve of Lipid Extraction

A washout curve of B&D extracts of herring roe and krill meal 1 is shown in this section. The objective was to find a formula to estimate the total lipid concentration as a function of the number of extractions, and to use this function to indicate the number of extractions needed to obtain high lipid yield.



	Herring roe, Original B&D	Herring roe, SINTEF B&D	Krill meal, B&D
Best-fit values			
Slope	-1.235 ± 0.07794	-1.820 ± 0.1790	-0.8400 ± 0.03464
Y-intercept when X=0.0	1.800 ± 0.1684	2.003 ± 0.3866	4.130 ± 0.07483
X-intercept when Y=0.0	1.457	1.101	4.917

	Herring roe, Original B&D	Herring roe, SINTEF B&D	Krill meal, B&D
R square	0.9960	0.9904	0.9983

Figure 3.6: Washout curve of herring roe and krill meal 1 extracts from three extractions by the B&D methods. The plotted concentration values represent the remaining lipids in the water phase after an extraction has been performed. The concentration values are given in natural logarithmic (LN)- values, estimated by linear regression. Extrapolations reveal the total lipid concentration of the samples. Raw data and calculations concerning the graph values are found in Appendix C and Appendix I.

ASE and Washout Curves

The use for this purpose rest on the equilibrium theory of liquid- liquid extraction. An equation was derived for the equilibrium constant. The equilibrium constant and the known lipid yield of each extraction can be used to calculate the lipid concentration left in the water phase, see Appendix I for detailed calculation of the washout curve, and Section 1.5.2. The equilibrium constant calculated, is dependent on the solvents being two immiscible phases[92]. Different from the B&D method, the ASE extraction is dependent on surface equilibrium between solvents and the adsorbed material within DE. Also, solubility and mass transfer effects is affected by increased temperature and pressure of ASE[82]. For these reasons, no washout curve is calculated from the ASE extracts.

Equations
The washout curve for herring roe and krill meal by B&D method and herring roe washout curve by SINTEF B&D, is shown in Figure 3.6. From the general equation of a linear curve y=ax+b (where a is the slope, b is the intercept, y is the natural logarithm (LN) of the lipid concentration in the water/methanol phase and x is the number of extractions), an equation of each sample is found as a linear regression line. The equation for krill meal 1 was y=-0.840x +4.130, for herring roe original B&D it was y=-1.235x+1.800 and for Herring roe with SINTEF B&D it is y=-1.820x+2.003. All the linear regression lines gave high correlation coefficients. This means that the point values lies close to the constructed straight line. This may be a good fit for explaining the behavior of the lipids extractions of these samples.

Herring Roe
Extrapolation of the regression lines is shown in Figure 3.6. It gives total concentration of lipids in Herring roe to be 6.049 ($e^{1.8}$) for the original B&D and 7.389 ($e^{2.003}$) for the SINTEF B&D. The total amount of extracted lipids from three extractions of herring roe is 4.93± ±0.20 g/100g sample (mean± SEM) for the original B&D. This was only c.1 g lipids less than the extrapolation value. The SINTEF B&D 4.89± 0.13 g/100g sample (mean± SEM) was missing c. 2.4g from the total extrapolation value. Earlier research of Herring roe revealed a total lipid concentration of Atlantic herring (*Clupea harengus*) of 3.5% wet weight and Baltic herring of 2-11% wet weight[48][7]. As discussed earlier, it is likely that the chloroform phase of SINTEF B&D is contaminated and that the extract contains some proteins, see section on B&D. In addition, the extrapolated value of these extraction regression lines yield too high lipid concentrations and pinpoints that this extract may contain more than just purified lipids.

Krill Meal 1
Ellingsen, Saether and Mohr (1986) determined that lipid content in various Atlantic krill species ranges from 12-50% (dry weight), depending on the season[85]. Research of Euphasia superba (dry weight) found from extraction by Folch that the lipid amount peaked to about 39.2% (dry weight) in the autumn[38]. The extrapolated value of Krill meal 1 is 62.178 ($e^{4.130}$), which is higher than these values. This may be due to that the lipid concentrations earlier found was extracted once. Hence, that an incomplete extraction of lipids has occurred. As mentioned earlier, pigments within the krill meal may participate into the chloroform phase. Consequently, it may give a non-purified lipid extract with overestimated values. However, from all the extractions performed the total lipid concentration obtained were 52.86± 0.43 g/100g sample (mean± SEM) for krill meal 1. It can be calculated from the regression line that X=4.917 when y=0 (cons= 1). This means that at least five extractions are needed to wash out most of the lipids (resulting in 98.39% lipid yield) from the water phase.

Some PL and polar lipids may form strong attractions to the proteins and to the organic tissue. These may be almost impossible to extract[37]. An approach concerning PL extraction, involves the removal of the methanol-water layer by aspiration and removal of the chloroform layer. The tissue is blended with chloroform and filtrated under

pressure. Research has showed that this method can yield some of the leftover polar lipids within the membrane[8].

General Comments

Testing of the maximal extractions to perform for complete lipid yield was not performed in this work because of limiting time issues. However, it is important to keep in mind that the B&D method may not be able to extract all PLs within the membrane. Therefore, small underestimations of the total lipids from the extrapolated values from the washout curve may occur.

The experiments resulted in variable slopes for the different regression lines for krill meal and herring roe. This means that the washout curves parameters of different samples may differ. To find the total lipid yield of each sample (extrapolated value of the washout curve), three extractions are needed.

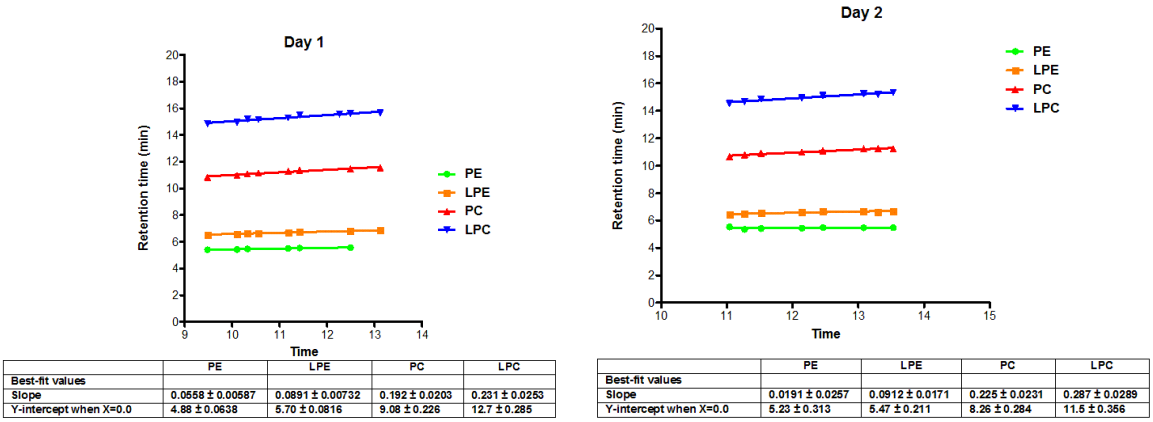
It is expected that most lipids, about 70% of the total lipids, are extracted from the first centrifugation of liquid-liquid extraction (B&D)[92]. Adsorption of organic phase to the tissue contributes to incomplete extraction. Earlier research of oils extraction by B&D state that the amount of lipids remaining in both the water phase and the tissue is negligible. However, polar lipids (PL) are only dissolved to a low degree in the chloroform phase. As a result, most of them remains in the tissue and water phase or form emulsions between the two phases[93]. The polar lipids as well as the remaining lipids in the absorbed chloroform phase may be more efficiently extracted when more extractions are performed. Especially samples possessing high concentration of phospholipids will benefit from increasing numbers of extractions. The washout curve shows improved efficiency of multiple extracts for the B&D extraction method. Also it is shown that the extraction efficiency declines. After four extractions of herring roe and six extractions of krill meal, very small amount of lipids are extracted. Anyway, there is a possibility that some of the phospholipids highly attached to the proteins and membranes, are extracted in later extractions.

3.3 Improvement of a PL class analysis by HPLC-CAD

This section presents the major steps of the improvement of a PL-class analysis by High Performance Liquid Chromatography with Charged Aerosol Detector (HPLC-CAD). First, observations of the HPLC chromatogram changes are displayed. Secondly, results from the first calibration are showed. A second calibration curve was analyzed followed by a CN-analysis. Then a solvent test of soy lecithin was performed. The third and final calibration curve is presented graphically with equations for all PL- class analyses employed in this study. Finally, a CN-analysis of the third calibration concentrations is shown.

3.3.1 Observations

Prior to calibration, tests of the reproducibility of the detector responses and retention times were conducted. Standards were used for this analysis.



	PE	LPE	PC	LPC
Best-fit values				
Slope	0.0558 ± 0.00587	0.0891 ± 0.00732	0.192 ± 0.0203	0.231 ± 0.0253
Y-intercept when X=0.0	4.88 ± 0.0638	5.70 ± 0.0816	9.08 ± 0.226	12.7 ± 0.285

	PE	LPE	PC	LPC
Best-fit values				
Slope	0.0191 ± 0.0257	0.0912 ± 0.0171	0.225 ± 0.0231	0.287 ± 0.0289
Y-intercept when X=0.0	5.23 ± 0.313	5.47 ± 0.211	8.26 ± 0.284	11.5 ± 0.356

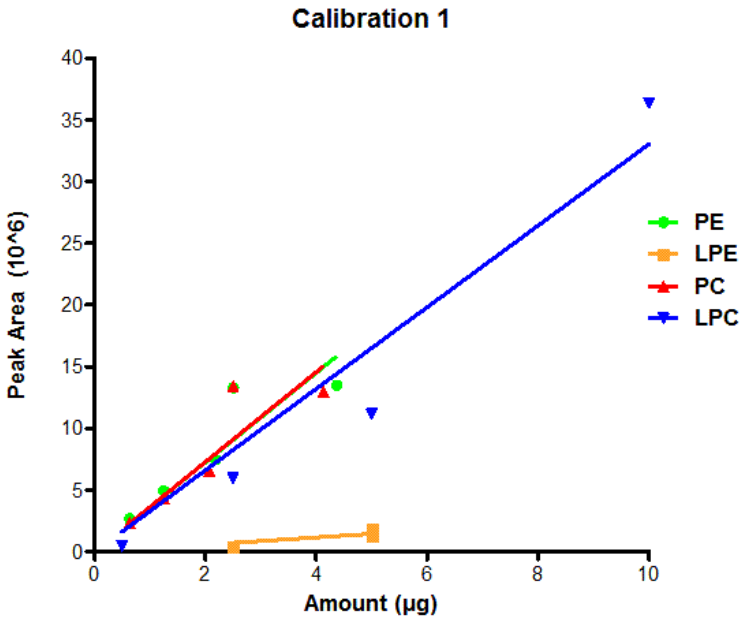
Figure 3.7: Retention time changes in the different standards as a function of increasing time during a day of analysis. Each data point represents an analysis and each analysis lasts for c. 22 minutes. The start of elution time is similar on both days because the column was rinsed prior to analysis (see Appendix E).

The solvent system, gradient mobile phase composition and the parameters of the HPLC instrument that was used was based on the recommendations of the HPLC/CAD manual. All PLs were well resolved in the solvent mix of the gradient system.

Retention time changes were observed from several chromatograms. Figure 3.7 shows the retention time changes as a function of time as the analysis is running. During day one (A) the linear increase of retention time varied from 0.05 for PE to 0.231 for LPC. Changes from day two (B) showed that the retention time increased with 0.019 for PE to 0.287 for LPC. Based on these observations, it was found that retention time increased with increasing polarity of the components. High loads of water causes diffusion processes in the column which change the chromatographic behavior[34]. The gradient solvent system increases to 13% towards the end of analysis (see Section 2). In this area, the most polar PL-classes are eluted. This is probably the reason for the increased retention time changes for more polar PL. Also, isopropanol caused higher levels of background noise at the end of the analysis[65]. To prevent elution of peaks within the area with high noise and to reduce the retention time changes, it was decided to rinse the column at the end of each analysis. The reproducibility of detector response and retention times was considered to be stable by these alterations of the method procedure.

3.3.2 First Calibration

At first different concentrations of standards in methanol: chloroform 1:2 were run, and the standard areas of the peaks were entered into the method. The calibration curve is only given in the amounts, 1-10μg, because in this area of the graph the detector response is linear. Increasing standard concentration received decreasing detector response.



	PE	LPE	PC	LPC
Best-fit values				
Slope	3.63 ± 0.433	0.293 ± 0.0471	3.65 ± 0.451	3.31 ± 0.343
Y-intercept when X=0.0	0.0	0.0	0.0	0.0

Figure 3.8: The first calibration curve with methanol: chloroform 1:2 obtained for HPLC PL-class analysis. Values are given in Table X, Appendix X. The linear curve represents detector response of peak area as a function of amount standard injected from an analysis (see Appendix E).

The PL-classes elute with increasing polarity. PE is the least polar component and LPC is the most polar component that is analyzed[37]. Each peak, formed by a single PL class, compose different saturated and unsaturated fatty acids and moieties in their molecules. This may be the reason for tailing and broad areas of some peaks[12].

The baseline is relatively smooth until about 4 minutes. After 4 minutes the baseline increases and plateau at 17 minutes. It has been shown that the effect of water as a solvent on the CAD response with no lipids injected, produce a higher baseline[65]. Therefore the effect of increasing baseline may be due to increasing water content of the gradient solvent system within this time range of analysis.

The first calibration curve is shown in Figure 3.8. Linear regression of the standard points show poor linearity, except for LPE. It has been found that CAD give linear calibration for concentrations below 10ppm Linear regression line is expected for HPLC-CAD when small amount of sample is detected[20][14]. Although the standards are different and separable, they share quite similar chemical compositions[37]. Based on this and also on other research results, an approximate calibration curve giving relatively comparable slopes for all PL classes are expected within the linear range[65]. The variable behavior of the standards may be a result of pollution or that standards were incomplete dissolved in solution. There was also a possibility that the concentrations made were inaccurate. A new calibration was therefore conducted.

Organic solvents are often required to make up some fraction of the eluent[20]. To evaluate the best pure solvent to use as a dissolvent of the standard PL, LPC was dissolved in chloroform and in isopropanol (1 mg/ml). At this time, LPC was the only available standard powder at SINTEF. Also, it was desirable to employ pure solvents to avoid errors involved with mixed solvent concentrations (example from pipetting and evaporation of solvents). Observations found that LPC with isopropanol received a clear solution while LPC with chloroform gave a cloudy solution. This indicates that the standard was not dissolved completely in chloroform. Isopropanol was therefore used as solvent for the PL standards in the next calibration.

3.3.3 Second Calibration

The second calibration to be evaluated analyzed new standards; purchased at Avantilipids, see Table 2.2, and dissolved in Isopropanol. At least four different amounts (different concentrations with 10μl injections) of each standard were analyzed. These standards are products from soy and egg yolk, due to either scares availability as well as the high cost of marine standards[4].

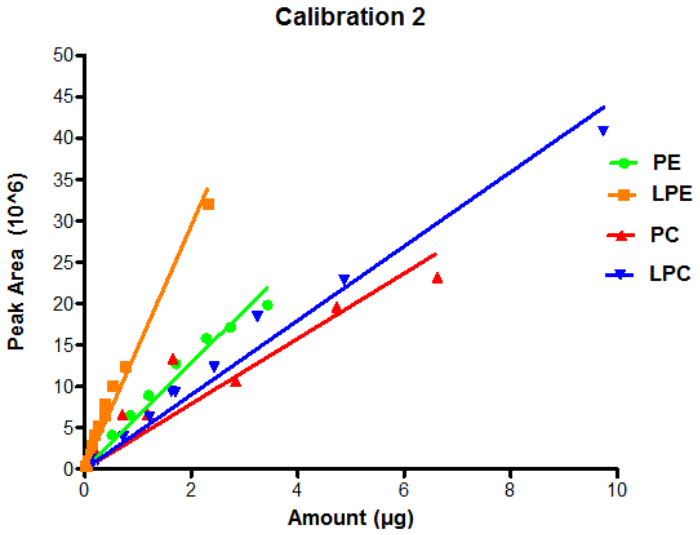
Results of the second calibration curves are shown in Figure 3.9. Linearity were achieved from the calibration curve regression lines of all standard samples analyzed. As discussed in the former section, the calibrated PL standards are expected to yield relatively similar slope values within the linear range[65]. However, unexpected high variability between the slope values of the different PL standards was obtained from the second calibration.

A relatively spread distribution in the points were observed. Unfortunately, there were variable slopes calculated for the calibrated lines, giving unexpected values.

3.3.4 CN Analysis of the Second Calibration

To decide the second calibration concentrations reliability and accuracy, a CN-analysis was performed.

CN-analysis was used to detect if the standard solutions were equally distributed in the solvent. By this analysis, evaluation of the solvent systems ability to dissolve the different standards was found. The CN-analysis is often used to determine the precise amount of carbon and nitrogen in a sample[30][50]. Therefore, it is assumed that the



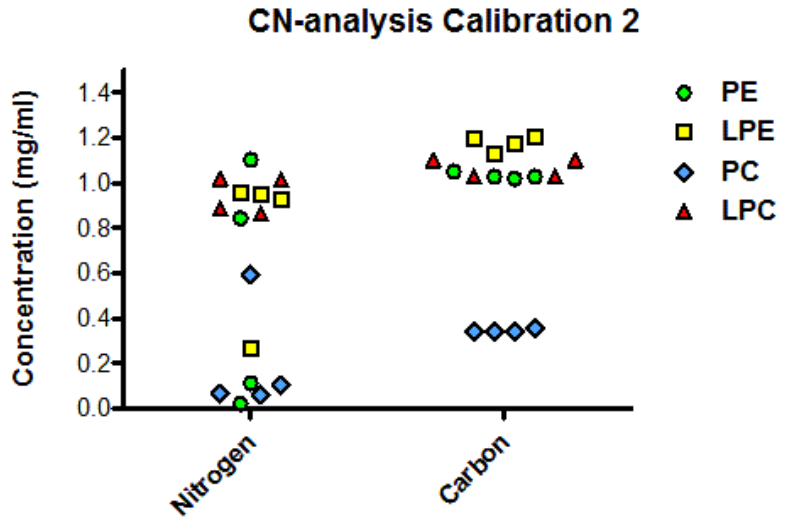
	PE	LPE	PC	LPC
Best-fit values				
Slope	6.40 ± 0.229	14.7 ± 0.545	3.94 ± 0.400	4.49 ± 0.163
Y-intercept when X=0.0	0.0	0.0	0.0	0.0

Figure 3.9: The second calibration curve of HPLC PL-class analysis by employing new standards with isopropanol. Abbreviations: PE,phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine (see Appendix E).

exact amounts dissolved in the solutions are found from CN-analysis. However, due to evaporation of solvents during sample preparation, it is suggested that this will not be a suited method for concentration determination.

The original concentration was supposed to be 1mg/ml. Huge variation in the concentrations from calibration 2 was detected. Some of the standards differed in orders of magnitude from the original concentration. For example, LPC gave concentrations of c.1mg/ml and PC gave concentrations of c. 0.1 mg/ml. It is crucial to attain similar concentrations of each standard analyzed. If this is the case, the standards give solutions that are highly reproducible and more reliable. This was not achieved from these solutions.

Research found that the CN-analysis can detect relatively precise ratios of the C: P[52]. Based on this, relatively similar ratios between C: N from all standards are expected. A variance test compared the difference between Carbon and Nitrogen concentrations of the different standard samples. The intention was to find if similar amount of standards were dissolved in the solvent system. The calibration proceeded until the different standard concentration values gave relatively comparable variation coefficients. The CN-analysis gave very different variation coefficients of the different standards (from



	PE	LPE	PC	LPC
Coefficient of variation	46.65%	28.94%	35.47%	8.38%

Figure 3.10: Scatter plot of carbon and nitrogen concentration from four different PL-class standards, obtained from CN-analysis of the second calibration. Each PL-class was analyzed four times. The variation coefficients of N and C concentrations are given in the table (see Appendix F).

46.7% to 8.3%). The profiles of the different PL-class concentrations of the second calibration did not exhibit clustering for Nitrogen and Carbon, see Figure 3.10. Since the concentration values were very different, it was questioned if the solvent, isopropanol, dissolved all the standards equally. Therefore, it was concluded that the concentrations of standards used in the second calibration curve did not obtain sustainable accuracy. New standards with higher precision was to be made.

3.3.5 Soy Lecithin Test

Soy Lecithin composes high amounts of Phospholipids. It was employed in a test analysis to find the most optimal solvent system for all phospholipids class component. Standard preparation is of great importance because almost all membrane soluble lipids are insoluble in water[87]. As stated in the first calibration section, organic solvents are the primarily solvents to dissolve PL. Due to the fact that the first and second calibration curves gave very different results, further investigations were performed to identify the chemical properties of the solvents for the purpose to dissolve the standards employed. Soy lecithin was dissolved in four different solvent systems that are known to dissolve phospholipids quite well, to find the superior solvent system[37].

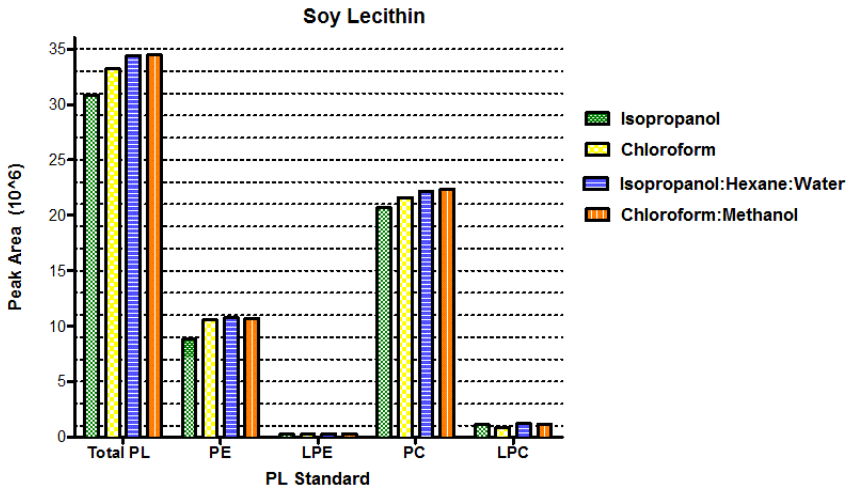


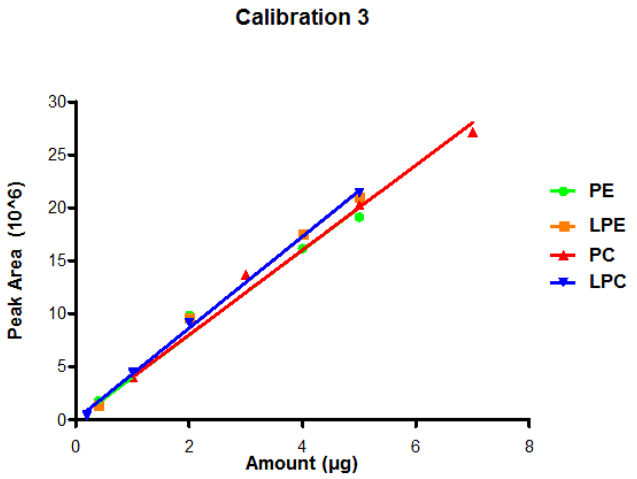
Figure 3.11: Detected peak area of the PL-class components in soy lecithin dissolved in four different solvent systems (n=1). Similar concentrations (1mg/ml) of Soy lecithin were dissolved in Isopropanol 100%, Chloroform 100%, Isopropanol: Hexane: Water 59:40:1 and Chloroform: Methanol 2:1. Appendix E shows the raw data.

Figure 3.11 shows the results of the peak area from detector responses of similar concentrations of each PL-class. Surprisingly, the test confirmed that all lipid classes that were dissolved in isopropanol, except for LPC, received detector response with lower peak areas. Relatively high concentrations of LPC were not dissolved completely in chloroform (see Calibration 1). Isopropanol and methanol are the most polar organic solvents and chloroform is the most non-polar solvent employed in this test[87]. An explanation to these observations may be that pure isopropanol dissolves the polar LPC completely, while the less polar PL, PE, LPE and PC is more dissolved in non-polar solvents, like pure chloroform.

The mixed solvents, methanol: chloroform and Isopropanol: water: hexane, gained similar and high detector responses of all PL-classes. On average their peak areas showed higher values, $4(10^6)$, than Isopropanol and higher values, $2(10^6)$, than chloroform. In addition, a mass balance between the total lipids detected for both solvent mixes are found. It seems that the mixed solvent systems were crucial for complete dissolvent of all PL-classes. Probably, the solvent mixes fulfill all properties of the different structural combinations of PL having LCFA on a phosphate head. Chloroform is the most volatile solvent in this test, being problematic since of evaporation causes inaccurate concentration analysis. Also, the detector, Corona CAD, is not compatible with volatile solvents, but this may not be a problem due to the minor injections of sample applied for analysis. Hexane: isopropanol: water 40: 59: 1 was chosen due to its appropriateness as a solvent for all PL-classes and its compatibility with the mobile phase and CAD detector. It was decided to employ similar solvent system to dissolve the sample extracts.

3.3.6 Third Calibration

Four analyses for different amounts of each standard were performed. The peak areas were integrated and the third and final calibration curve was established.



	PE	LPE	PC	LPC
Best-fit values				
Slope	3.9982 ± 0.17578	4.3186 ± 0.10254	4.0032 ± 0.11825	4.3224 ± 0.073864
Y-intercept when X=0.0	0.0	0.0	0.0	0.0

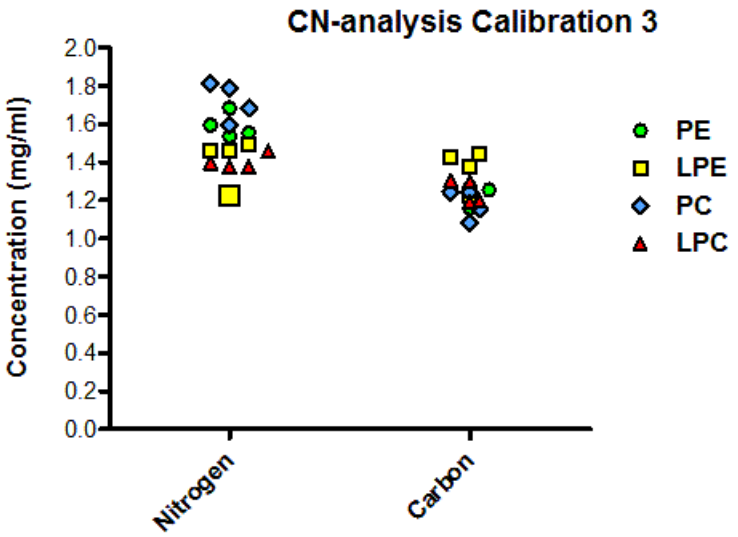
Figure 3.12: Calibration curve of four phospholipid-classes by HPLC-CAD analysis. Performed for the third and final time. PE: Phosphatidylethanolamine, LPE: Lysophosphatidylethanolamine, PC: Phosphatidylcholine, LPC: Lysophosphatidylcholine. The calibration lines are determined by linear regression trough each point of peak area as a function of standard phospholipid-class weights. Raw data are presented in Appendix E.

Figure 3.12 shows that the response of peak area as a function of amount standards injected, was linear for the HPLC PL-classes calibration curve. Negligible, but small deviations from linearity was observed. The slope and intercept with standard deviations of each standard calibration curve is shown in the table. The different phospholipids classes require separate calibration curve[47]. Expected values of similar intercept and small differences within the slope values were obtained.

The relationship between the peak areas and the solute concentration of the standards are described by the following equation: y=ax+ b, in which a is the constant arising from sample amount, x, analyzed and b is 0 (the solvent constant) and y is the peak area integrated from analysis. From the HPLC-calibration curve the equations of each PL-class is: PE; y= 3.99*x, LPE; y=4.316*x, PC; 4.00*x and LPC; 4.32*x.

3.3.7 CN-Analysis of the Third Calibration

A CN-analysis of the four standard concentrations was conducted. It was employed to secure the reproducibility and accuracy for the concentrations of the final calibration curve.



	PE	LPE	PC	LPC
Coefficient of variation	17.95%	2.12%	26.30%	8.22%

Figure 3.13: Scatter plot of the standard concentrations of the third calibration by CN-analysis. The coefficient of variation compares the Nitrogen and Carbon concentration means and SEM of each PL-class. Small variations indicate higher precision and more likely true concentrations made (see Appendix F).

The original concentrations made were 1mg/ml. This CN-analysis gave much higher concentration values for all standards (see Figure 3.13). As mentioned earlier, evaporation of solvents may occur during sample preparation prior to analysis. In addition, the solvent system used composed some fairly volatile solvents, mainly hexane. The major loss of volatile solvent prior to analysis may result in higher concentrations than originally made.

The CN-analyses are used to find the exact amount of C: N ratio, as this may be a precise measurement[30]. Earlier, it was emphasized that the standards contained different fatty acids. The predominant species were used to calculate the concentrations from the carbon content given from CN-analysis. Because some of the fatty acids are excluded from concentration calculations, the concentrations are approximate values. Therefore it is expected that there are some variation between the carbon and nitrogen concentrations. Small values of the variation coefficients of each sample imply that there are small differences within the samples. These results indicate that the Calibration 3

curve could be reliably used for quantitative analysis of PL classes. Clustering of the distribution from both Carbon and Nitrogen of all the standards, reveals that even amounts of PL-classes are dissolved within the solvent system.

The concentrations were prepared in great detail. Also, there is an uncertainty related to solvent evaporation. Therefore, it was decided to use the original concentrations for calculation. Furthermore, the curves of the different PLs are quite similar. As stated earlier, although the standards are different and separable, they share quite similar chemical compositions[37]. Based on this and also on other research results, an approximate calibration curve giving relatively comparable slopes for all PL classes are expected within the linear range[65]. From this, indications that the standards are equally distributed in the solvent system are likely. The third calibration was approved to represent the calibration curve for HPLC PL-class analysis.

The detector is responding differently to the concentrations of different PL standards[65]. Therefore it is crucial to calibrate each standard that is used for analysis. Unknown components within a sample are determined from the calibration curve by finding the amount corresponding to the peak area, found from each equation. The retention time value of the peak determines which calibration curve to, referring the qualitative analysis of the samples components. Retention time windows were set for each standard calibration. This allows the peak to drift slightly and still be identified. The chemical nature of the marine samples composes more unsaturated fatty acids compared to the plant standards employed and earlier research found that this feature gave shorter and broader peaks[42].

3.4 Lipid Class Analysis

A lipid class analysis of the B&D extracts performed by Iatroscan TLC-FID is presented in the first section. Next, a washout curve of the PL and TG concentrations obtained by Iatroscan analysis is presented for krill meal 1 and herring roe. In the end, the PL concentrations of B&D extracts obtained by HPLC is presented and compared to the PL concentrations analyzed by Iatroscan.

3.4.1 Lipid Class Analysis Results

The results of lipid class analysis of the B&D extracts are carried out in this section. These analyses were conducted to attain an evaluation of the sample composition. The chemical behavior of the samples towards different extraction methods and solvents are discussed. B&D accomplishes a complete extraction of a vide range of lipid classes as well as being an established method[58]. Therefore, extracts obtained from B&D were analyzed by Iatroscan TLC-FID. The ASE extracts were not prioritized due to time limitations.

Lipid class composition of B&D extracts from different samples is viewed in Figure 3.14 and Table 3.4. One of the most striking feature of these profiles are the large peaks of Polar lipids (PL) concentrations of the roe extracts. Another striking feature is that the meal extracts contain more Triglycerides (TG) than the roe extracts. This is expected

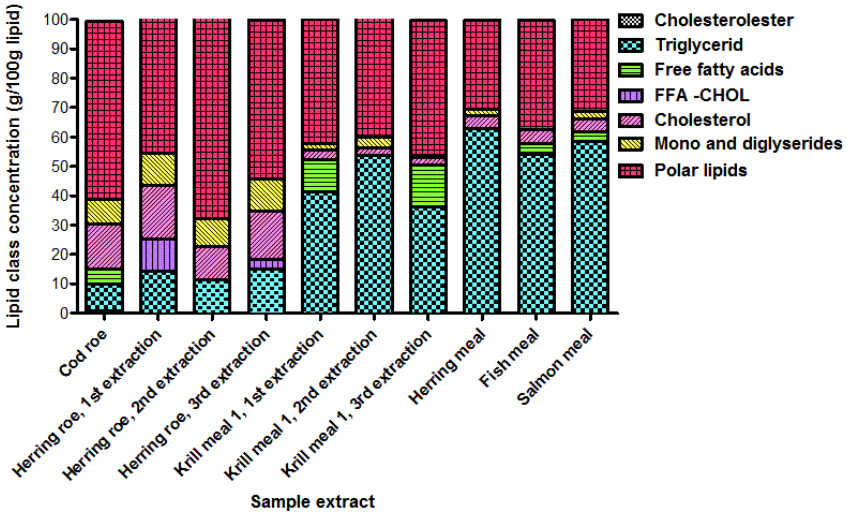


Figure 3.14: Lipid class analysis of the B&D extracts by Iatroscan TLC-FID. Mean lipid class composition of the B&D sample extracts analyzed.

because, with few exceptions, the predominant components of lipid classes in fish roe are TG and PL[7]. All major lipid classes presented in the majority of marine samples were analyzed. The dominant neutral lipids in fish are CE, TG, FFA and CHOL[73].

Roe

PL serves as the main energy source and therefore constitutes the majority of lipids. The fatty acid composition of polar lipids in fish roe are roughly 50% polyunsaturated fatty acids, 29% saturated and 19% monounsaturated fatty acids. The expected lipid class profiles are 71-76% PL, 5.9-6.8% CHOL and 12-13% TG and 5-6% FFA in cod roe. For herring roe the expected composition is and 68-89%PL, 3.6-9.1% CHOL, 6-16% TG and 5.7% FFA[7]. In general, lower PL values, higher CHOL levels and similar TG and FFA levels were found compared to the expected values. The major difference is that there are no expected mono and diglycerides, which was found in this experiment.

Mono and diglycerides occur only as a trace in most fish, unless extencive lipilysis has occurred[73]. The roe extracts compose high amounts of mono and diglycerides, indicating that lipolysis may have occurred. Also, overlap between the PL and the mono and diglycerides was observed. From this, a decrease in values of PL and an increase in values of mono and diglycerides may occur. When summing these values the total is c. 69 in cod roe and between c. 56-77 in herring roe (depending of the extraction number), which is similar to the expected values. Another issue it that methanol can cause problems in extracts. It can react with samples in an esterificaton reaction with carboxcylic groups[16].

Table 3.4: Table of lipid concentration values (g/100g lipid). Appendix 5 shows the raw data. Abbreviations; Cholesterol ester (CE), Triglycerides (TG), Free fatty acids (FFA), Cholesterol (CHOL), mono- and diglycerides and Polar lipids (PL). FFA-CHOL represents non-separable peaks spreading across both the free fatty acid and cholesterol retention time of the chromatogram.

Sample	PL	Mono-diglycerides	CHOL	FFA-CHOL	FFA	TG	CE	Total yield
Cod roe	60.28	8.67	15.42	0.00	4.48	9.39	0.56	98.80
Herring roe, 1 extr	45.67	10.94	18.32	11.52	0.00	15.71	0.00	102.16
Herring roe, 2 extr	67.87	9.41	11.58	0.00	0.00	10.67	0.00	99.53
Herring roe, 3 extr	53.98	10.96	16.59	3.85	0.00	14.20	0.00	99.58
Herring meal	30.49	2.06	4.38	0.00	0.00	62.57	0.00	99.50
Fish meal	37.15	0.00	4.68	0.00	3.81	55.16	0.00	100.79
Salmon meal	31.10	2.65	4.56	0.00	3.12	56.17	0.00	97.59
Krillmeal 1, 1 extr	42.08	2.46	3.19	0.00	10.40	42.01	0.00	100.14
Krillmeal 1, 2 extr	40.27	3.55	2.46	0.00	0.00	52.37	0.00	98.64
Krillmeal 1, 3 extr	45.96	0.66	2.74	0.00	13.83	35.47	0.00	98.67

Krill Meal 1

Estimations of other types of krill meals revealed a PL content of 9.33g and neutral lipids of 15.58g from a total lipid concentration of 25.54g[103]. These values do not fit with the krill meal 1, both in lipid concentration and lipid class composition. The compositions of the meals depend on the raw material, the species, the season and how it is processed. Application for patentees of the krill meals was considered during this work. Therefore, scarce information about the products was shared. Comparisons of these meals are therefore considered not to be validated. Total lipids of krill, E. Superba, varied from 8 to 36% lipids of TG and between 54 to 58% lipids of PL during winter season[9], indicating huge biological variations within the same species within a season.

Despite the fact that the sample extracts were stored at -80°C, degradation may occur during time-consuming analysis and thawing. Decomposition of lipid components can affect the accurate measurement of lipid class composition in a sample. Degradation of phospholipids, for example PE and PC is signified by an increase in free fatty acids[86]. The high FFA values of the analyzed krill meals are probably due to degradation of PL. It can result in decreased values of PL and increased values of FFA.

Fish Meal, Salmon Meal and Herring Meal

A fish meal lipid analysis only found 1.29g PL and 6.38g neutral lipids from a total lipid concentration of 8.87g[103]. The results obtained in this thesis were 37.15g/100g lipid PL of fish meal. The salmon and herring meals are quite similar and not significantly different from the fish meal. Overestimation of the polar lipid components in the meals

is probably caused by inclusion of pigments and antioxidants within this peak. The polar lipids peak can also contain some monoglycerides[75].

General comments

Although thin layers of the chromarods are quite homogeneous, there were small variations within the FID response among the rods for similar samples analyzed, especially for krill meal and cod roe. These samples composed salt or antioxidants coloring agents, which may result in minimized elution and a lowering of the useful lifetime of the rod. The peak shape and area is affected by the chemical nature of the sample. In fish samples with highly unsaturated fatty acids this feature is more pronounced, giving shorter and vide peaks[42]. This is an effort that describes why some of the separated peaks are broad, deformed and exhibits tailing.

When working with lipids there is always a chance that oxidation occurs. Oxidation can impact the lipid analysis by making the lipids less polar than originally. The lipids will therefore elute at a different time in the chromatogram. Lower chemical stability of the unsaturated substances in the biological samples is a possible explanation of a higher variation and standard deviations within some of the sample lipid classes. Reproducibility of the integration is a factor that has a negative effect on the reproducibility of analysis by TLC-FID[60]. Triglycerides generate a curvilinear relationship with sample loads[42]. Fixing the chromatogram baselines for different analyses resulted in variable integration areas of similar samples. The mass balance is almost 100% for the average value of each sample. The samples resulting in more or less than 100% lipids are probably due to individual fixing of the graphs and baselines.

Herring roe and krill meal 1 were extracted three times. The analysis results shoved that the second extraction contained higher amount of PL. In addition, there were more FFA-CHOL in the first and second extractions. The krill meal 1 contained more FFA in the first and third extraction and more TG in the second extraction. The PL composition was about the same in all three extractions of krill meal 1. This may show that not only PL, but also TG and other non-polar lipid classes remain in the tissue and water/methanol phase after the B&D extraction is performed. Also, increasing extractions yield increasing concentrations of more than just PLs.

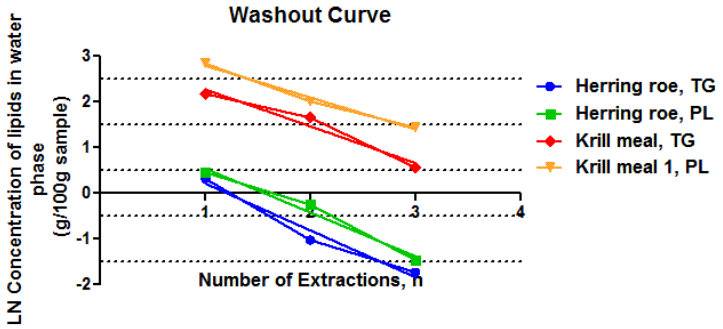
The lipid class composition of the various samples reveals why different methods yield different lipid concentrations. The fish meal, salmon meal and herring meal extracts compose more than 50% non-polar lipids. Non-polar solvents dissolve these sample components best. This is probably why the method employing non-polar solvents, example ASE chloroform and B&D, yield higher lipid concentrations of these samples. In contrast, a method employing polar solvents, for example ASE ethanol yield lower concentrations. On the other hand, the sample extracts of krill meal and roe composed more than 50% polar lipids. This favors the ASE ethanol method. The B&D method may exclude some polar lipids, especially when only one extraction is performed[93], see the Section 1.5.2. Therefore the composition amount may favor the non-polar lipids. On the other hand, the Iatroscan methods analyze not only polar lipids, but also all polar components in the extracts. Small polar components like coloring or antioxidants may

contribute to overestimate the amount polar lipids.

Unfortunately, quantification analysis by Iatroscan is highly affected by the spotting technique of the lab worker. Spreading of the sample on the chromarods result in broader bands and lower FID responses[88]. Several analyses were performed before obtaining acceptable reproducibility of samples application with syringe. Hence, the method is time-consuming and less efficient due to hard work for obtaining few results. Therefore no other method sample extracts were analyzed by this method.

3.4.2 Washout Curve of TG and PL Lipid Classes by Iatroscan

A washout curve is calculated from average PL and TG concentration values. It is derived from a general equilibrium constant equation. Comparison of extrapolation values from these washout curves and the extraction curves by B&D washout curve was conducted to state if mass balance were obtained.



	Herring roe, TG	Herring roe, PL	Krill meal, TG	Krill meal 1, PL
Best-fit values				
Slope	-1.025 ± 0.1826	-0.9608 ± 0.1489	-0.8056 ± 0.1643	-0.6970 ± 0.07690
Y-intercept when X=0.0	1.238 ± 0.3944	1.502 ± 0.3216	3.077 ± 0.3550	3.492 ± 0.1661
X-intercept when Y=0.0	1.208	1.564	3.820	5.009

	Herring roe, TG	Herring roe, PL	Krill meal, TG	Krill meal 1, PL
R square	0.9693	0.9766	0.9600	0.9880

Figure 3.15: Washout curve for the Triglycerides (TG) and the phospholipids (PL) of herring roe and krill meal analyzed by Iatroscan and HPLC. The remaining lipids in the water phase is calculated (see Appendix 5 and Appendix I). Natural logarithm (LN) of the water phase is plotted as a function of remaining lipids in the water phase after each extraction number. Extrapolations of this curve find the LN of the total concentration of this lipid component of the sample.

Figure 3.15 shows that on average the dominant lipid classes are TG and PL for both krill meal 1 and herring roe. Quantitative evaluation of total lipid concentration of each sample obtained by extrapolation values, presume relative responses to the regression lines. As a result, the total lipid amount estimated may only give approximate values.

Herring Roe
Extrapolation of Herring roe PL is 4.49g/100g sample (e1.502) and of TG it is 3.45g/100g sample (e1.238). The sum yields 7.94g/100g sample. The total lipids given from the original B&D washout curve is 6.049g/100g sample. There may be an overestimation within the regression line calculations giving the fact that these are approximate values. In addition, the errors and deviations are unknown. On the other hand, it was earlier stated that B&D might not extract all PL. The PLs that are not extracted can be ignored in the extrapolated value of total lipids extracted.

Krill Meal 1
Extrapolation of the washout curve regression line reveals the total amount of the lipid class in a sample. From extrapolations of the Krill meal the total amount of TG is 21.69g/100g lipid (e3.077) and PL is 32.85g/100g lipid (e3.492). The sum of these values is 54.54g/100g sample. The sum of the leftover free fatty acids, cholesterol and mono and diglyserides are 7.32g/100g sample. These make up the rest of the lipids in the krill meal extracts (the extrapolated value is not calculated because these values are so small). Overall total lipid concentrations of these values are 61.86g/100g sample. The extrapolated value of total lipids from Krill meal is 62.18g/100g sample. see extraction section. These results provide a mass balance between the extrapolated values of B&D and Iatroscan analysis. In addition, the mass balance emphases that the extrapolated values almost certainly represent the exact values in a sample.

From the linear regression it is possible to calculate the number of extractions needed to receive a high yield of PL or TG from the samples employed. In herring roe there are small amounts of both TG and PL extracted after one extraction. The Krill meal 1 gives high PL and TG values after many extractions.

3.4.3 Analysis by HPLC Method vs. Iatroscan TLC-FID method

Comparison of the total PL concentration in B&D extracts analyzed by Iatroscan and HPLC methods is presented in this section. The polar lipid composition of Iatroscan is directly translated into PL concentration, and is therefore an approximation. In this section the PL concentrations of HPLC analysis is only employed for comparison to the Iatroscan results. The HPLC results is presented and discussed in more detail in the next chapter. Expected values of the PL concentrations within each sample extract are presented in this chapter. The main objective was to evaluate the accuracy of the method and the PL values obtained.

Figure 3.16 presents the PL concentrations of the different B&D extracts obtained by HPLC and Iatroscan.

PL Extracts of the Meals
The methods and the samples were overall significant ($P<0.05$). All PL concentrations of meals analyzed by Iatroscan were significantly greater ($P<0.0001$) than by HPLC. From the graph, it seems that the Iatroscan TLC-FID approach in general yield higher total PL values than HPLC. These differences increases with increasing lipid content.

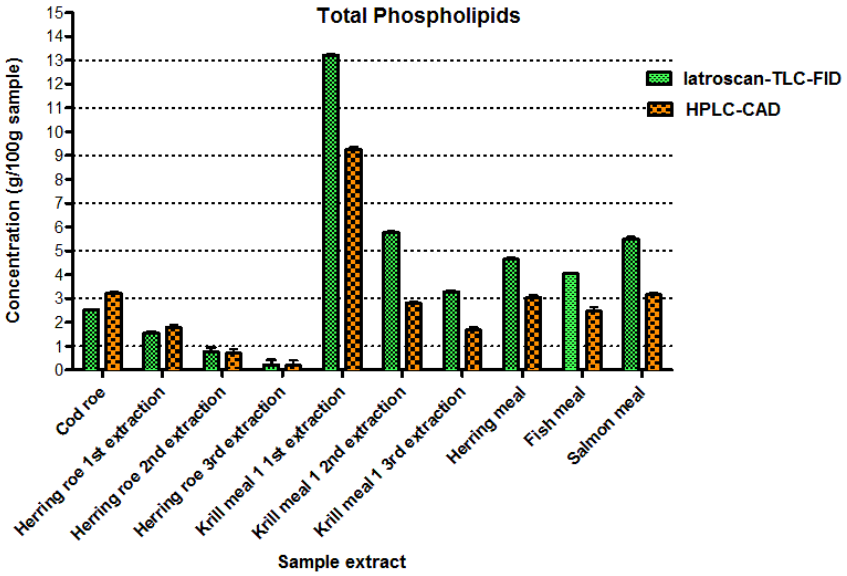


Figure 3.16: Comparison of the total PL concentration in different sample extracts of B&D by Iatroscan TLC-FID and HPLC-CAD. The bar-values represent mean (±SEM, n=4) for Iatroscan and mean (±SEM, n=6) for HPLC. In this graph it is assumed that all polar lipids of Iatroscan analysis is phospholipids (see Appendix G and Appendix 5).

Studies of aquatic invertebrates and fatty fishes conclude that the Iatroscan TLC-FID tend to underestimate total lipids. The distribution of each lipid class is affected by this and is therefore causing an overestimation of the other lipid classes[56]. This may explain why the polar lipid classes of the Iatroscan analysis are significantly higher than for the HPLC in the samples with high lipid content. It has been introduced earlier that pigments may contribute to an overestimation of polar lipids. Among all the samples, Krill meal gave the deepest stain on the Chromarods, followed by salmon meal, fish meal and herring meal. This indicates that these samples, especially the Krill meal, contained relatively high concentrations of pigments. Earlier research has showed that the extraction of freeze-dried meat balls received less pigments in the extract[14]. This may be a solution to the overestimation of the Polar lipids by Iatroscan.

PL Extracts of the Roes

PL concentration of cod roe that is analyzed by HPLC is significantly greater than by Iatroscan ($P<0.01$). No significant differences for the PL concentrations of herring roe analyzed by HPLC and Iatroscan were observed. HPLC and Iatroscan gave similar values. This indicates that the choice of method does not affect the determination of total PL concentration in herring roe extracs. Also, the mass balance between herring roe PL concentrations by HPLC and by Iatroscan suggests that this may be the true

value of PL concentration. The HPLC PL-concentration yielded 76.4% PL in cod roe and about 46-62% PL in herring roe (depending on the extraction number). According to the expected values, the cod roe obtained similar values (71-76%) and the herring roe values were underestimated (68-89%)[7].The herring roe contains salt, which may be the reason for lower extraction yield. Attractions between the salt and polar or charged components may cause crystallization and precipitation to some extent[92]. Thus, preventing the phospholipids to be analyzed. As a result, precipitation before application to chromarods may be a reason for the underestimation of PL concentration found by Iatroscan analysis.

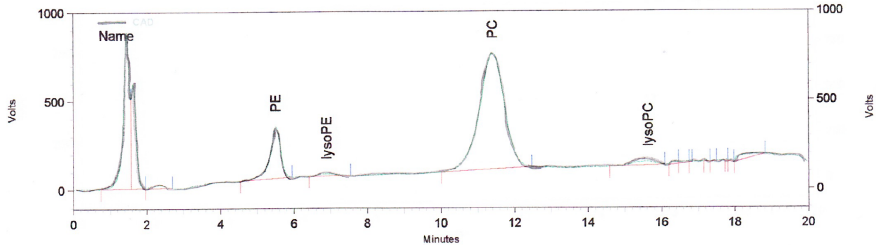


Figure 3.17: Chromatogram of HPLC separation of Herring roe. extracts by B&D. Peaks: 1,PE; 2, LPC; 3, PC; 4,LPC. The sum of the concentrations of all phospholipids class components in a sample are plotted as/resemble the total PL concentration of the sample. See Appendix G

Compared to Iatroscan, HPLC provides sharper and narrower peaks that is less affected by the unsaturation of the fatty acids in the sample[42]. The integrated peak areas are therefore easier to estimate correctly for the HPLC method, giving a smaller uncertainty of the method and samples analyzed. An example of HPLC separations are shown in Figure 3.17. Prior research comparing TLC-FID and HPLC concluded that the HPLC method correlated the best-calculated and experimented values. Higher results of TLC-FID may be due to the retention principle of adsorption without specific interactions of functional groups[109]. Based on this, it was decided to employ the HPLC B&D results as references in the HPLC-PL class analysis.

3.5 Phospholipid Analysis

The sample extracts from all the extractions performed was analyzed by HPLC-CAD, to find its PL-class distribution and total PL concentration. In this chapter the PL concentrations by Bligh and Dyer original and the SINTEF method are compared at first. In the next section, different extraction methods are introduced to eliminate methods that give a lower yield of PL by the first extraction than the reference method. Then, three extractions are performed on samples having a high PL concentration employing the remaining methods. A hypothesis of ASE Chloroform: methanol was tested. Finally, a washout curve of PL concentrations obtained by B&D with tree numbers of extractions is presented. An observation that was not further investigated is presented in the last

section.

3.5.1 PL Class Analysis Results

HPLC-CAD was employed for all PL-class analysis.

The data, given in Table 3.5, represents all the HPLC analyses performed in this thesis. The total phospholipid concentration is the sum of the four lipid-class concentrations found from HPLC analysis. The last table row gives the total PL concentration extracted from the samples. Qualitative fatty acid composition within the PL-classes was not tested in this thesis due to time limitations. As discussed earlier, the positive contribution of increased diffusion rates by increased temperature does not change the FA.

There are many challenges to HPLC-CAD PL-class analysis. Some of these are the limited amount of solvents to employ and that it is universal and does not detect FAME or saturation of the lipids[65].Due to high sensitivity, solvent impurities are detected as noise[20]. This emphasizes the importance of having pure solvents and samples. In Section 3.4.3, it was discovered retention time changes, and baseline noise in the chromatograms. These are described more detailed in Section 1.6.2.

Note that the total PL concentrations are measured as extracted PL of the sample, while the PL class concentrations are measured as the PL amount within the extracted lipids (gram per 100 gram lipid).

The expected PL values for all extraction samples were discussed in section 2,X and compared to values analyzed by Iatroscan. According to the expected values, the B&D extracts analyzed by HPLC was considered to yield accurate PL concentrations. The expected values of total PL concentration lie within the B&D extracts (for some samples three extractions are crucial), which is set as reference in this section. In addition the B&D is an established method worldwide, trusted to give high yields of PL[44].

3.5.2 Bligh and Dyer Methods

A comparison between PL concentration obtained by SINTEF B&D and the Original B&D was analyzed from three extractions. The aim was to evaluate the overall differences between the methods. Herring roe was employed due to its high water content. The high water content alters the solvent proportions of the SINTEF B&D method, differing from the original method[8].

The content of total phospholipids concentration in different extracts of the two separate B&D methods is viewed in Figure 3.18, A.

Table 3.5: PL-class amount by HPLC in all extracts

HPLC results for all extractions performed						
Sample	PL class (g/100g lipid mean ±SEM)				Total PL (g/100g lipid mean ±SEM)	Total PL (g/100g sample mean ± SEM)
	PE	LPE	PC	LPC		
B&D						
Cod roe	14.78 ± 0.24	1.07 0.07	58.66 ± 0.76	1.86 ± 0.10	76.37 ± 0.81	3.21 ± 0.04
Hering roe, 1 extr	7.59 ± 0.32	±	44.20 ± 0.43	0.65 ± 0.07	52.43 ± 0.54	1.78 ± 0.04
Hering roe, 2 extr	9.35 ± 0.25	±	52.64 ± 0.98	±	61.99 ± 1.01	0.70 ± 0.14
Hering roe, 3 extr	6.34 ± 0.32	±	39.76 ± 0.70	±	46.10 ± 0.77	0.19 ± 0.16
Krill meal 1, 1 extr	1.90 ± 0.04	0.44 ± 0.03	25.04 ± 0.82	2.11 ± 0.13	29.49 ± 0.83	9.26 ± 0.04
Krill meal 1, 2 extr	1.43 ± 0.07	±	16.65 ± 0.46	1.54 ± 0.07	19.62 ± 0.48	2.81 ± 0.04
Krill meal 1, 3 extr	1.55 ± 0.04	±	19.05 ± 0.43	2.30 ± 0.17	22.90 ± 0.47	1.63 ± 0.04
Fish meal	5.64 ± 0.47	1.01 ± 0.12	14.35 ± 1.05	1.64 ± 0.08	22.63 ± 1.16	2.46 ± 0.06
Herring meal	3.04 ± 0.19	0.40 ± 0.03	14.97 ± 0.30	1.53 ± 0.06	19.94 ± 0.37	3.04 ± 0.04
Salmon meal	3.04 ± 0.21	0.54 ± 0.03	12.61 ± 0.26	1.66 ± 0.09	17.85 ± 0.34	3.16 ± 0.04
Modified SINTEF B&D						
Herring roe,1 extr	9.78 ± 0.18		53.64 ± 1.09	1.08 ± 0.03	64.49 ± 0.48	2.51 ± 0.03
Herring roe,2 extr	8.89 ± 0.13		51.70 ± 1.87	±	60.58 ± 0.77	0.53 ± 0.05
Herring roe,3 extr	5.16 ± 0.12		34.00 ± 1.37	0.30 ± 0.03	39.45 ± 0.57	0.08 ± 0.06
ASE Chloroform						
Cod roe	3.27 0.28	0.37 0.06	21.10 ± 0.95		24.74 ± 0.99	0.36 ± 0.05
Fish meal	1.02 ± 0.07	0.24 ± 0.02	3.86 ± 0.20		5.13 ± 0.21	0.77 ± 0.05
Hering meal	3.13 ± 0.19	0.26 ± 0.02	9.63 ± 0.42	0.64 ± 0.03	13.65 ± 0.47	1.39 ± 0.05
Salmon meal	0.55 ± 0.02	0.37 ± 0.24	6.13 ± 0.09		7.05 ± 0.25	1.12 ± 0.05
ASE Ethanol						
Cod roe	1.22 0.09		4.59 ± 0.15	0.32 ± 0.02	6.12 ± 0.18	0.60 ± 0.04
Herring roe,1 extr	3.48 ± 0.35	0.32 ± 0.01	17.34 ± 0.17	0.53 ± 0.02	21.67 ± 0.39	1.67 ± 0.04
Hering roe, 2 extr	5.91 ± 0.24	0.31 ± 0.02	24.41 ± 0.57	0.65 ± 0.07	31.28 ± 0.63	0.84 ± 0.04
Herring roe,3 extr	8.20 ± 0.36	0.58 ± 0.03	38.69 ± 0.66	1.13 ± 0.06	48.59 ± 0.75	0.45 ± 0.04
Krill meal 1, 1 extr	3.90 ± 0.54	1.01 ± 0.22	26.53 ± 0.44	3.41 ± 0.33	34.85 ± 0.80	10.46 ± 0.04
Krill meal 1, 2 extr	2.36 ± 0.28	0.54 ± 0.03	21.52 ± 1.25	3.86 ± 0.22	28.28 ± 1.30	2.72 ± 0.06
Krill meal 1, 3 extr	2.37 ± 0.20	1.63 ± 0.19	15.40 ± 0.36	2.57 ± 0.18	21.97 ± 0.49	0.46 ± 0.04
Krill meal 2, 1 extr	3.75 ± 0.13	0.52 ± 0.02	40.35 ± 0.43	0.81 ± 0.08	45.42 ± 0.46	10.24 ± 0.04
Krill meal 2, 2 extr	2.94 ± 0.11	0.36 ± 0.04	54.17 ± 0.31	2.17 ± 0.12	59.64 ± 0.35	10.26 ± 0.03
Krill meal 2, 2 extr	4.86 ± 0.22	0.65 ± 0.08	42.45 ± 0.69	1.14 ± 0.17	49.09 ± 0.75	3.44 ± 0.04
Fish meal	5.88 ± 0.13	4.51 ± 0.30	12.45 ± 0.18	2.85 ± 0.13	25.68 ± 0.40	3.66 ± 0.04
Herring meal	1.33 ± 0.06	0.38 ± 0.01	3.96 ± 0.19	±	5.67 ± 0.20	0.93 ± 0.05
Salmon meal	5.35 ± 0.09	5.12 ± 0.30	17.55 ± 0.22	2.76 ± 0.14	30.77 ± 0.41	4.85 ± 0.04
ASE Chloroform: methanol 2:1						
Hering roe, 1 extr	3.78 ± 0.08	±	22.96 ± 0.27	0.62 ± 0.04	27.36 ± 0.28	0.91 ± 0.04
Hering roe, 2 extr	7.81 ± 0.26	0.54 ± 0.03	18.95 ± 0.38	0.50 ± 0.01	27.80 ± 0.46	0.96 ± 0.04
Hering roe, 3 extr	8.15 ± 0.24	0.95 ± 0.01	36.51 ± 0.42	0.76 ± 0.04	46.36 ± 0.49	0.42 ± 0.04
Krill meal 2, 1 extr	6.08 ± 0.07	±	60.08 ± 0.80	±	66.15 ± 0.80	15.58 ± 0.04
Krill meal 2, 2 extr	3.41 ± 0.10	0.65 ± 0.06	35.52 ± 0.66	0.69 ± 0.08	40.27 ± 0.68	6.89 ± 0.04
Krill meal 2, 3 extr	3.79 ± 0.23	0.69 ± 0.07	44.59 ± 0.62	0.87 ± 0.05	49.93 ± 0.67	3.27 ± 0.04

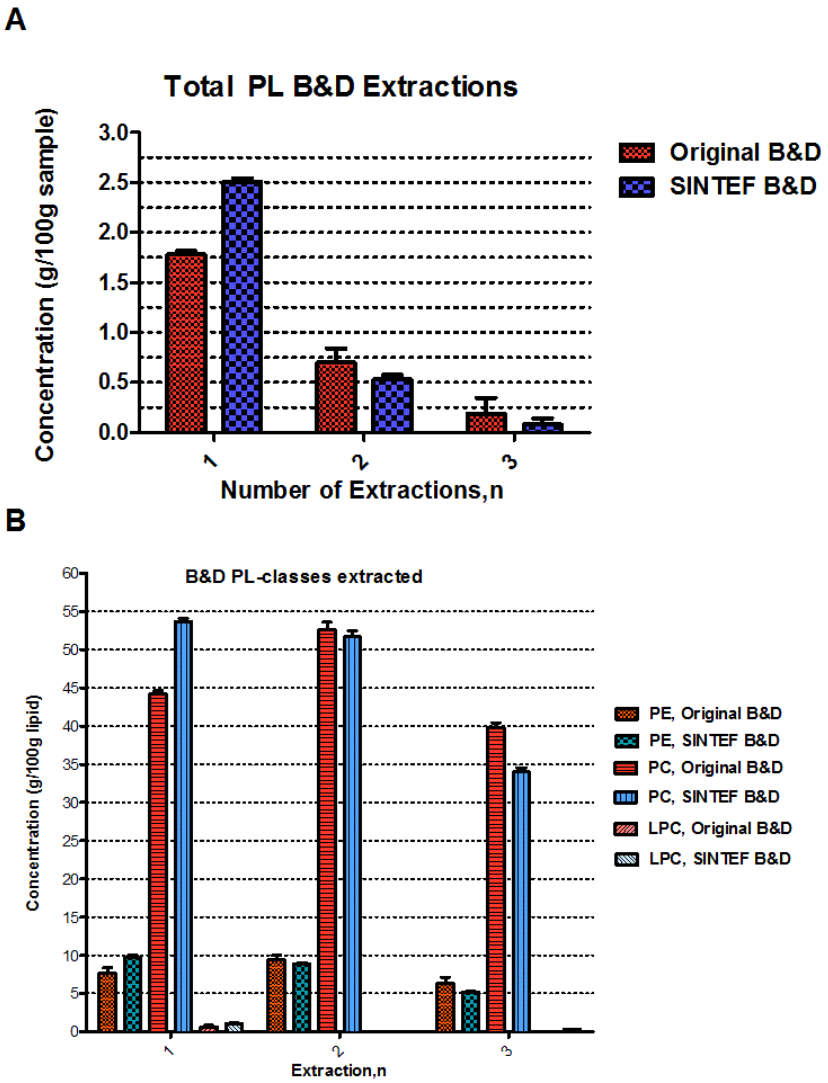


Figure 3.18: Phospholipid (PL) concentrations from three extractions by B&D. The values are given in mean (±SEM, n=6) A. Total concentration (g/100g sample) of Phospholipids extracted by B&D SINTEF and Original method. B. PL-class composition of the extracts by SINTEF and Original B&D methods. The raw data is presented in Appendix G.

First Extraction

The difference between total phospholipids concentrations of the first extractions was considered significantly higher for the SINTEF method ($P<0.0001$, $t=5.571$). At the

beginning of this study it was decided to employ the Original B&D method, for further analysis. The SINTEF B&D method was dismissed. Theory[8] states that biphasic systems of similar solvent proportions like SINTEF B&D compose a polluted chloroform phase. The SINTEF B&D chloroform phase probably contains 10-20% of methanol/ water. Experimental data showed that the SINTEF B&D yielding higher PL concentrations from its first extraction. Mixed solvent conditions of the chloroform phase may influence the PL yield positively. Higher polarity properties of the mixed solvents within the chloroform phase may contribute to dissolve more PL. Mixed solvent systems of different polarity express the amphiphilic properties of PLs. This makes a favorable environment for dissolving variable PL-classes. PC is the dominant PL class of all biological membranes. It has cone constructions with a big, dominant polar head, being one of the most polar PL-classes[37], see Figure 1.7. Therefore it is expected that a higher PL yield have more PC within its extracts. From another point of view, more proteins are likely to dissolve in the polluted chloroform phase. This is due to higher polarity of the solvent phase. Some of the PL is bound or makes strong connections to Proteins in the polluted chloroform phase, making them less susceptible[10].

Second, Third and Total Extraction

The relationship between the second and third concentration of extractions by the compared methods was considered not significant ($P>0.05$,second; $t=1.297$, third; $t=0.8394$). Overall the methods were considered not significantly different on a 99% CI (ANOVA, $P=0.0566$). Thus, the methods extracted similar total lipids concentrations from three extractions it was found that the SINTEF B&D approach received higher concentrations of phospholipids from the first extraction. Distribution of phospholipids classes within the extracted lipids is showed in Figure 3.19, B. Note that within the first extraction the amount of PC with the SINTEF B&D approach is greater than with the Original B&D approach. No difference in the other phospholipid classes of each number of extractions was evident between methods.

Decisions made from a theoretical aspect of the B&D research concluded that the original B&D was the most reliable method. The purity of the chloroform phase of original B&D was not analyzed. It is assumed that 100% chloroform and lipids in this phase. All B&D methods employed in the rest of this chapter represent the original B&D approach.

3.5.3 The Effect of Extraction Methods on the PL Yield

PL concentrations of marine samples representing different quality were performed to evaluate the ASE chloroform and ASE ethanol methods in comparison to B&D. The aim was to determine the PL concentrations within the extracts and to find which method that gave the highest PL concentrations. Most PL are soluble in chloroform and ethanol[37].Therefore, these solvents were employed in two different ASE extraction methods to discover which yielded the most PL.

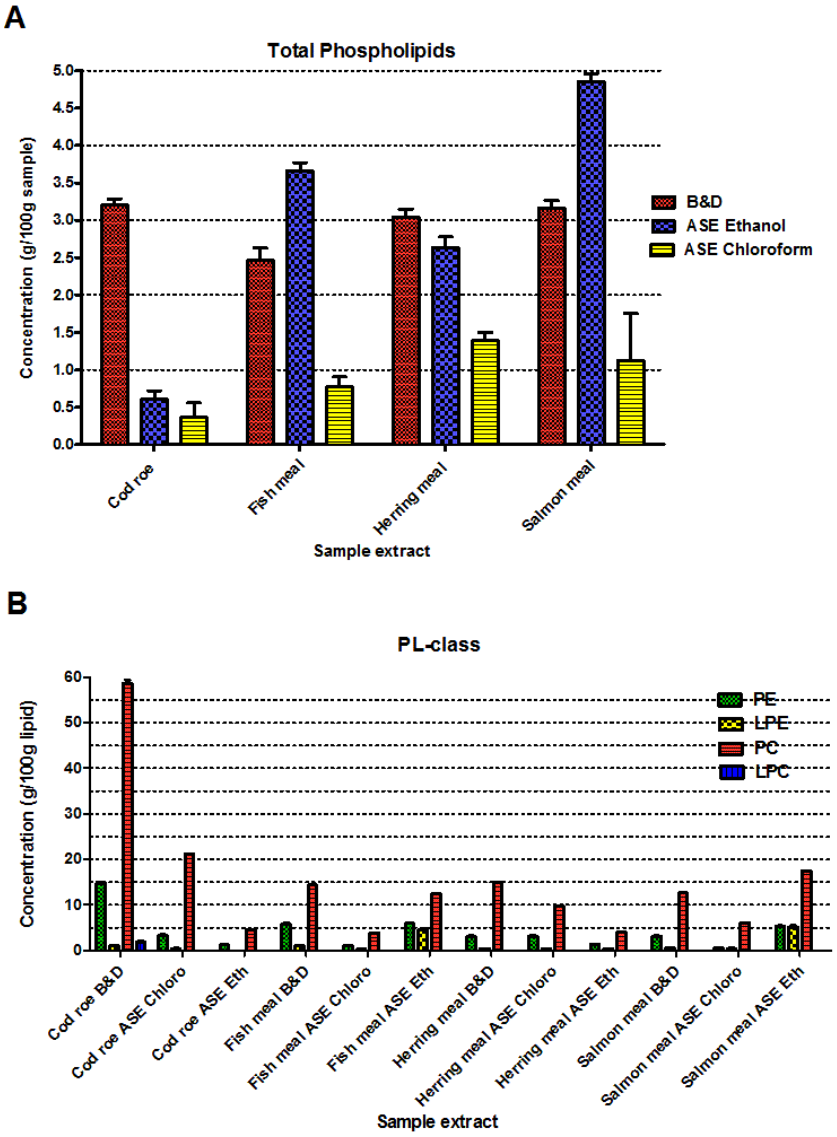


Figure 3.19: PL concentrations of different samples extracted by different methods: ASE ethanol, ASE chloroform and B&D. The bar values are given in mean (± SEM, n=6). A. Total PL concentration by the different methods. B. PL-class distribution of the lipid extracts by the different methods. The raw data is shown in Appendix G.

Comparison of phospholipids concentrations of the samples by one extraction is viewed in Figure 3.19, A.

ASE Chloroform

Most samples obtained significantly different PL yield for the methods employed ($P<0.0001$). The ASE chloroform approach yielded significantly lower PL concentrations in comparison to all B&D extracts (cod roe; $P<0.0001$; $t=9.1$, herring meal; $P<0.0001$ $t=5.3$, fish meal; $P<0.0001$, $t=5.4$, salmon meal; $P<0.0001$, $t=6.5$). In comparison to ASE ethanol there were no significant difference between cod roe extracts ($P>0.05$, $t=0.8$). The chloroform method is not interchangeable with the ethanol and B&D method for optimal PL concentration yield. As discussed earlier, chloroform favors non-polar lipids due to its non-polar solvent properties. ASE extractions (100°C) on a variety of fish tissue found that the extracts using chloroform recovered less of the more polar, unsaturated fatty acids[22]. It is therefore expected that the polar PL yield low concentrations in chloroform.

ASE Ethanol

The extracts of salmon meal ($P<0.0001$, $t=11.93$) and herring meal ($P<0.01$, $t=0.01$) were significantly higher for ASE ethanol in comparison to ASE chloroform. There are no significant differences between the PL concentrations of herring meal ($P>0.05$,$t=1.3$) by B&D and by ASE ethanol methods. By contrast, the PL concentrations of the meal from extracts by ASE ethanol, except for herring meal, were significantly higher than by the B&D method (fish meal; $P<0.01$,$t=1.4$ and salmon meal; $P<0.0001$,$t=5.6$). Ethanol is a polar organic solvent and dissolves polar components[87]. The meal samples are processed in high temperatures and conditions. It is assumed that most cell structures are broken and that PL components are more accessible to react with solvents. In addition, increased diffusion rate within ASE may result in more extracted PLs. Figure X, B shows that all the major PL classes are detected in all the meal samples. The most polar PLs, PC and LPC, yield higher concentrations for ASE ethanol extractions than for the B&D extraction. The lower yield from B&D extracts may be due to lower solubility for the more hydrophilic PL that was detected in this experiment. Also, B&D may experience significant losses of acidic PL[51]. However, the acidic PLs are not detected in this analysis. Therefore they do not affect the calculated total PL concentration. Anyway, they may contribute to increase lipid yield in the total lipid extraction of ASE ethanol.

B&D

The cod roe extracts of B&D provided the highest PL concentrations, significantly greater than ASE ethanol ($P<0.0001$, $t=8.6$). In contrast to the other sample extracts, elevated numbers of PL extraction is obtained by B&D. This may be due to the vigorous mixing of samples and solvents by the B&D procedure. Interactions between the solvents and sample are weaker than the strong bonding within the membrane structure. Therefore low concentrations are dissolved in the solvent, see next section for more detailed explanations. The relationship between methods varied among samples and indicated a sample-method interaction. From the lipid class analysis of the samples it was shown that the roe samples and the krill samples contained high amounts of PL. The fish meals contain higher amounts of non-polar lipids, see Section X. This is an effort to explain

why the roe sample, although having a low total lipid yield, gave high PL concentrations. According to theory, this is expected[7].

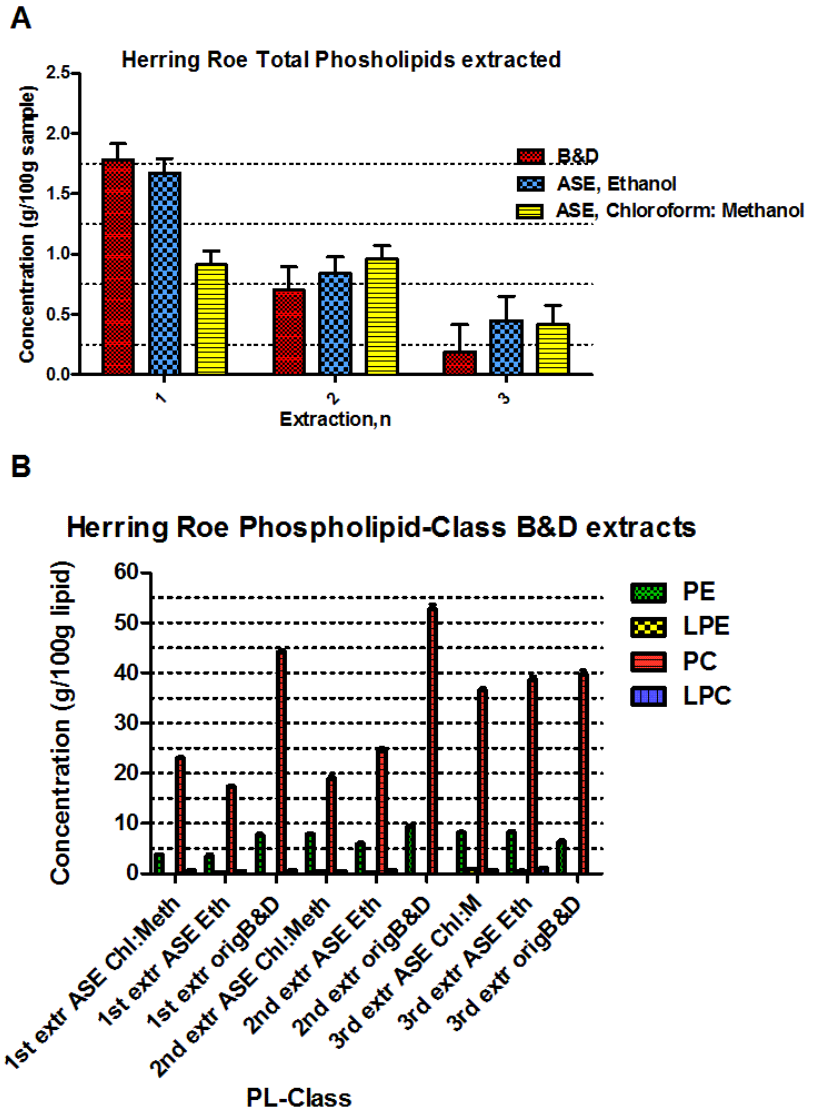


Figure 3.20: PL concentration in the extractions obtained by B&D, ASE ethanol and ASE chloroform: methanol methods. The bar values are given in means (±SEM, n=6). A. The total PL concentration by the different methods. B. PL-class distribution of the extracts obtained by different methods. The raw data is presented in Appendix G.

From the data and statistical analysis obtained from these extractions it was decided

not to use the ASE chloroform method in further research.

3.5.4 The Effect of Three Extractions on the PL Yield

The PL concentrations from three extractions of two different krill meals and herring roe by ASE ethanol, ASE chloroform: methanol and B&D was compared. The samples were chosen due to their high concentrations of PLs. The aim was to evaluate the method yielding the highest PL concentration after three extractions. In addition, the aim was to determine if three extractions of the chosen samples gave sufficiently higher yields of PLs. Data obtained from earlier experiments were used to design of a new experiment in order to test a hypothesis, the ASE chloroform: methanol method.

3.5.4.1 Herring Roe

The Hypothesis
It was hypothesized that an ASE method with similar solvent proportions as B&D would give similar phospholipid concentrations as the B&D method. This was tested on krill meal and herring roe. The main intention was to break the membrane structure to free the PLs from excessive interactions that hindered extraction. Unfortunately, chemical disruption of the membrane structure before analysis was unattainable. ASE requires dry samples for proper and complete extraction of lipids, see Section 2.5. However, methanol: chloroform in proportions 1:2 is able to break the membrane structure[93]. The mix composes both polar and non-polar properties. This is a desirable environment for amphiphilc characters such as PL to dissolve[87]. Based on this, Methanol: chloroform was employed as solvent system in the new method. Prior to the extraction the samples were crushed. It was an attempt to replace the mechanical force of ultraturrax to break the membrane structure. The samples were frozen to achieve small crystal structures in powder form after being crushed.

Comparison between three different extraction methods on herring roe with three numbers of extractions is viewed in Figure 3.20, A.

ASE Ethanol
ASE Chloroform: methanol represents the hypothesis testing. The first extraction of PL concentrations was considered significantly higher for ASE ethanol ($P<0.05$,$t=3.4$) and B&D ($P<0.01$,$t=3.9$) in comparison to ASE chloroform: methanol. As mentioned earlier, this may be due to the polar properties of ethanol. More PL are dissolved in this solvent. See next section

B&D
The difference between ASE chloroform: methanol and B&D PL concentration of the first extraction was considered not significant. There were no differences between the second and third extraction yields of the three methods ($P>0.05$, third; $t=1.2$, second;

t=0.1). However, after three extractions there are no significant differences between any of the methods (P>0.05,t=0.2).

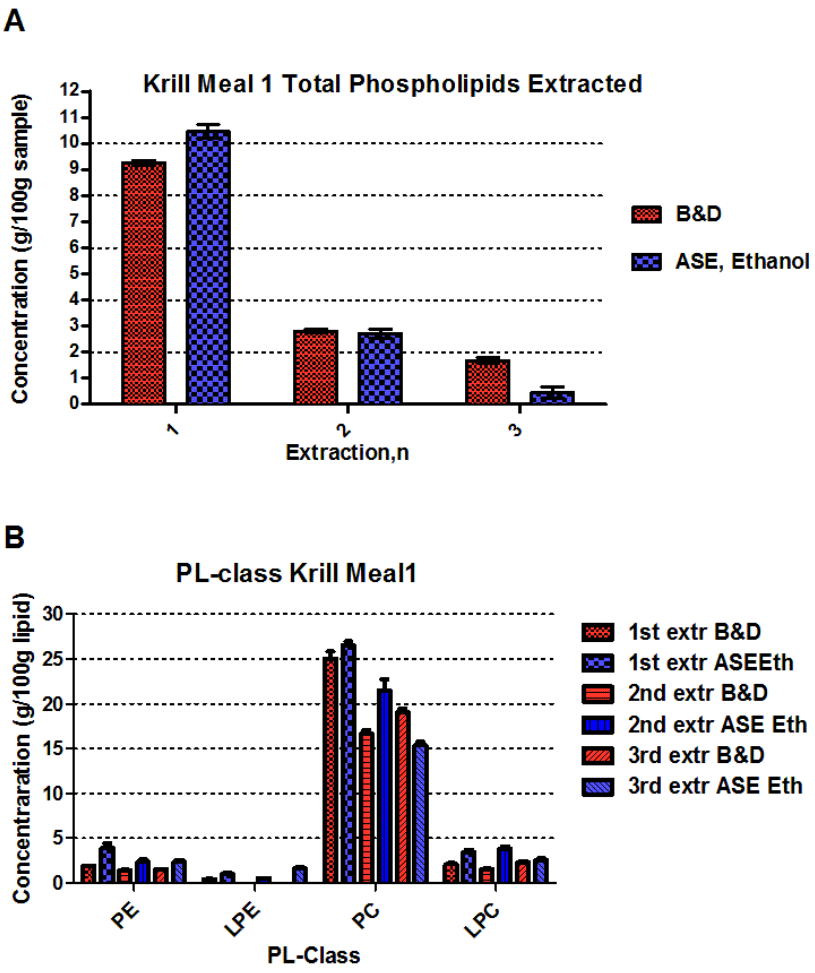


Figure 3.21: PL concentrations from three extractions of Krill meal 1 by ASE ethanol and B&D. The bar values are given in mean (± SEM, n=6) A: The total PL concentration by the different methods. B. PL-class composition of extracts by the different methods. Raw data is seen in Appendix G.

ASE Methanol: Chloroform

From the first extraction, the ASE chloroform: methanol method yields the lowest PL concentration. This indicates that the ASE chloroform: methanol method gave underestimated total PL. Surprisingly, after three extractions the ASE chloroform: methanol

method yields similar PL concentrations as B&D. In general, three number of extractions of herring roe by ASE ethanol, ASE chloroform: methanol and B&D methods estimates the total PL concentration equally well, as no overall statistical differences between the methods were found, see Section 3.2 for explanation of the ASE chloroform: methanol extraction.

3.5.4.2 Krill Meal 1

Figure 3.21, A shows the total Phospholipids concentrations of Krill meal 1 extracts by B&D and ASE ethanol.

ASE Ethanol
The difference of the mean for all extracted PL concentrations by each method is considered not significant (P=0.797). Anyhow, note that the PL concentration of ASE ethanol is significantly higher in the first extraction (t-test, P<0.0001,t=4.904). Several patterns of PL-class compositions between the two methods and their number of extractions are depicted in Figure 3.21, B. It is shown that the concentration of LPC, PE and LPE is higher for the ASE ethanol method. This can make it a more suitable method for all PL-class extractions detected in this thesis. The total amount of PC extracted is similar in both methods when three numbers of extractions are conducted. According to theory, PE and PC are supposed to be almost completely extracted by B&D. The yield of the more polar PLs are incomplete[51]. Also, ASE ethanol dissolves more polar components. The observation that the PE gave higher yield in ASE ethanol than B&D needs to be researched in more detail. Anyway, this may be ignorant due to the small amounts of PE present in the sample.

B&D
Reversely, ASE ethanol is significantly lower by the third extraction (t-test, P<0.0001, t=4.958) in comparison to the PL concentrations of B&D. The total lipid concentration extracted by B&D was higher than the ASE ethanol, see Section 3.2.3.2. Despite this observation, the extracted amount of PL is similar to the B&D method. This reveals that there are fewer extracted non-polar lipids within the ASE ethanol extract compared to the B&D extract. A new research on extraction of Antarctic krill, E. Superba, found that ethanol: acetone 1:1 in a one step extraction gave high extraction efficiency of PL, polar non-phospholipids and TG. In addition, in the right proportions (1:30 krill: solvent) it extracted higher antioxidant amounts than Folch[33]. Based on these experiments, it is suggested that ethanol and ethanol: acetone 1:1 may be good solvents for lipid extraction of krill and krill meal.

Earlier research of Antarctic krill (*E. Superba*), collected in December and extracted by Folch method, showed that the main PL classes were: PC; 33-36%, PE; 5-6% of total lipids[28]. According to NOFIMA krill oil contains 34-35% g/100g oil of PC[106]. The Krill meal used in this thesis is not completely comparable to whole krill (see Section 3.2.3.2). Anyway, it pinpoints a distribution of composition between the dominant PL-classes in Krill raw material of similar species. The ASE ethanol yield same amount of PC

from first extraction. Unexpected high concentrations of LPC and LPE are detected. The LPC and FFA are hydrolytic degradation products of its corresponding PL[61]. From the TLC-FID analysis the Krill meal, as well as many other samples, composed too high FFA values. As stated earlier, increased values of FFA, LPC and LPE indicate that enzymatic degradation of krill PL has occurred.

3.5.4.3 Krill Meal 2

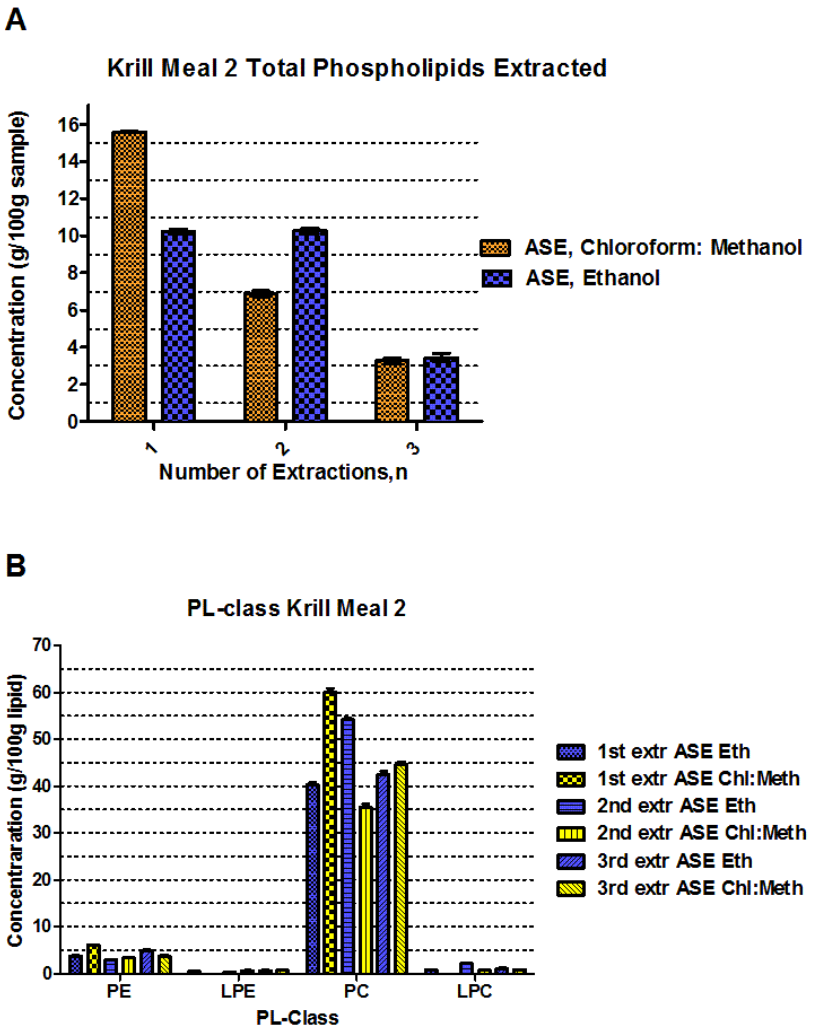


Figure 3.22: PL concentrations from three extractions of Krill meal 2 from by ASE ethanol and ASE chloroform: methanol. The bar values are given in mean (±SEM, n=6) A: Total PL concentration of the different methods. B. PL-class composition of extracts by different methods. The raw data for is listed in Appendix G.

A new krill meal, krill meal 2, was purchased. It contained high amounts of PL, and was therefore employed for testing of the hypothesis method. Since high PL concentration results were obtained from Krill meal 1, ASE ethanol was employed in further work. The next experiment considered pertains to a comparison between two extraction methods, ASE ethanol and ASE chloroform: methanol. PL concentrations of Krill meal

2 extracts were analyzed.

The data is plotted in Figure 3.22.

ASE Chloroform:Methanol

For the first extraction, ASE chloroform: methanol is significantly greater than ASE ethanol ($P<0.0001$, $t=25.4$). Also, after three extractions the total PL concentration of ASE chloroform: methanol is 25.7± 0.2 (mean± SEM) and 23.9± 0.3 (mean± SEM). A significant greater PL concentration of ASE chloroform: methanol than of ASE ethanol ($P<0.0001$) was obtained. Figure 3.22, B illustrates the PL-class composition within the total PL concentrations. The ASE chloroform: methanol tends to give higher values of PE and PC, while the ASE ethanol yield higher LPC values. Research found that ASE (100°C) with chloroform: methanol of all FA from various fish tissues recovered higher FA amounts than when chloroform was applied[22].

ASE Ethanol

However, the ASE ethanol method is significantly greater than the ASE chloroform: methanol for the second extraction ($P<0.0001$, $t=16.0$). Furthermore, the two methods show no difference in the third extraction ($P>0.05$, $t=0.8$).

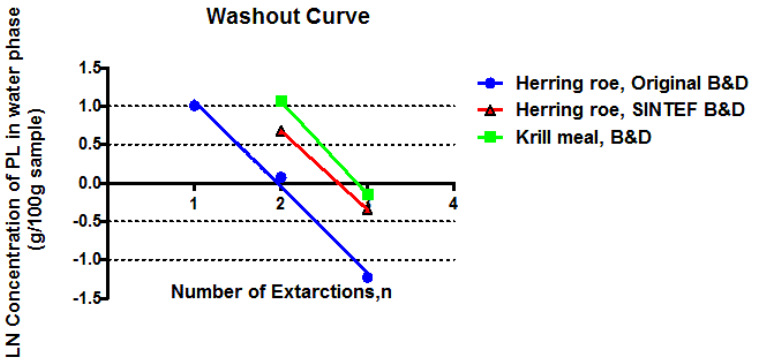
General Comments

These results suggest that the ASE chloroform: methanol method was found to be a very efficient. It may be an even better way to detect the phospholipids concentrations within a sample of krill meal. For three extractions performed, the method is considered appropriate for herring roe PL recovery. A challenge to the ASE chloroform: methanol method is that it can extract non-lipid components and may therefore receive polluted extracts[22]. Another concern is that this solvent system is health hazardous and poisonous. Therefore, it cannot be applied for extractions for food or medical applications. Anyway, ASE ethanol was found to yield great amounts of phospholipids. Hence, it can be a good alternative for phospholipids extractions.

It has been discovered that two successive extractions by PLE (140°C) chlorofom: methanol 2:1 achieved a total recovery of PC, PE and PI from soy, egg yolk and calf brain. In comparison, four extractions were necessary to recover all PL by Folch[110]. Herring roe yielded small amounts of PLs after one extraction. Krill meal 1 achieved high concentrations of PLs by 2 extractions of both B&D and ASE ethanol. After three extractions it seems like most of the PL are extracted. Krill meal 2 gave high PL concentration for all three extractions performed. The experimental results of this work suggest that B&D and ASE ethanol yield high concentrations after 3 extractions. More extractions are necessary to achieve total recovery of PL within these samples. The number of extractions necessary to extract most PLs within the herring roe and krill meal 1, is presented in the washout curve, see next section.

3.5.5 Washout Curve of Phospholipids Extractions

Experiments from the section above showed the extracted amount of phospholipids from three extractions of herring roe and krill meal. From calculations of the extracted B&D phospholipids concentration, a washout curve was constructed. The aim of these calculations was to find a mathematical formula for the approximate PL concentration within a sample. In addition, the number of extractions needed to perform a maximum yield can be calculated from the linear regression.



	Herring roe, Original B&D	Herring roe, SINTEF B&D	Krill meal, B&D
Best-fit values			
Slope	-1.119 ± 0.1071	-1.026 ±	-1.212 ±
Y-intercept when X=0.0	2.190 ± 0.2314	2.734 ±	3.491 ±
X-intercept when Y=0.0	1.958	2.665	2.880

	Herring roe, Original B&D	Herring roe, SINTEF B&D	Krill meal, B&D
R square	0.9909	1.000	1.000

Figure 3.23: Washout curve from the total PL extracts of krill meal 1 and herring roe by B&D and B&D SINTEF methods of HPLC analysis. See Appendix G and Appendix I.

The washout curve of the Phospholipids concentration in herring roe and krill meal 1 is illustrated in Figure 3.23. The logarithm of the phospholipids concentration in the water/methanol phase was plotted as a function of extraction numbers performed. Linearity was obtained for all sample curves. The first extraction points calculated from both Krill meal 1 and Herring roe was rejected. This is because the first points give values that are not representative, yielding extremely high extrapolation values and giving non-linearity of the regression line.

The washout curve is only valid for the B&D extraction method, due to specific chemical and physical properties the performance by this method. Therefore, the tree extractions performed by B&D method are the only samples that can be calculated correctly by washout curve principles.

In these curves, the slope is similar to the equilibrium constant value. The intercept

is similar to the total phospholipid concentration of the sample extract. To extract most of the phospholipids within the sample, 2-3 extractions are necessary for all samples.

Herring Roe
Extrapolations for the Herring roe phospholipids curve are 8.94 g/100g sample ($e^{2.190}$) for the original B&D and 15.39g/100g sample ($e^{2.734}$) for the SINTEF B&D. Compared to the washout curves obtained by Iatroscan measurements extrapolations of the herring roe by Original B&D were 4.48g/100g sample. The herring roe did not receive mass balance between any of the washout curve extrapolation values, see iatroscan for discussion.

Krill Meal 1
The krill meal 1 extrapolation curve reveals 32.79g/100g sample ($e^{3.491}$). These values indicate the total PL concentration of the samples. From the Iatroscan washout curve the extrapolation values of krill meal 1 was 32.85g/100g sample. These results state a mass balance are calculated for krill meal 1, indicating that this is probably the true value of total phospholipids within the sample.

3.5.6 Observations of Unknown Peaks in Some Chromatograms

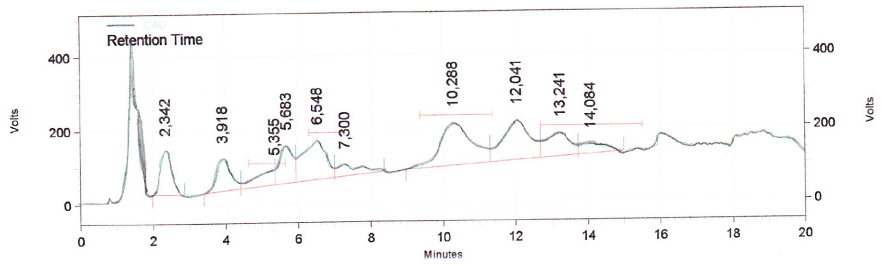


Figure 3.24: HPLC chromatogram of PL-class analysis by ASE ethanol Krill meal 1 extracts.

Recoveries of many PLs, especially PS and PI, are only about 50% from B&D first extractions. Many of the polar lipids remain in the water phase or in the tissue. Many techniques lack overall sensitivity and relatively rare PLs that are present in minor quantities are seldom detected[105]. As mentioned earlier, for the B&D extractions the PLs having more acidic properties (PI, PA and cardiolipin) are often either lost in the upper phase or extracted from the microsomal suspension, leading to significant losses of these PLs. The reason is that these PLs are insoluble in chloroform: methanol. However, the major PL classes, that is PE and PC is present in the extract fraction of B&D[51].

In this study the B&D method obtained high concentrations of phospholipids from increasing number of extractions. From Figure 3.17, a typical chromatogram of B&D analysis of Krill meal 1 is shown. For all three extractions, two to four peaks occurred, representing PE, LPE, PC or LPC. No other peaks were detected, indicating that the

PL-classes of small quantities within the sample were not extracted.

From Figure 3.24 a typical chromatogram showing the analysis of PL-classes in the third krill meal extraction by ASE ethanol. There are many peaks overlapping. The HPLC method is specifically developed for PL-class detection, and the mobile phase is supposed to dissolve most types, depending on its composition. Also, the PL classes are relatively similar in comparison to other molecules. These observations suggest that the other peaks detected represent unknown PL-classes within the extract. Increasing number of extractions gave increasing umber of peaks. Anyway, the use of ethanol as a solvent of PLs has been studied earlier. They found that PC dissolves excellent in ethanol, while PE is only partial dissolved. The more acidic PL, PI and PA was only partially insoluble[87]. It is speculated that this ASE ethanol method may extract different PL classes due to increased temperature and pressure.

Unfortunately, no other PL-class standards were available during this research. Each PL-class needs a single calibration curve and the elution time varies with the methods, the solvent proportion and the mix. The unknown peaks were therefore not identified. Similar observations of the Herring roe was found, but Krill meal received larger peaks and are therefore applied to illustrate the findings.

3.5.7 PH Test

environment. The main objective was to get higher PL yield from a single extraction. Considerations of manipulating the PL isoelectric point (pI) were accounted.

Extraction of lipids by a liquid-liquid (ex B&D) extraction method will separate the lipids from their originally bound proteins and carbohydrates. The acidic or basic proteins and carbohydrates are more polar and dissolve in water. The more non-polar lipids dissolve in an organic solvent. The organic solvent containing the lipids in the extract, whereas the polar reagents remain in the sample matrix and polar environment[102].

From a theoretical point of view the PL behaves as a non-polar lipid at its pI. PE pka(amino) is 9.3 and pka(phosphate) is 1.9, and for PC pka(tetra amino group) it is 11.0 and pka(phosphate) it is 1.7. The exact pI for PE is 1.9+9.3/2=5.6 and pI for PC is 11.0+1.7/2=6.35[37]. This means that the molecule is fully dissociated and has both a positive and negative charge. The sum of the charges in the molecule is overall zero. In the PE molecule, 99% is dissociated in the pH range 3.9-7.3, and in PC it is between pH 3.7-9. Within this range the physiochemical properties of PL are more likely not ionic[94]. The interactions between the proteins and PL are weaker in this pH area in general and it is easier to extract them.

Studies show that in pH 8, 36% PL was removed from soy proteins and less was removed when pH 2.5 was experimented. This is probably because the molecule has stronger interactions with the proteins when it has an overall negative charge[3]. Lab experiments revealed that the pH in krill meal 2 was 6.4 and in herring roe was 7.2. These pH values lie within the pI area and therefore it is not necessary to employ buffers.

Another approach to this challenge, which was not experimented in this thesis, is to perform an acid and base heat hydrolysis. The main idea is that treatment with acid or base heat hydrolysis the proteins and carbohydrates, releasing the bound lipids. Newer

research performed an acid and base heat hydrolysis on foods followed by extraction of lipids witch received excellent fat recoveries[102]. There is however a challenge to the hydrolysis procedure, because phospholipids are amphiphilc and no research has shown that the PL concentration increases within the organic solvent. It is crucial that the pH added to achieve acid or base hydrolysis is higher than the pI of the PL. A very low or high pH will change the overall charge of the PL. A charged PL may react with other charged molecules, example carbohydrates and proteins. In addition a charged PL is more polar and may not dissolve in an organic solvent. This is especially the case of LyzoPL and the most polar PL because they are structurally dominated by their polar heads[37].

3.6 Summary of Results and Discussion

This study composed five aims:

1. to evaluate the significance of the mono and biphase solvent proportions during lipid extraction by B&D.
2. to study the capacity of different solvent systems in ASE to extract marine lipids and PLs.
3. to investigate the effects of three extraction steps on the recover of total lipid and PL amount.
4. to evaluate the efficiency for complete lipid recovery of extraction steps by B&D using washout curves.
5. to study the possibilities to improve a method for analyzing phospholipids classes of marine lipids by HPLC-CAD.

An effort to relate the aims and the experimental data as well as mentioning some areas of applications is given in the text below.

1. Significance of the mono and biphase solvent proportions during lipid extraction by B&D.
Comparison of herring roe exactions by the two B&D approaches found that there were no differences in both PL and lipid concentration after three extractions. In the first extraction, the SINTEF approach yielded the highest concentration of both PL and lipids. Based on the B&D theory[8], it can be argued that the solvent proportions of SINTEF B&D contained solvent proportions with a contaminated chloroform phase. The contaminated chloroform phase is a more favorable environment to dissolve PL. This may explain why the SINTEF approach achieved increased PL values from first extraction. Since theory states that the solvent proportions of the SINTEF approach may give polluted extracts, the original B&D was considered to be the most suited approach in further research and as a reference.

It is important to obtain a reliable reference method to estimate the true lipid concentration. Also, it is desirable that the extraction method achieves complete separation of lipids and non-lipids, in case of further applications of the extract.

Table 3.6: Overview of the methods that gave the highest lipid and PL recovery for different samples.

Sample	Lipids	PL
Herring meal	B&D, ASE ethanol	ASE ethanol
Fish meal	ASE chloroform, B&D	ASE ethanol
Salmon meal	B&D	ASE ethanol
Cod roe	ASE ethanol	B&D

2. Capacity of different solvent systems in ASE to extract marine lipids and PLs.
In general, depending on the sample, ASE ethanol, ASE chloroform or B&D was the favored method to yield the highest lipid amount. ASE ethanol gave higher PL concentrations for all meals than B&D. ASE chloroform gave sufficiently lower PL concentrations than all B&D samples. Cod roe achieved the highest lipid concentration with ASE ethanol and the highest PL concentrations using B&D method. Table 3.6 shows an overview of this summary. According to theory as well as the Iatroscan analysis, the meals composed most TG (¿54g/100g lipid), while the dominant lipid class of roe was PL (45-68 g/100g lipid). Ethanol is a polar organic solvent and it favors to dissolve PL, especially PC[37]. The increased PL concentration of B&D is probably due to the vigorous mixing that may destroy many associations between cell constituents and therefore dissolves more PL[93]. Research on extraction of lipids from krill found that ethanol: acetone solvents gave higher lipid concentrations than Folch[33]. Also, industrial production of lecithin often uses ethanol as a solvent[37]. It is therefore suggested that the ASE ethanol method is an acceptable method to yield high PL concentrations.

Efficient and optimal methods for determination of total lipid concentration simplify industrial work. An automatic method is less expensive, less time consuming and reduces challenges related to health problems for lab workers. ASE can analyze smaller volumes of both samples and solvents. This enhances the ability for scientists to 1) conduct inexpensive, yet reliable, analyses 2) to estimate lipid content in organisms with low lipid levels or small sample size when the sample volume is scarce. However, the machine is expensive and requires special equipment to function properly.

3. Effects of three extraction steps on the recover of total lipid and PL amount.
Three step extraction of herring roe yielded maximum expected value of total lipids for ASE ethanol (c.11g/100g sample)[7]. The ASE chloroform: methanol yielded similar lipid concentration as B&D for herring roe after three extractions (c. 8g/100g sample).

Similar PL yield was achieved from all the studied methods, ASE ethanol, ASE methanol: chloroform and B&D, after three extractions.

By three extractions, krill meal 1 achieved higher lipid concentration from B&D than ASE ethanol. The krill meal 1 gave higher PL concentration from ASE ethanol in the first extraction while B&D gave the highest concentration in the third extraction. Overall there were no differences between these two methods. This may be due to the mixing of ultraturrax, and that more unpolar solvents are dissolved in the chloroform phase of B&D.

After three extractions, there were no significant differences between the extract yield of both PL and lipids of krill meal 2 by ASE methanol: chloroform and ASE ethanol. However, after three extraction steps the ASE chloroform: methanol gave greater PL yield. Unfortunately this method may extract non-lipid components. Thus, overestimations of total lipids may occur[22]. Therefore, it is difficult to conclude if the method is better. The comparison of Iatroscan and HPLC PL speculate that this can be an issue. Experiments performed on krill meal and herring roe suggest that three extractions are needed to extract most PL by ASE ethanol, ASE chloroform: methanol and B&D. Research on plant and animal samples found that two extractions by PLC (140°C) with chloroform: methanol 2:1 recovered all PL and lipids. Four extractions by Folch achieved similar results[110].

The difference in the PL-concentration within each extract should be of commercial interest because an extraction procedure that yields the highest concentration would be an indication of the sample or product having healthier benefits. More efficient methods to extract all lipids and PL will take full advantage of the raw material available. A negative aspect is that the solvents may be expensive, and with three extractions relatively large volumes are needed. There mignt be a storage problem, since the hazardous solvents represent environmental treats.

Table 3.7: Extrapolation values (g/100g sample) from the extractions performed in this work.

Sample	Extrapolation PL by Iatroscan	Extrapolation PL by HPLC	Extrapolation TG by Iatroscan	Extrapolation Total lipids By B&D
Herring roe, original B&D	4.5	8.9	3.5	6.0
Herring roe, SINTEF B&D	-	15.4	-	7.4
Krill meal 1	32.2	32.8	21.7	62.2

4. Efficiency of extraction steps by B&D washout curves for complete lipid recovery.
Table 3.7 shows the estimated values of total lipid, TG and PL. These values are obtained by washout curve linear regressions. The sum of TG and PL by Iatroscan gave 7.9g/100g sample. When the FFA and mono- and diglyserideds are added to the Iatroscan TG and PL, the sum is 61.9g/100g sample. From the PL extrapolation values of krill meal 1, PL is estimated to be c.32-33 g/100g sample. This gives mass balance between the calculated total lipids of krill meal 1. Indications that the extrapolation values are more likely to be true values within the sample are stronger. However, comparison of HPLC and Iatroscan analysis gave higher PL values for the meal extracts. Overestimation by Iatroscan is possible when high amounts of pigments are analyzed similar to the PL[7]. The original B&D method achieved expected values of total PL[7], indicating that the extrapolated value of SINTEF B&D is somewhat overestimated. On the other hand, this difference may be due to incomplete extraction of acidic PL by B&D[51].

Research stated early that B&D had a discriminatory effect toward acidic PLs. This may have been the reason for lower extraction yield[51].

The data obtained and the calculation of the washout curve imply that at least three extractions are necessary for complete phospholipids recovery of samples composing high amounts of phospholipids, independent of the total lipid amount of the sample. The discriminatory effect of biphasic solvent systems is decreasing with increasing number of extractions. The ASE methods also yield higher concentrations with increasing number of extractions. These methods have the advantage of being automatic and less labor intensive. Therefore the time consumed for one or three extractions are ignored, as compared to the B&D method, which is labor intensive. Increasing number of extractions by B&D can result in long and hard days of work with health hazardous solvents.

Washout curves is a simple approach to find the extractions needed to yield high PL and lipid yields and may be interesting for optimal exploitation of samples composing variable PL and lipids in small amounts. Also, it can be used to evaluate the efficiency of each extract. The number of efficient extraction steps extractions to perform for total lipid yield of different samples may also be found.

5. The possibilities for improving a method analyzing phospholipids classes of marine lipids by HPLC-CAD.
To improve the PL-class analysis of HPLC-CAD for marine lipids, three extractions were carried out. This was necessary to obtain acceptable and reproducible values of each standard. Indications that the standard concentrations were equally distributed in the solvents system were obtained from CN-analysis. See Section X.

Reliable analytical procedures for the characterization of PL-classes can be needed in both basic research as well as in industry[74]. Employment of this method may serve as quality insurance for commercial products, drugs and foods.

Chapter 4

Conclusion

By one extraction step, a modified method with higher water content within its solvent proportions achieved higher lipid and PL recovery for herring roe. Unfortunately, the theory states that the solvent proportions of the method applied may result in impurities of proteins within the extracts. The experiments indicate that the significance of solvent proportions had no impact on the lipid and PL recovery when three extractions were performed.

Observations of solvent systems capacity, using ethanol and chloroform, found that ASE ethanol gave higher PL recovery of meal samples than the B&D method. Furthermore, B&D achieved superior PL amounts of roe with one extraction step. As expected, the optimal solvent system for lipid recovery is dependent on the lipid composition of the sample. Samples high in TG favored non-polar solvent systems, like chloroform. In contrast, samples high in PL favored more polar solvents, like ethanol.

Experiments indicate that for samples high in PL and lipids, such as krill meal, yield sufficient amount of lipids and PL after three extractions. ASE ethanol, ASE chloroform: methanol and B&D are methods that can be used for several extractions with this objective. On the other hand, increasing extraction steps may be costly and more time consuming. The use of higher amounts of solvents may also be an issue.

Extrapolations of the washout curve of B&D extracts may give the total concentration of a component within a sample. Estimations of efficient lipid extraction steps can be obtained from this curve by linear regression. In this study, mass balance was obtained for krill meal 1, but not for herring roe.

A suitable PL-class HPLC- CAD has been improved by calibration.

Chapter 5

Further Work

- Study the most convenient temperature for extraction of PL with different solvents.
- Use other solvents with ASE to research the maximal PL and lipid yield, for example ethanol:acetone.
- Determine oxidation and fatty acid content of the extracts from the methods evaluated in this work.
- Determine extraction efficiency for other samples, for example dry krill.
- Develop fatty acid and lipid class analysis methods by HPLC-CAD.
- Calibrate other standards PL-classes to explore if the ASE ethanol extraction yields several PL that was not detected in this study.

Appendix A

Measurement of Water Content

Table 1 shows the raw data for determination of water content in the different samples applied in this work.

Table 1 Water content of the sample applied in this work

Sample	Paralell, n	Weight test tube (g)	Weight test tube + sample (g)	Weight sample (g)	Weight test tube + dried sample (g)	Weight dried sample (g)	Weight water content (g)	Water content (g/100g sample)	Average water content (g/100g sample)
Fish meal	1	4.9598	5.6598	0.7000	5.6047	0.6449	0.0551	7.8714	7.93
	2	5.0019	5.9053	0.9034	5.8338	0.8319	0.0715	7.9145	
	3	4.9136	5.8812	0.9676	5.8037	0.8901	0.0775	8.0095	
Salmon meal	1	5.0003	5.8697	0.8694	5.8130	0.8127	0.0567	6.5217	6.52
	2	4.9831	5.7252	0.7421	5.6770	0.6939	0.0482	6.4951	
	3	4.9606	5.7160	0.7554	5.6666	0.7060	0.0494	6.5396	
Herring meal	1	4.9192	5.8025	0.8833	5.7247	0.8055	0.0778	8.8079	8.85
	2	4.9435	5.7113	0.7678	5.6434	0.6999	0.0679	8.8434	
	3	4.9650	5.8166	0.8516	5.7409	0.7759	0.0757	8.8891	
Krill meal 1	1	4.9450	5.6598	0.7148	5.6092	0.6642	0.0506	7.0789	6.72
	2	4.9193	5.6615	0.7422	5.6110	0.6917	0.0505	6.8041	
	3	4.9519	5.7448	0.7929	5.6951	0.7432	0.0497	6.2681	
Krill meal 2	1	4.9176	5.7082	0.7906	5.6950	0.7774	0.0132	1.6696	1.54
	2	4.9638	5.9664	1.0026	5.9497	0.9859	0.0167	1.6657	
	3	5.2007	6.3132	1.1125	6.2990	1.0983	0.0142	1.2764	
Herring roe	1	4.9702	6.4328	1.4626	5.4006	0.4304	1.0322	70.5730	68.08
	2	4.9857	6.3773	1.3916	5.4430	0.4573	0.9343	67.1385	
	3	4.9671	6.3625	1.3954	5.4343	0.4672	0.9282	66.5186	
Cod roe	1	4.9809	6.1024	1.1215	5.1902	0.2093	0.9122	81.3375	77.63
	2	4.9604	5.9908	1.0304	5.1917	0.2313	0.7991	77.5524	
	3	4.9594	6.0664	1.1070	5.2471	0.2877	0.8193	74.0108	

Appendix B

Standard Deviation of Methods

Raw data used to calculate the standard deviations (STD) of different methods are given in this Appendix. Table 1 shows the STD for ASE, Table 2 for Iatroscan and table 3-6 show the STD and relative STD for HPLC.

Table 1. ASE METHOD STD

Sample Krill meal 2
Solvent Ethanol
Method ASE, std method

Sample nr	Weight sample (g)	Extracted lipids+solvent (ml)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)
1	20.01	114.01	17.9255	18.0066	0.0811	23.10
2	20.01	114.01	12.2459	12.3277	0.0818	23.30
3	20.06	105.70	12.3710	12.4525	0.0815	21.47
4	20.06	105.70	12.3710	12.4549	0.0839	22.10
5	20.01	108.85	12.4287	12.5115	0.0828	22.52
6	20.01	108.85	12.3780	12.4609	0.0829	22.55
7	20.06	106.43	12.2513	12.3321	0.0808	21.43
8	20.06	106.43	12.4088	12.4912	0.0824	21.86
9	20.02	105.72	12.1886	12.2782	0.0896	23.66
10	20.02	105.72	12.2929	12.3817	0.0888	23.46
11	20.02	114.32	12.2259	12.3108	0.0849	24.24
12	20.02	114.32	12.2079	12.2924	0.0845	24.13
13	20.02	116.02	12.2091	12.2845	0.0754	21.85
14	20.02	116.02	12.2896	12.3678	0.0782	22.66
15	20.02	114.12	12.4156	12.4908	0.0752	21.43
16	20.02	114.12	17.4734	17.5479	0.0745	21.23
17	20.05	114.35	12.4630	12.5508	0.0878	25.04
18	20.05	114.35	12.2851	12.3698	0.0847	24.15
19	20.03	104.99	12.2697	12.3554	0.0857	22.46
20	20.03	104.99	12.4599	12.5420	0.0821	21.52
21	20.01	114.37	17.9301	18.0123	0.0822	23.49
22	20.01	114.37	12.3655	12.4481	0.0826	23.61
23	20.06	107.74	12.3710	12.4545	0.0835	22.42
24	20.06	107.74	12.5679	12.6503	0.0824	22.13
25	20.02	110.19	12.3287	12.4106	0.0819	22.54
26	20.02	110.19	17.9235	18.0081	0.0846	23.28
					Average	22.76
					STD	1.03
					SEM	0.20

Table 2 Iatroscan STD

Sample	**Standards**												
Number	1	2	3	4	5	6	7	8	9	10	Average	STD	SEM
Lipid class (g/100g lipid)													
Cholesterolester	25.46	27.08	25.30	26.36	25.34	26.52	25.79	25.91	26.28	26.98	26.10	0.65	0.20
Triglycerid	17.35	16.42	17.81	17.39	16.90	16.27	17.03	17.54	16.09	16.97	16.98	0.57	0.18
Free fatty acids	10.97	12.44	12.09	10.27	12.39	11.96	12.41	12.52	10.69	10.99	11.67	0.85	0.27
Cholesterol	40.04	38.75	37.87	39.86	38.66	40.00	39.82	37.52	37.86	38.70	38.91	0.97	0.31
Polar lipids	5.48	5.36	7.31	5.22	5.81	4.82	5.01	6.12	7.48	6.34	5.90	0.92	0.29
Total	99.30	100.05	100.38	99.10	99.10	99.57	100.06	99.61	98.40	99.98	99.55		

Table 3 HPLC STD Krillmeal, 1st extr

Consentration,mg/ml	1
injected µl	15
Consentration µg /µl	15
Method	ASE, 100% ethanol,100°C

Phospholipidclass amount in sample,µg contnet in sample injected (µg/5µl)								
Phospholipid class	1	2	3	4	5	6	Average	STD
PE	0.83	0.95	1.05	0.70	0.68	0.74	0.82	0.15
LPE	0.18	0.22	0.15	0.22	0.17	0.19	0.19	0.03
PC	4.57	4.47	4.59	3.96	4.11	4.25	4.33	0.26
LPC	0.73	0.88	0.76	0.64	0.66	0.52	0.70	0.12

Table 4 HPLC STD Krillmeal, 1st extr

Consentration,mg/ml	1
injected µl	10
Consentration µg /10µl	10
Method	ASE, 100% ethanol,1

Phospholipidclass amount in sample,µg content in sample injected (µg/5µl)												
Phospholipid class	1	2	3	4	5	6	7	8	9	10	Average	STD
PE	0.22	0.23	0.40	0.41	0.40	0.39	0.51	0.50	0.48	0.54	0.41	0.11
LPE	0.05	0.05	0.06	0.09	0.10	0.08	0.12	0.16	0.16	0.16	0.10	0.04
PC	2.57	2.54	2.84	2.86	2.95	2.90	2.60	2.81	2.65	2.77	2.75	0.15
LPC	0.25	0.34	0.46	0.50	0.57	0.51	0.26	0.40	0.33	0.36	0.40	0.11
Total PL(sum)											3.66	

	PE	PC	LPC
STD for 3 samples:	0.10	0.16	0.11
STD for 6 samples:	0.09	0.17	0.12
STD for 10 samples:	0.11	0.15	0.11

Table 5 HPLC STD Krillmeal, 1st extr

Consentration,mg/ml 1

injected µl 7

Consentration µg /µl 7

Method ASE, 100% ethanol,1

Phospholipidclass amount in sample,µg content in sample injected (µg/5µl)								
Phospholipid class	1	2	3	4	5	6	Average	STD
PE	0.1100	0.1020	0.1030	0.0900	0.0920	0.0800	0.10	0.01
LPE	0.0540	0.0500	0.0630	0.0490	0.0530	0.0620	0.06	0.01
PC	2.1610	2.0650	2.1090	1.9990	1.9720	2.0440	2.06	0.07
LPC	0.3410	0.3670	0.3210	0.3680	0.3320	0.3380	0.34	0.02

Total PL(sum)

% of total lipid

Table 6 HPLC STD Krillmeal, 1st extr

Consentration,mg/ml 1

injected µl 5

Consentration µg /µl 5

Method ASE, 100% ethanol,1

Phospholipidclass amount in sample,µg content in sample injected (µg/5µl)								
Phospholipid class	1	2	3	4	5	6	Average	STD
PE	0.0540	0.0980	0.0600	0.0420	0.0570	0.0490	0.06	0.02
LPE	0.0490	0.0240	0.0540	0.0300	0.0330	0.0260	0.04	0.01
PC	1.2480	1.5680	1.4360	1.2110	1.4020	1.1820	1.34	0.15
LPC	0.0800	0.1380	0.1300	0.2160	0.2610	0.2540	0.18	0.07

	Standard deviation of average amount i			
Amount injected (µl)	PE	LPE	PC	LPC
5	0.0197	0.0125	0.1517	0.0743
7	0.0109	0.0060	0.0698	0.0191
10	0.1092	0.0446	0.1470	0.1087
15	0.1473	0.0281	0.2604	0.1209

Appendix C

Extraction by Bligh and Dyer

The raw data obtained from Bligh and Dyer extractions are presented in Table 1-7.

Table 1. Bligh and Dyer extractions of herring roe

Extraction nr (n)	Sample nr	Weight sample (g)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)	Average	STD	SEM
1	1	10.05	15.9455	15.9617	0.0162	3.22	3.39	0.28	0.11
	2	10.04	15.8414	15.8593	0.0179	3.57			
	3	10.11	15.6264	15.6439	0.0175	3.46			
	4	10.10	15.5603	15.5789	0.0186	3.68			
	5	10.01	15.7430	15.7576	0.0146	2.92			
	6	10.04	15.6786	15.6961	0.0175	3.49			
2	1	10.05	15.5799	15.5839	0.0040	0.80	1.13	0.38	0.15
	2	10.04	15.6210	15.6240	0.0030	0.60			
	3	10.11	15.7648	15.7731	0.0083	1.64			
	4	10.10	15.8809	15.8874	0.0065	1.29			
	5	10.01	15.8981	15.9039	0.0058	1.16			
	6	10.04	15.7009	15.7073	0.0064	1.27			
3	1	10.05	15.8415	15.8433	0.0018	0.36	0.42	0.16	0.06
	2	10.04	15.8208	15.8219	0.0011	0.22			
	3	10.11	15.9326	15.9348	0.0022	0.44			
	4	10.10	15.6441	15.6462	0.0021	0.42			
	5	10.01	15.8024	15.8059	0.0035	0.70			
	6	10.04	14.8961	14.8980	0.0019	0.38			
						Total lipids extracted	4.93	0.49	0.2008

Table 2. Modified SINTEF B&D extractions by herring roe

Extraction nr (n)	Sample nr	Weight sample (g)	Weight test tube (g)	Weight lipids+ test	Weight lipids (g)	Lipid concentration (g/100g sample)	Average	STD	SEM
1	1	5.02	12.2762	12.2863	0.0101	4.02	3.83	0.30	0.12
	2	5.05	12.2335	12.2436	0.0101	4.00			
	3	5.00	12.2237	12.2341	0.0104	4.16			
	4	5.05	12.2285	12.2382	0.0097	3.84			
	5	5.04	18.0361	18.0445	0.0084	3.33			
	6	5.04	12.3469	12.3561	0.0092	3.65			
2	1	5.02	12.1716	12.1737	0.0021	0.84	0.85	0.09	0.04
	2	5.05	17.8636	17.8657	0.0021	0.83			
	3	5.00	12.4477	12.4495	0.0018	0.72			
	4	5.05	12.1432	12.1457	0.0025	0.99			
	5	5.04	12.3497	12.3520	0.0023	0.91			
	6	5.04	12.1804	12.1824	0.0020	0.79			
3	1	5.02	10.6376	10.6381	0.0005	0.20	0.21	0.03	0.01
	2	5.05	10.1667	10.1673	0.0006	0.24			
	3	5.00	10.7018	10.7022	0.0004	0.16			
	4	5.05	10.8215	10.8221	0.0006	0.24			
	5	5.04	10.7961	10.7966	0.0005	0.20			
	6	5.04	10.4602	10.4608	0.0006	0.24			
						Total lipid extracted	4.89	0.32	0.13

Table 3. B&D extractions of fish meal

Sample nr	Weight sample (g)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)
1	5.03	12.1090	12.1367	0.0277	11.01
2	5.04	12.5318	12.5593	0.0275	10.91
3	5.02	12.2790	12.3064	0.0274	10.92
4	5.05	12.1788	12.2065	0.0277	10.97
5	5.04	12.3823	12.4096	0.0273	10.83
6	5.05	12.3784	12.4053	0.0269	10.65
				Average	10.88
				STD	0.13
				SEM	0.05

Table 4. B&D extractions of herring meal

Sample nr	Weight sample (g)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)
1	5.02	15.7643	15.8029	0.0386	15.38
2	5.00	14.8961	14.9335	0.0374	14.96
3	5.02	15.8024	15.8404	0.0380	15.14
4	5.02	15.6438	15.6827	0.0389	15.50
5	5.06	15.8207	15.8584	0.0377	14.90
6	5.02	15.6704	15.7100	0.0396	15.78
7	5.00	15.7432	15.7812	0.0380	15.20
8	5.00	15.5800	15.6177	0.0377	15.08
9	5.00	15.8414	15.8799	0.0385	15.40
10	5.01	15.7414	15.7794	0.0380	15.17
11	5.04	15.8805	15.9199	0.0394	15.63
12	5.01	15.8414	15.8789	0.0375	14.97
				Average	15.26
				STD	0.28
				SEM	0.00

Table 5. B&D extractions of Salmon meal

Sample nr	Weight sample (g)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)
1	5.04	15.9321	15.9766	0.0445	17.6587
2	5.03	15.7129	15.7570	0.0441	17.5000
3	5.02	14.9782	15.0232	0.0450	17.9283
				Average	17.6957
				STD	0.2165
				SEM	0.1250

Table 6. B&D extractions of Cod roe

Sample nr	Weight sample (g)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)
1	10.02	15.8976	15.9189	0.0213	4.2515
2	10.04	15.8481	15.8690	0.0209	4.1633
3	10.01	15.9511	15.9720	0.0209	4.1758
				Average	4.1969
				STD	0.0477
				SEM	0.0275

Table 7. B&D extractions of krill meal 1

Sample nr	Weight sample (g)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)	Average	STD	SEM
1	5.02	15.7888	15.8684	0.0796	31.71	31.40	0.36	0.15
2	5.03	15.5334	15.6133	0.0799	31.77			
3	5.02	15.8609	15.9403	0.0794	31.63			
4	5.04	15.7776	15.8559	0.0783	31.07			
5	5.01	15.6521	15.7296	0.0775	30.94			
6	5.05	15.9809	16.0598	0.0789	31.25			
1	5.02	12.9114	12.9451	0.0337	13.43	14.33	0.66	0.27
2	5.03	12.9202	12.9588	0.0386	15.35			
3	5.02	12.8411	12.8763	0.0352	14.02			
4	5.04	12.8786	12.9148	0.0362	14.37			
5	5.01	12.9300	12.9670	0.0370	14.76			
6	5.05	12.7998	12.8353	0.0355	14.06			
1	5.02	12.6937	12.7127	0.0190	7.57	7.13	0.73	0.30
2	5.03	12.8649	12.8840	0.0191	7.59			
3	5.02	12.8698	12.8847	0.0149	5.94			
4	5.04	12.8141	12.8320	0.0179	7.10			
5	5.01	12.7987	12.8154	0.0167	6.67			
6	5.05	12.8521	12.8721	0.0200	7.92			
					Total lipid extracted	52.86	1.05	0.4288

Appendix D

Extraction by ASE

Raw data from ASE extractions are viewed in Table 1-13.

Table 1. ASE chloroform extractions of fish meal

Sample nr	Weight sample	Extracted lipids+solvent	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)
1	20.01	124.41	15.6781	15.7249	0.0468
2	20.01	124.41	15.8715	15.9184	0.0469
3	20.01	122.25	15.7002	15.7482	0.0480
4	20.01	122.25	15.7967	15.8453	0.0486
5	20.01	120.85	15.8201	15.8724	0.0523
6	20.01	120.85	15.6631	15.7152	0.0521
					Average
					STD
					SEM

Table 2. ASE chloroform extractions of herring meal

Sample nr	Weight sample	Extracted lipids+solvent	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)
1	20.01	124.16	15.7560	15.7892	0.0332
2	20.01	124.16	15.7116	15.7448	0.0332
3	20.01	122.32	15.8937	15.9272	0.0335
4	20.01	122.32	15.7072	15.7405	0.0333
5	20.01	120.41	15.8983	15.9316	0.0333
6	20.01	120.41	15.5200	15.5536	0.0336
					Average
					STD
					SEM

Table 3. ASE chloroform extractions of salmon meal

Sample nr	Weight sample (g)	Extracted lipids+solvent (ml)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)
1	20.01	103.08	15.8800	15.9444	0.0644	16.59
2	20.01	103.08	15.7639	15.8289	0.0650	16.74
3	20.01	74.02	15.8297	15.9059	0.0762	14.09
4	20.01	74.02	15.5597	15.6365	0.0768	14.20
5	20.01	106.07	15.7411	15.8040	0.0629	16.67
6	20.01	106.07	15.6697	15.7330	0.0633	16.78
					Average	15.85
					STD	1.32
					SEM	0.54

Table 4. ASE chloroform extractions of cod roe

Sample nr	Weight sample	Extracted lipids+solvent	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)
1	20.01	63.20	12.8739	12.8827	0.0088	1.39
2	20.01	63.20	12.9160	12.9245	0.0085	1.34
3	20.01	82.21	12.7724	12.7800	0.0076	1.56
4	20.01	82.21	12.7624	12.7701	0.0077	1.58
5	20.00	76.07	12.8218	12.8293	0.0075	1.43
6	20.00	76.07	12.8868	12.8941	0.0073	1.39
					Average	1.45
					STD	0.10
					SEM	0.04

Table 5. ASE ethanol extractions of herring meal

Sample nr	Weight sample (g)	Extracted lipids+solvent (ml)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)
1	20.01	111.32	19.3259	19.3913	0.0654	18.19
2	20.01	111.32	12.0259	12.0805	0.0546	15.19
3	20.01	111.32	12.0437	12.1003	0.0566	15.74
					Average	16.37
					STD	1.60
					SEM	0.92

Table 6. ASE ethanol extractions of fish meal

Sample nr	Weight sample	Extracted lipids+solvent	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)
1	20.05	126.43	10.8627	10.9032	0.04050	12.77
2	20.05	126.43	10.9901	11.0304	0.04030	12.71
3	20.04	124.02	10.6616	10.7114	0.04980	15.41
4	20.04	124.02	10.9126	10.9621	0.04950	15.32
5	20.01	112.97	10.8434	10.8954	0.05200	14.68
6	20.01	112.97	10.2016	10.2539	0.05230	14.76
					Average	14.27
					STD	1.23
					SEM	0.50

Table 7. ASE ethanol extractions of salmon meal

Sample nr	Weight sample (g)	Extracted lipids+solvent (ml)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)
1	20.05	106.13	10.9923	11.0462	0.05390	14.27
2	20.05	106.13	10.9535	11.0072	0.05370	14.21
3	20.04	108.37	10.8828	10.9396	0.05680	15.36
4	20.04	108.37	12.2777	12.3341	0.05640	15.25
5	20.06	123.41	10.9145	10.9716	0.05710	17.56
6	20.06	123.41	12.0187	12.0771	0.05840	17.96
					Average	15.77
					STD	1.62
					SEM	0.66

Table 8. ASE ethanol extractions of cod roe

Sample nr	Weight sample (g)	Extracted lipids+solvent (ml)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)
1	20.06	126.53	10.8604	10.8888	0.02840	8.96
2	20.06	126.53	10.9716	11.0004	0.02880	9.08
3	20.07	127.19	10.7771	10.8086	0.03150	9.98
4	20.07	127.19	10.7978	10.8288	0.03100	9.82
5	20.05	119.20	10.8648	10.8996	0.03480	10.34
6	20.05	119.20	11.9523	11.9867	0.03440	10.23
					Average	9.74
					STD	0.59
					SEM	0.24

Table 9. ASE ethanol extractions of krill meal 1

N	Sample nr	Weight sample (g)	Extracted lipids+solvent (ml)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)	Average	STD	SEM
1	1	20.01	121.17	19.1697	19.2675	0.0978	29.61	30.01	1.19	0.69
	2	20.01	121.17	19.3699	19.4671	0.0972	29.43			
	3	20.01	121.17	19.1553	19.2601	0.1048	31.73			
	4	20.01	123.03	12.6204	12.7191	0.0987	30.34			
	5	20.01	123.03	12.7354	12.8352	0.0998	30.68			
	6	20.01	123.03	12.0274	12.1193	0.0919	28.24			
2	1	20.01	119.83	12.0693	12.1001	0.0308	9.22	9.61	0.77	0.45
	2	20.01	119.83	19.4772	19.5093	0.0321	9.61			
	3	20.01	119.83	19.5630	19.5922	0.0292	8.74			
	4	20.01	125.01	19.7343	19.7682	0.0339	10.59			
	5	20.01	125.01	19.6345	19.6680	0.0335	10.47			
	6	20.01	125.01	12.4327	12.4615	0.0288	9.00			
3	1	20.01	120.92	19.5009	19.5101	0.0092	2.78	2.11	0.35	0.20
	2	20.01	120.92	19.7324	19.7394	0.0070	2.12			
	3	20.01	120.92	19.8012	19.8079	0.0067	2.02			
	4	20.01	118.54	19.3640	19.3698	0.0058	1.72			
	5	20.01	118.54	12.8432	12.8501	0.0069	2.04			
	6	20.01	118.54	12.1157	12.1224	0.0067	1.98			

Table 10. ASE ethanol extractions of krill meal 2

N	Sample nr	Weight sample (g)	Extracted lipids+solvent (ml)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)	Average	STD
1	1	20.01	114.01	17.9255	18.0066	0.0811	23.10	22.55	0.91
	2	20.01	114.01	12.4523	12.5311	0.0788	22.45		
	3	20.06	105.70	12.3710	12.4525	0.0815	21.47		
	4	20.06	105.70	12.3623	12.4449	0.0826	21.76		
	5	20.01	108.85	12.4287	12.5115	0.0828	22.52		
	6	20.01	108.85	12.3245	12.4127	0.0882	23.99		
2	1	20.01	114.11	10.7027	10.7670	0.0643	18.33	17.20	0.75
	2	20.01	114.11	10.8634	10.9212	0.0578	16.48		
	3	20.06	105.23	10.6402	10.7037	0.0635	16.66		
	4	20.06	105.23	10.7230	10.7897	0.0667	17.49		
	5	20.01	108.96	11.0648	11.1297	0.0649	17.67		
	6	20.01	108.96	10.8423	10.9032	0.0609	16.58		
3	1	20.01	114.05	10.7203	10.7506	0.0303	8.63	7.00	0.98
	2	20.01	114.05	11.0311	11.0573	0.0262	7.47		
	3	20.06	105.92	10.8653	10.8899	0.0246	6.49		
	4	20.06	105.92	10.6732	10.6958	0.0226	5.97		
	5	20.01	108.76	10.9935	11.0165	0.0230	6.25		
	6	20.01	108.76	10.8743	10.9007	0.0264	7.17		

Table 11. ASE ethanol extractions of herring roe

N	Sample nr	Weight sample (g)	Extracted lipids+solvent (ml)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)	Average	STD	SEM
1	1	20.03	101.14	12.4287	12.4580	0.0293	7.40	7.69	0.37	0.22
	2	20.03	101.14	12.3689	12.4001	0.0312	7.88			
	3	20.01	98.39	12.2256	12.2546	0.029	7.13			
	4	20.01	98.39	12.3663	12.3976	0.0313	7.70			
	5	20.01	108.25	12.2893	12.3194	0.0301	8.14			
	6	20.01	108.25	17.4727	17.5020	0.0293	7.93			
2	1	20.03	114.16	12.2733	12.2831	0.0098	2.79	2.69	0.09	0.05
	2	20.03	114.16	12.2691	12.2790	0.0099	2.82			
	3	20.01	105.44	12.2848	12.2946	0.0098	2.58			
	4	20.01	105.44	12.2850	12.2951	0.0101	2.66			
	5	20.01	108.32	12.2841	12.2939	0.0098	2.65			
	6	20.01	108.32	12.3777	12.3875	0.0098	2.65			
3	1	20.03	114.09	12.2510	12.2550	0.0040	1.14	0.92	0.20	0.11
	2	20.03	114.09	12.4634	12.4662	0.0028	0.80			
	3	20.01	105.26	12.4083	12.4126	0.0043	1.13			
	4	20.01	105.26	17.7473	17.7507	0.0034	0.89			
	5	20.01	108.77	12.2086	12.2109	0.0023	0.63			
	6	20.01	108.77	12.2747	12.2781	0.0034	0.92			

Table 12. ASE methanol: chloroform extractions of herring roe

N	Sample nr	Weight sample (g)	Extracted lipids+solvent (ml)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)	Average	STD	SEM
1	1	20.03	78.13	12.2323	12.2504	0.0181	3.53	3.32	0.43	0.25
	2	20.03	78.13	17.7671	17.7853	0.0182	3.55			
	3	20.08	66.38	12.1713	12.1878	0.0165	2.73			
	4	20.08	66.38	17.9426	17.9595	0.0169	2.79			
	5	20.04	79.86	12.2749	12.2931	0.0182	3.63			
	6	20.04	79.86	12.3432	12.3616	0.0184	3.67			
2	1	20.03	90.02	12.3078	12.3212	0.0134	3.01	3.46	0.37	0.21
	2	20.03	90.02	12.1258	12.1393	0.0135	3.03			
	3	20.08	87.25	17.4938	17.5115	0.0177	3.85			
	4	20.08	87.25	12.2632	12.2809	0.0177	3.85			
	5	20.04	91.12	12.2363	12.2518	0.0155	3.52			
	6	20.04	91.12	12.2969	12.3122	0.0153	3.48			
3	1	20.03	89.57	11.8757	11.8787	0.0030	0.67	0.91	0.16	0.09
	2	20.03	89.57	12.2632	12.2666	0.0034	0.76			
	3	20.08	85.97	12.1326	12.1376	0.0050	1.07			
	4	20.08	85.97	9.9836	9.9885	0.0049	1.05			
	5	20.04	90.14	12.3165	12.3208	0.0043	0.97			
	6	20.04	90.14	12.2658	12.2700	0.0042	0.94			

Table 13. ASE methanol: chloroform extractions of krill meal 2

N	Sample nr	Weight sample (g)	Extracted lipids+solvent (ml)	Weight test tube (g)	Weight lipids+ test tube (g)	Weight lipids (g)	Lipid concentration (g/100g sample)	Average	STD	SEM
1	1	20.04	87.30	12.2282	12.3391	0.1109	24.16	23.55	0.32	0.19
	2	20.04	87.30	12.4225	12.5291	0.1066	23.22			
	3	20.05	84.94	17.5219	17.6321	0.1102	23.34			
	4	20.05	84.94	12.1829	12.2940	0.1111	23.53			
	5	20.06	83.88	12.1137	12.2263	0.1126	23.54			
	6	20.06	83.88	12.2576	12.3699	0.1123	23.48			
2	1	20.04	89.51	12.3199	12.3961	0.0762	17.02	17.11	0.36	0.21
	2	20.04	89.51	12.4493	12.5241	0.0748	16.70			
	3	20.05	91.12	12.2267	12.3036	0.0769	17.47			
	4	20.05	91.12	12.3626	12.4402	0.0776	17.63			
	5	20.06	90.49	17.5427	17.6175	0.0748	16.87			
	6	20.06	90.49	12.2596	12.3347	0.0751	16.94			
3	1	20.04	86.35	12.2292	12.2573	0.0281	6.05	6.55	0.44	0.25
	2	20.04	86.35	12.2788	12.3075	0.0287	6.18			
	3	20.05	87.51	12.1666	12.1964	0.0298	6.50			
	4	20.05	87.51	17.8130	17.8425	0.0295	6.44			
	5	20.06	89.38	12.2370	12.2685	0.0315	7.02			
	6	20.06	89.38	12.4273	12.4593	0.0320	7.13			

Appendix E

Calibration

Detailed informatin about retention time changes is viewed in Table 1-2.
The standard concenatrations and peak areas from calibration 1-3 is viewed in Table 3-5

Table 1. Retention time changes, day 1

time	PE	LPE	PC	LPC	Comment
9.48	5.401	6.531	10.839	14.856	Mix 1:1:1:1
10.10	5.426	6.571	10.971	14.947	
10.32	5.465	6.635	11.086	15.207	
10.55		6.630	11.156	15.167	PE peak not in frame, injected 20 µl
11.18	5.503	6.705	11.289	15.289	
11.42	5.530	6.750	11.371	15.501	
12.04					Empty run before Mix 2:1:2:1
12.26				15.561	1µl injected," vide and short peaks"
12.49	5.561	6.818	11.474	15.614	
13.12		6.838	11.536	15.664	PE peak not in frame, injected 20 µl

Table 2.Retention time changes in day 2

time	PE	LPE	PC	LPC	Comment
11.04	5.532	6.440	10.643	14.537	Frozen from -80 deg,mix 1:1:1:1
11.27	5.358	6.471	10.801	14.679	Mix 1:1:1:1 heated in water-bath
11.52	5.420	6.560	10.919	14.866	
12.14	5.441	6.593	10.994	14.942	
12.46	5.483	6.676	11.088	15.114	
13.08	5.481	6.678	11.234	15.246	1µl injected," vide and short peaks"
13.30		6.636	11.234	15.196	PE peak not in frame, injected 20 µl
13.53	5.488	6.690	11.239	15.337	

Table 3. Calibration 1

Sample/ St	Cons, mg/ml	Injected, µl	Amount injected,µg	Peak area	Peak area (/10^6)
PE	0.250	10	1.25	4936678	4.936678
PE	0.250	5	0.63	2677986	2.677986
PE	0.500	10	2.50	13304967	13.304967
PE	0.437	5	2.18	7491663	7.491663
PE	0.437	10	4.37	13491360	13.49136
PC	0.250	5	0.63	2363738	2.363738
PC	0.250	10	1.25	4322428	4.322428
PC	0.413	5	2.07	6521078	6.521078
PC	0.500	10	2.50	13446153	13.446153
PC	0.413	10	4.13	13008471	13.008471
LPE	0.500	5	2.50	453255	0.453255
LPE	1.000	5	5.00	1249234	1.249234
LPE	0.500	10	5.00	1818283	1.818283
LPC	0.500	1	0.50	476904	0.476904
LPC	0.500	5	2.50	5948105	5.948105
LPC	0.500	10	5.00	11162414	11.162414
LPC	1.000	10	10.00	36369065	36.369065

Table 4. Calibration 2

µg	Peak area (* 10^6)				
	PE	PC	LPE	LPC	
0.019		0.373524			Mix 2:2:1:1
0.026		0.375346			Mix 1:1:1:1
0.058		1.005065			Single
0.097		1.782404			
0.135		2.878573			
0.162				0.807677	
0.172	1.252962				
0.193		4.115099			
0.229	1.631198				
0.237			1.714425		
0.244				1.146237	
0.257		5.209894			
0.385		6.4592720			
0.386		7.954988			
0.513		10.103306			
0.515	4.108613				
0.710			6.669414		
0.729				3.877296	
0.770		12.457920			
0.858	6.513086				
1.183			6.627550		
1.201	8.924807				
1.218				6.274958	
1.623				9.407467	
1.656			13.347460		
1.705				9.318646	
1.715	12.736275				
2.287	15.822277				
2.310		32.142747			
2.435				12.347721	
2.744	17.135050				
2.838			10.602340		
3.247				18.404440	
3.432	19.829125				
4.730			19.607094		
4.870				22.836596	
6.622			23.195426		
9.740				40.830595	

Table 5. Soy lecithin analysis with different solvents

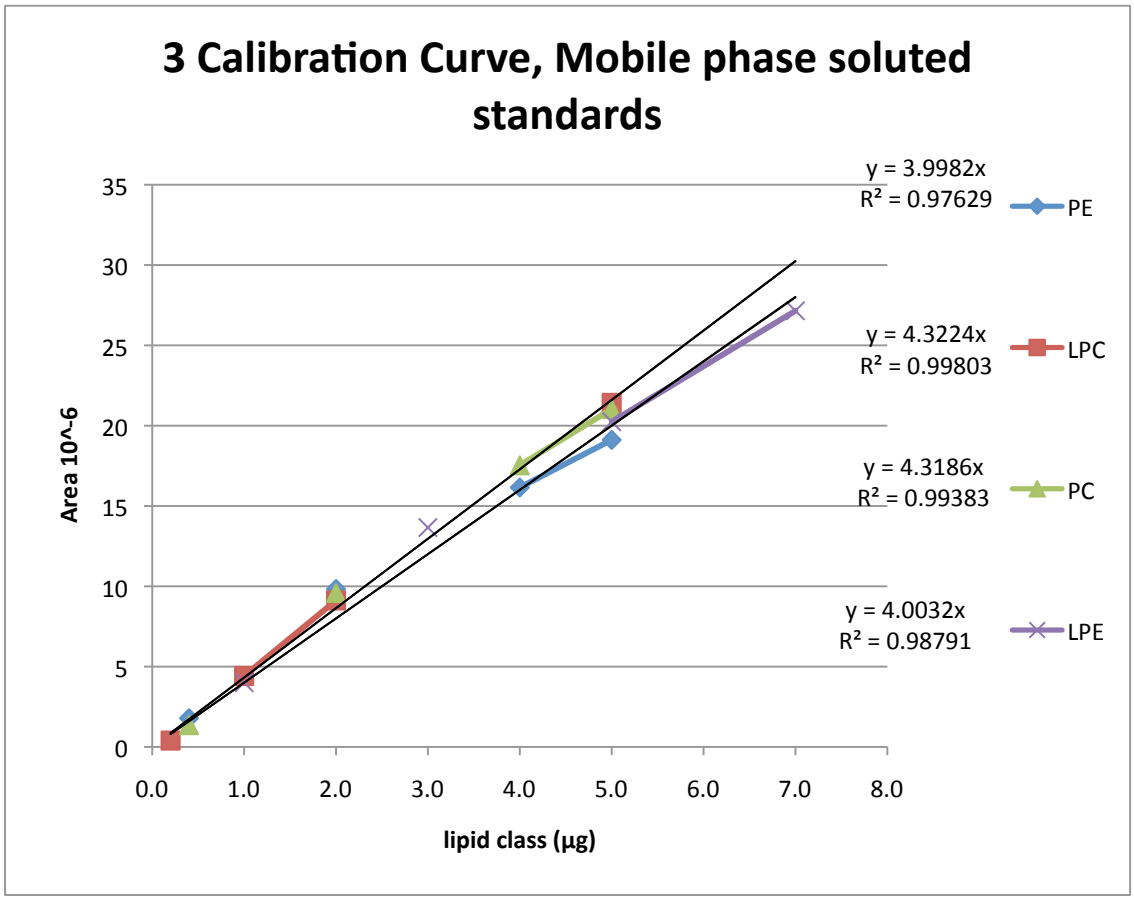
	Peak area (/1000000)			
Solvent	PE	LPE	PC	LPC
Isopropanol	8.811365	0.228419	20.679380	1.098738
Chloroform	10.554293	0.229000	21.605523	0.808813
Isopropanol:Hexane:Water(59:40:1)	10.758599	0.276258	22.196949	1.197395
Chloroform:Methanol(2:1)	10.692070	0.299322	22.368740	1.135054

Incomplete dissolved in solution

Injected 10µl of each sample
1mg/ml consentrations

Table 6. Calibration 3

µg	PE	PC	LPE	LPC	
0.2				0.396926	Mix 2:2:1
0.4	1.788772	1.355489			
1			4.002637	4.419771	
2	9.828719	9.590471		9.121200	
3			13.658124		
4	16.152475	17.532116			
5	19.115152	21.035217	20.242661	21.420582	Single 1mg/ml
7			27.153562		



Appendix F

CN-Analysis

The raw data obtained from CN-analysis is presented in Figure 1-4. Table 1 and 3 shows the Nitrogen concentrations. Table 2 and 4 shows the C concentrations. A calculation example is presented below table 1.

Table 1. CN-analysis of N amounts in Calibration 2 standards

Sample	Weight(mg)		Mm= 14.01g/mol						
	Nitrogen	Carbon	n (N)=n (PL)	Sample applied(L)	Mm PL(g/mol)	Consentration (mg/ml)	Average consentration	STD	SEM
PE									
1	0.001618	0.067416	1.1549E-07	0.0001	730.453	0.84359	0.52	0.54	0.27
2	0.000215	0.065971	1.5346E-08	0.0001	730.453	0.11210			
3	0.002116	0.065916	1.5103E-07	0.0001	730.453	1.10324			
4	0.000040	0.065407	2.85510E-09	0.0001	730.453	0.02086			
LPE									
1	0.002823	0.064397	2.015E-07	0.0001	471.609	0.95029	0.95	0.02	0.01
2	0.002848	0.062785	2.0328E-07	0.0001	471.609	0.95870			
3	0.002758	0.063884	1.9686E-07	0.0001	471.609	0.92841			
4	0.000785	0.060194	5.6031E-08	0.0001	471.609	0.26425			
PC									
1	0.000189	0.023167	1.349E-08	0.0001	775.037	0.10456	0.21	0.26	0.13
2	0.001072	0.022158	7.6517E-08	0.0001	775.037	0.59303			
3	0.000121	0.022158	8.6367E-09	0.0001	775.037	0.06694			
4	0.000109	0.022158	7.7802E-09	0.0001	775.037	0.06030			
LPC									
1	0.002421	0.061864	1.7281E-07	0.0001	513.564	0.88746	0.95	0.08	0.04
2	0.002781	0.061864	1.985E-07	0.0001	513.564	1.01943			
3	0.002769	0.057891	1.9764E-07	0.0001	512.564	1.01305			
4	0.002362	0.057788	1.6859E-07	0.0001	513.564	0.86584			

Originally the consentration was made to be 1mg/ml for each sample

Example of calculation procedure:

1) m(N)=0.001618mg

n(mol)= m(g)/Mm(g/mol) Mm(N)=14.01g/mol

Finding n(N)= n(PL)

0.000001618g/14.01g/n 1.1549E-07 mol

2)

Finding the PL mass (g) Mm(PL)=730.453g/mol

3)

Calculating the consentration of the sample in 100µl

g/L=mg/mL

8.4359*10^-5g/0.0001L= 0.84359mg/mL

Table 2. CN-analysis of C amounts in Calibration 2 standards

Sample	Weight(mg)		Mm= 14.01g/mol						
	Nitrogen	Carbon	n (N)=n (PL)	Sample applied(L)	Mm PL(g/mol)	Consentration (mg/ml)	Average consentration	STD	SEM
PE									
1	0.001618	0.067416	1.4391E-07	0.0001	730.453	1.05118	1.03	0.01	0.01
2	0.000215	0.065971	1.4082E-07	0.0001	730.453	1.02864			
3	0.002116	0.065916	1.4071E-07	0.0001	730.453	1.02779			
4	0.000040	0.065407	1.3962E-07	0.0001	730.453	1.01985			
LPE									
1	0.002823	0.064397	2.5529E-07	0.0001	471.609	1.20396	1.19	0.02	0.01
2	0.002848	0.062785	2.489E-07	0.0001	471.609	1.17383			
3	0.002758	0.063884	2.5325E-07	0.0001	471.609	1.19437			
4	0.000785	0.060194	2.3863E-07	0.0001	471.609	1.12538			
PC									
1	0.000189	0.023167	4.592E-08	0.0001	775.037	0.35590	0.34	0.01	0.00
2	0.001072	0.022158	4.392E-08	0.0001	775.037	0.34040			
3	0.000121	0.022158	4.392E-08	0.0001	775.037	0.34040			
4	0.000109	0.022158	4.392E-08	0.0001	775.037	0.34040			
LPC									
1	0.002421	0.061864	2.1459E-07	0.0001	513.564	1.10206	1.07	0.04	0.02
2	0.002781	0.061864	2.1459E-07	0.0001	513.564	1.10206			
3	0.002769	0.057891	2.0081E-07	0.0001	512.564	1.02928			
4	0.002362	0.057788	2.0045E-07	0.0001	513.564	1.02945			

Table 3. CN-analysis of N amounts in Calibration 3 standards

Sample	Weight(mg)		Mm= 14.01g/mol						
	Nitrogen	Carbon	n (N)=n (PL)	Sample applied(L)	Mm PL(g/mol)	Consentration (mg/ml)	Average consentration (mg/ml)	STD	SEM
PE									
1	0.003229	0.074227	2.3048E-07	0.0001	730.453	1.68354	1.59	0.07	0.03
2	0.002944	0.080409	2.1014E-07	0.0001	730.453	1.53494			
3	0.003058	0.080406	2.1827E-07	0.0001	730.453	1.59438			
4	0.002980	0.081311	2.1271E-07	0.0001	730.453	1.55371			
LPE									
1	0.004347	0.076329	3.1028E-07	0.0001	471.609	1.46330	1.41	0.13	0.07
2	0.00443	0.073761	3.1620E-07	0.0001	471.609	1.49124			
3	0.003635	0.065572	2.5946E-07	0.0001	471.609	1.22363			
4	0.004345	0.077145	3.1014E-07	0.0001	471.609	1.46263			
PC									
1	0.002883	0.070498	2.0578E-07	0.0001	775.037	1.59488	1.72	0.10	0.05
2	0.003278	0.075129	2.3398E-07	0.0001	775.037	1.81340			
3	0.003235	0.080878	2.3091E-07	0.0001	775.037	1.78961			
4	0.003041	0.080915	2.1706E-07	0.0001	775.037	1.68229			
LPC									
1	0.003807	0.067108	2.7173E-07	0.0001	513.564	1.39553	1.40	0.04	0.02
2	0.003973	0.072855	2.8358E-07	0.0001	513.564	1.45638			
3	0.003759	0.073288	2.6831E-07	0.0001	512.564	1.37525			
4	0.003753	0.066851	2.6788E-07	0.0001	513.564	1.37574			

Table 4. CN-analysis of C amounts in Calibration 3 standards

Sample	Weight(mg)		Mm=12.012g/mol						
	Nitrogen	Carbon	n C/x=n (PL)	Sample applied(L)	Mm PL(g/mol)	Consentration (mg/ml)	Average consentration (mg/ml)	STD	SEM
PE									
1		0.074227	1.58446E-07	0.0001	730.453	1.15738	1.23	0.05	0.03
2		0.080409	1.71642E-07	0.0001	730.453	1.25377			
3		0.080406	1.71636E-07	0.0001	730.453	1.25372			
4		0.081311	1.73568E-07	0.0001	730.453	1.26783			
LPE									
1		0.076329	3.0259E-07	0.0001	471.609	1.42704	1.37	0.10	0.06
2		0.073761	2.9241E-07	0.0001	471.609	1.37903			
3		0.065572	2.59946E-07	0.0001	471.609	1.22593			
4		0.077145	3.05825E-07	0.0001	471.609	1.44230			
PC									
1		0.070498	1.39737E-07	0.0001	775.037	1.08302	1.18	0.08	0.04
2		0.075129	1.48917E-07	0.0001	775.037	1.15416			
3		0.080878	1.60312E-07	0.0001	775.037	1.24248			
4		0.080915	1.60385E-07	0.0001	775.037	1.24305			
LPC									
1		0.067108	2.32781E-07	0.0001	513.564	1.19548	1.25	0.06	0.03
2		0.072855	2.52716E-07	0.0001	513.564	1.29786			
3		0.073288	2.54218E-07	0.0001	512.564	1.30303			
4		0.066851	2.3189E-07	0.0001	513.564	1.19090			

Appendix G

HPLC-CAD PL-Class Analysis

Raw data from HPLC-CAD analysis of PL-classes is presented in Table 1-37. The data were given in µg amount.

Table 1 HPLC PL Herring roe, 1st extr

Consentration,mg/ml 1
injected µl 10
Consentration µg /10µl 10
Method Bligh and Dyer

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.7242	0.6381	0.8035	0.8043	0.7268	0.8554	0.76	0.08
PC	4.3571	4.2954	4.5194	4.3780	4.3914	4.5770	4.42	0.11
LPC	0.0815	0.0331	0.0821	0.0597	0.0702	0.0654	0.07	0.02
Total PL(sum)							5.24	

Table 2 HPLC PL Herring, 2nd extr

Consentration,mg/ml 1
injected µl 10
Consentration µg /10µl 10
Method Bligh and Dyer

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	1.0171	0.9377	0.9450	0.8400	0.9740	0.8984	0.94	0.06
PC	5.6085	5.0525	5.2029	4.9624	5.4080	5.3468	5.26	0.24
Total PL(sum)							6.20	

Table 3 HPLC PL Herring roe, 3rd extr

Consentration,mg/ml 1
injected µl 10
Consentration µg /10µl 10
Method Bligh and Dyer

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.7213	0.7062	0.6773	0.6116	0.5446	0.5438	0.63	0.08
PC	4.2590	3.9297	4.0931	3.7870	3.8780	3.9098	3.98	0.17
Total PL(sum)							4.61	

Table 4 HPLC PL Herring roe, 1st extr

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method Bligh and Dyer, SINTEF

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.9864	0.9161	1.0380	0.9871	0.9963	0.9424	0.98	0.04
PC	5.4060	5.2124	5.3890	5.5009	5.3104	Rejected	5.36	0.11
LPC	0.1092	0.1012	0.1119	0.1027	0.1056	0.1184	0.11	0.01
Total PL(sum)							6.45	

Table 5 HPLC PL Herring roe, 2nd extr

Method Bligh and Dyer, SINTEF

Consentration, mg/ml 1

injected, µl 10

Consentration µg /10µl 10

Phospholipidclass measured amount in sample (µg)

Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.8470	0.9099	0.9257	0.8591	0.8757	0.9128	0.89	0.03
PC	4.9390	4.9762	5.2415	5.3836	5.1299	5.3456	5.17	0.19
Total PL(sum)							6.06	

Table 6 HPLC PL Herring roe, 3rd extr

Method Bligh and Dyer, SINTEF

Consentration, mg/ml 1

injected, µl 10

Consentration µg /10µl 10

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.4918	0.4883	0.5329	0.5300	0.5609	0.4893	0.52	0.03
PC	3.3982	3.3989	3.4148	3.6434	3.2511	3.2910	3.40	0.14
LPC	0.0285	0.0302	0.0328	0.0256	0.0221	0.0397	0.03	0.01
Total PL(sum)							3.94	

Table 7 HPLC PL Krillmeal, 1st extr

Method Bligh and Dyer

Consentration, mg/ml 1

injected, µl 10

Consentration µg /10µl 10

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.1839	0.1957	0.1893	0.1810	0.2057	0.1863	0.19	0.01
LPE	0.0375	0.0478	0.0498	0.0463	0.0319	0.0483	0.04	0.01
PC	2.4190	2.5371	2.3809	2.8734	2.5124	2.3040	2.50	0.20
LPC	0.1978	0.1971	0.2461	0.1871	0.1800	0.2567	0.21	0.03
Total PL(sum)							2.95	

Table 8 HPLC PL Krillmeal, 2nd extr

Method Bligh and Dyer

Consentration, mg/ml 1

injected, µl 10

Consentration µg /10µl 10

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.1443	0.1167	0.1499	0.1651	0.1549	0.1291	0.14	0.02
PC	1.5943	1.8100	1.7999	1.5689	1.6546	1.5590	1.66	0.11
LPC	0.1666	0.1381	0.1361	0.1678	0.1711	0.1450	0.15	0.02
Total PL(sum)							1.96	

Table 9 HPLC PL Krillmeal, 3rd extr

Method Bligh and Dyer

Consentration, mg/ml 1

injected, µl 10

Consentration µg /10µl 10

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.1658	0.1620	0.1508	0.1621	0.1465	0.1419	0.15	0.01
PC	1.8033	2.0112	2.0384	1.8024	1.9407	1.8326	1.90	0.11
LPC	0.3054	0.1955	0.2159	0.2449	0.2291	0.1906	0.23	0.04
Total PL(sum)							2.29	

Table 10 HPLC PL Fish meal

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method Bligh and Dyer

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.4864	0.7288	0.6718	0.4759	0.5677	0.4520	0.56	0.11
LPE	0.0789	0.0971	0.1072	0.1139	0.1457	0.0622	0.10	0.03
PC	1.1557	1.3187	1.5729	1.5321	1.1999	1.8288	1.43	0.26
LPC	0.1564	0.1903	0.1805	0.1324	0.1561	0.1691	0.16	0.02
Total PL(sum)							2.26	

Table 11 HPLC PL Herring meal

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method Bligh and Dyer

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.2586	0.2773	0.3915	0.2758	0.3090	0.3134	0.30	0.05
LPE	0.0446	0.0501	0.0327	0.0300	0.0411	0.0390	0.04	0.01
PC	1.5355	1.5031	1.4618	1.5712	1.5259	1.5244	1.52	0.04
LPC	0.1427	0.1576	0.1616	0.1299	0.1595	0.1647	0.15	0.01
Total PL(sum)							2.02	

Table 12 HPLC PL Salmon meal

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method Bligh and Dyer

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE+PI	0.7411	0.6686	0.6823	0.5449	0.5218	0.4915	0.6084	0.10
LPE	0.1319	0.1009	0.1046	0.1006	0.1117	0.0988	0.1081	0.01
PC	2.7063	2.5674	2.5946	2.4508	2.4470	2.3602	2.5210	0.13
LPC	0.3759	0.3612	0.3557	0.3354	0.2936	0.2706	0.3321	0.04
Total PL(sum)							3.5695	

Table 13 HPLC PL Cod roe

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method Bligh and Dyer

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	(100PA)	4	5	6	Average	Std.dev.
PE	1.4109	1.5238	1.4853	1.4215	1.5621	1.4608	1.48	0.06
LPE	0.1007	0.0930	0.1044	0.1255	0.0895	0.1301	0.11	0.02
PC	5.7022	6.0509	5.9127	5.6842	6.1130	5.7316	5.87	0.19
LPC	0.1839	0.1626	0.1882	0.2231	0.1587	0.2006	0.19	0.02
Total PL(sum)							7.64	

Table 14 HPLC PL Herring meal

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method ASE, 100% Chloroform,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE+?	0.5530	0.7355	0.6491	0.5661	0.5203	0.7244	0.6247	0.09
LPE	0.0610	0.0501	0.0403	0.0501	0.0478	0.0592	0.0514	0.01
PC	2.0342	2.1369	1.9868	1.9244	1.5290	1.9449	1.9261	0.21
LPC	0.1491	0.1066	0.1193	0.1399	0.1277	0.1257	0.1280	0.02
Total PL(sum)							2.73	

Table 15 HPLC PL Krill meal 1

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method ASE, 100% chloroform,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.3190	0.2844	0.2782	0.2344	0.3114	0.2622	0.28	0.03
LPE	0.0381	0.0420	0.0507	0.0389	0.0537	0.0371	0.04	0.01
PC	2.9043	2.7295	2.7992	2.4999	2.3610	2.3260	2.60	0.24
LPC	0.2338	0.2117	0.1721	0.1941	0.1533	0.1622	0.19	0.03
Total PL(sum)							3.12	

Table 16 HPLC PL Salmon meal

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method ASE, 100% chloroform,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.1278	0.1068	0.1077	0.0997	0.1157	0.0998	0.11	0.01
LPE	0.0285	0.0284	0.0268	0.0256	0.0291	0.3090	0.07	0.11
PC	1.2389	1.2889	1.2427	1.1592	1.1977	1.2245	1.23	0.04
Total PL(sum)							1.41	

Table 17 HPLC PL Cod roe

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% chloroform,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.2200	0.3817	0.3848	0.3031	0.3833	0.2900	0.3271	0.07
LPE	0.0308	0.0213	0.0616	0.0403	0.0441	0.0241	0.0370	0.01
PC	1.7609	2.1497	2.1306	1.9512	2.4324	2.2330	2.1096	0.23
Total PL(sum)							2.47	

Table 18 HPLC PL Fishmeal

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method ASE, 100% chloroform,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE+PI	0.1626	0.2236	0.2403	0.1806	0.2391	0.1805	0.20	0.03
LPE	0.0620	0.0345	0.0588	0.0400	0.0399	0.0573	0.05	0.01
PC	0.7629	0.8853	0.7066	0.6782	0.8990	0.6996	0.77	0.10
Total PL(sum)							1.03	

Table 19 HPLC PL Krillmeal 1, 1st extr

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.2213	0.2293	0.398	0.513	0.495	0.481	0.3896	0.1332
LPE	0.0459	0.0547	0.064	0.119	0.156	0.164	0.1006	0.0527
PC	2.5716	2.5444	2.841	2.697	2.613	2.652	2.6532	0.1071
LPC	0.2506	0.3434	0.463	0.261	0.398	0.33312	0.3415	0.0810
Total PL(sum)							3.48	

Table 20 HPLC PL Krillmeal 1, 2nd extr

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.1523	0.2269	0.1879	0.3130	0.2137	0.3214	0.24	0.07
LPE	0.0515	0.0478	0.0647	0.0568	0.0499	0.0501	0.05	0.01
PC	1.6218	2.2203	2.3004	2.3417	1.9720	2.4570	2.15	0.31
LPC	0.4050	0.3536	0.3114	0.4670	0.3690	0.4093	0.39	0.05
Total PL(sum)							2.83	

Table 21 HPLC PL Krillmeal 1, 3rd extr

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.1975	0.1951	0.2470	0.3028	0.1907	0.2879	0.21	0.05
LPE	0.1068	0.1621	0.1854	0.1099	0.1994	0.2137	0.15	0.05
PC	1.5592	1.5856	1.3881	1.6082	1.4867	1.6110	1.54	0.09
LPC	0.2169	0.2161	0.2191	0.2789	0.2993	0.3137	0.22	0.05
Total PL(sum)							2.12	

Table 22 HPLC PL Herring meal

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.7470	0.6360	0.7119	0.8235	0.8938	0.9126	0.79	0.11
LPE	0.4697	0.3508	0.4015	0.4729	0.3541	0.2698	0.39	0.08
PC	1.9883	1.7735	1.4155	2.0190	1.7979	1.6623	1.78	0.22
LPC	0.2086	0.2661	0.2341	0.2727	0.2638	0.3039	0.26	0.03
Total PL(sum)							3.21	

Table 23 HPLC PL Fish meal

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	1.1464	1.1308	1.1121	1.2926	1.1745	1.1964	1.1754	0.0648
LPE	0.7561	0.8404	1.1042	0.9700	1.0076	0.7313	0.9016	0.1489
PC	2.4530	2.6099	2.3738	2.4631	2.5765	2.4592	2.4892	0.0877
LPC	0.5850	0.5258	0.5825	0.5280	0.6782	0.5156	0.5692	0.0613
Total PL(sum)							5.14	

Table 24 HPLC PL Salmon meal

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	1.0484	1.0435	1.0658	1.1376	1.1045	1.0134	1.05	0.05
LPE	1.0257	1.1772	1.1808	0.9270	1.0347	0.7986	1.13	0.15
PC	3.3798	3.6721	3.5494	3.4683	3.5708	3.4153	3.53	0.11
LPC	0.5571	0.6160	0.5245	0.4659	0.6421	0.5004	0.57	0.07
Total PL(sum)							6.28	

% of total lipid

Table 25 HPLC PL Cod roe

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE+?	0.3149	0.2758	0.2289	0.2363	0.1965	0.2078	0.24	0.04
PC	0.9124	0.8564	0.8461	0.8822	1.0346	0.9723	0.92	0.07
LPC	0.0519	0.0689	0.0651	0.0622	0.0816	0.0523	0.06	0.01
Total PL(sum)							1.22	

Table 26 HPLC PL Herring roe, 1 ex

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method ASE, 100% ethan

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.537	0.569	0.579	0.773	0.725	0.989	0.70	0.17
LPE	0.070	0.059	0.067	0.054	0.073	0.061	0.06	0.01
PC	3.534	3.472	3.395	3.470	3.582	3.358	3.47	0.08
LPC	0.098	0.094	0.111	0.105	0.116	0.104	0.10	0.01
Total PL(sum)							4.33	0.19

Table 27 HPLC PL Herring roe, 2 ex

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	1.091	1.141	1.118	1.153	1.416	1.169	1.18	0.12
LPE	0.065	0.060	0.064	0.078	0.048	0.060	0.06	0.01
PC	4.868	4.584	4.516	5.155	5.177	4.985	4.88	0.28
LPC	0.090	0.085	0.142	0.165	0.149	0.150	0.13	0.03
Total PL(sum)							6.25	0.31

Table 28 HPLC PL Herring roe, 3 ex

Consentration,mg/ml 2

injected µl 10

Consentration µg /10µl 20

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	1.472	1.490	1.491	1.730	1.872	1.784	1.64	0.18
LPE	0.117	0.099	0.097	0.118	0.124	0.131	0.11	0.01
PC	7.436	7.566	7.363	8.094	8.069	7.892	7.74	0.32
LPC	0.184	0.200	0.253	0.248	0.213	0.257	0.23	0.03
Total PL(sum)							9.72	0.37

Table 29 HPLC PL Krillmeal 2, 1 ex

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE+?	0.3850	0.4100	0.3510	0.4098	0.3450	0.3470	0.37	0.03
LPE	0.0500	0.0490	0.0420	0.0534	0.0560	0.0590	0.05	0.01
PC	4.0710	4.1210	3.9340	3.9990	3.9080	4.1780	4.04	0.11
LPC	0.0630	0.0700	0.0650	0.0810	0.1100	0.0960	0.08	0.02
Total PL(sum)							4.54	0.11

Table 30 HPLC PL Krillmeal 2, 2 ex

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.2780	0.3210	0.2900	0.2510	0.2980	0.3240	0.29	0.03
LPE	0.0400	0.0487	0.0380	0.0399	0.0250	0.0205	0.04	0.01
PC	5.3580	5.5110	5.4700	5.3210	5.3780	5.4650	5.42	0.08
LPC	0.1840	0.2130	0.2520	0.1980	0.2560	0.2000	0.22	0.03
Total PL(sum)							5.96	0.09

Table 31 HPLC PL Krillmeal 2, 3 ex

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% ethanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.4500	0.4980	0.5660	0.5078	0.4900	0.4056	0.49	0.05
LPE	0.0730	0.0567	0.0620	0.0970	0.0380	0.0600	0.06	0.02
PC	4.0000	4.2456	4.3070	4.5010	4.2870	4.1290	4.24	0.17
LPC	0.0690	0.0897	0.1800	0.1000	0.1430	0.0990	0.11	0.04
Total PL(sum)							4.91	0.18

Table 32 HPLC PL Krillmeal 2, 1 ex

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% chloroform:methanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.633	0.612	0.591	0.609	0.613	0.587	0.61	0.02
PC	5.872	5.929	5.902	5.887	6.377	6.079	6.01	0.20
Total PL(sum)							6.62	

% of total lipid

Table 33 HPLC PL Krillmeal 2, 2 ex

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% chloroform:methanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.379	0.338	0.319	0.352	0.347	0.310	0.34	0.02
LPE	0.088	0.059	0.078	0.050	0.050	0.067	0.07	0.02
PC	3.584	3.497	3.295	3.605	3.792	3.537	3.55	0.16
LPC	0.086	0.102	0.059	0.056	0.050	0.062	0.07	0.02
Total PL(sum)							4.03	0.17

Table 34 HPLC PL Krillmeal 2, 3 ex

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% chloroform:methanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.337	0.349	0.300	0.432	0.420	0.434	0.38	0.06
LPE	0.074	0.084	0.065	0.038	0.080	0.075	0.07	0.02
PC	4.500	4.225	4.366	4.435	4.633	4.594	4.46	0.15
LPC	0.091	0.101	0.093	0.077	0.065	0.092	0.09	0.01
Total PL(sum)							4.99	0.16

Table 35 HPLC PL Herring roe, 1 ex

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% chloroform:methanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE+?	0.390	0.363	0.372	0.348	0.396	0.397	0.38	0.02
PC	2.280	2.269	2.249	2.240	2.319	2.418	2.30	0.07
LPC	0.062	0.051	0.068	0.051	0.071	0.070	0.06	0.01
Total PL(sum)							2.74	1.21

Table 36 HPLC PL Herring roe, 2 ex

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% chloroform:me

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.817	0.744	0.875	0.715	0.817	0.720	0.78	0.06
LPE	0.048	0.050	0.052	0.055	0.053	0.066	0.05	0.01
PC	1.999	1.851	1.783	1.815	1.999	1.922	1.89	0.09
LPC	0.052	0.044	0.049	0.052	0.051	0.049	0.05	0.00
Total PL(sum)							2.78	0.11

Table 37 HPLC PL Herring roe, 3 ex

Consentration,mg/ml 1

injected µl 10

Consentration µg /10µl 10

Method ASE, 100% chloroform:methanol,100°C

Phospholipidclass measured amount in sample (µg)								
Phospholipid class	1	2	3	4	5	6	Average	Std.dev.
PE	0.844	0.731	0.849	0.896	0.788	0.783	0.82	0.06
LPE	0.092	0.099	0.095	0.097	0.090	0.094	0.09	0.00
PC	3.725	3.567	3.558	3.564	3.690	3.802	3.65	0.10
LPC	0.076	0.091	0.078	0.067	0.075	0.066	0.08	0.01
Total PL(sum)							4.64	0.12

Appendix H

Iatroscan TLC-FID Lipid-Class Analysis

Raw data of lipid class composition by Iatroscan is presented in Table 1-12.

Table 1. Lipid composition of herring roe 1 extracts by Iatroscan

Sample	Herring roe, 1st extraction						
Number	177	114	167	138	Average	STD	SEM
Lipid class (g/100g lipid)							
Cholesterolester	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Triglycerid	15.56	13.24	12.85	15.71	14.34	1.50	0.8
Free fatty acids	0.00	0.00	0.00	0.00	0.00	0.00	0.0
FFA -CHOL	12.06	9.83	9.56	11.52	10.74	1.23	0.6
Cholesterol	19.01	18.39	17.90	17.97	18.32	0.51	0.3
Mono and diglyserides	9.54	12.93	11.65	9.63	10.94	1.65	0.8
Polar lipids	44.84	45.63	47.05	45.18	45.67	0.97	0.5
							fa
Total	101.00	100.03	99.00	100.01	100.01		

Table 2. Lipid composition of herring roe 2 extracts by Iatroscan

Sample	Herring roe, 2nd extraction						
Number	285	178	168	115	Average	STD	SEM
Lipid class (g/100g lipid)							
Cholesterolester	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Triglycerid	10.26	11.83	12.12	10.67	11.22	0.90	0.4
Free fatty acids	0.00	0.00	0.00	0.00	0.00	0.00	0.0
FFA -CHOL	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Cholesterol	11.65	10.33	13.72	10.63	11.58	1.54	0.8
Mono and diglyserides	9.72	8.73	9.23	9.96	9.41	0.55	0.3
Polar lipids	68.38	69.12	65.33	68.66	67.87	1.72	0.9
Total	100.01	100.00	100.40	99.92	100.08		

Table 3. Lipid composition of herring roe 3 extracts by Iatroscan

Sample	Herring roe, 3rd extrcation						
Number	169	179	116	26	Average	STD	SEM
Lipid class (g/100g lipid)							
Cholesterolester	0.00	0.00	0.00	0.00	0.00	0.00	
Triglycerid	14.98	16.20	14.06	14.20	14.86	0.98	0.5
Free fatty acids	0.00	0.00	0.00	0.00	0.00	0.00	0.0
FFA -CHOL	3.50	2.88	3.71	3.85	3.48	0.43	0.2
Cholesterol	16.03	16.70	17.65	15.97	16.59	0.78	0.4
Mono and diglyserides	9.85	9.84	12.02	12.12	10.96	1.29	0.6
Polar lipids	54.64	55.15	52.56	53.57	53.98	1.15	0.6
Total	98.99	100.77	100.00	99.71	99.87		

Table 4. Lipid composition of salmon meal extracts by latroscan

Sample	**Salmon meal**						
Number	40	319	313	255	Average	STD	SEM
Lipid class (g/100g lipid)							
Cholesterolester	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Triglycerid	56.01	62.14	60.06	56.17	58.59	3.01	1.5
Free fatty acids	2.58	3.55	3.12	3.12	3.09	0.40	0.2
FFA -CHOL	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Cholesterol	3.45	5.25	5.34	4.21	4.56	0.90	0.5
Mono and diglyserides	2.59	2.83	2.58	2.58	2.65	0.12	0.1
Polar lipids	35.30	26.10	28.90	34.09	31.10	4.34	2.2
Total	99.93	99.86	100.00	100.17	99.99		

Table 5. Lipid composition of cod roe extracts by latroscan

Sample	**Cod roe**						
Number	210	316	37	278	Average	STD	SEM
Lipid class (g/100g lipid)							
Cholesterolester	0.95	0.67	1.12	0.56	0.83	0.26	0.1
Triglycerid	9.86	7.19	10.50	9.39	9.24	1.44	0.7
Free fatty acids	4.56	4.56	5.61	4.48	4.80	0.54	0.3
FFA -CHOL	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Cholesterol	13.64	15.85	15.52	16.66	15.42	1.28	0.6
Mono and diglyserides	9.92	8.50	7.62	8.65	8.67	0.95	0.5
Polar lipids	58.38	62.24	59.63	60.88	60.28	1.66	0.8
Total	97.32	99.00	100.00	100.62	99.24		

Table 6. Lipid composition of herring meal extracts by latroscan

Sample	**Herring meal**						
Number	119	314	39	209	Average	STD	SEM
Lipid class (g/100g lipid)							
Cholesterolester	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Triglycerid	63.10	64.68	61.01	62.57	62.84	1.51	0.8
Free fatty acids	0.00	0.00	0.00	0.00	0.00	0.00	0.0
FFA -CHOL	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Cholesterol	5.08	4.41	3.13	4.92	4.38	0.89	0.4
Mono and diglyserides	2.53	1.62	2.08	2.01	2.06	0.37	0.2
Polar lipids	29.29	29.30	33.37	29.99	30.49	1.95	1.0
Total	100.00	100.00	99.59	99.49	99.77		

Table 7. Lipid composition of krill meal 1 extracts by Iatroscan

Sample	Krill meal, 1st extraction						
Number	281	135	204	483	Average	STD	SEM
Lipid class (g/100g lipid)							
Cholesterolester	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Triglycerid	42.19	40.78	40.04	42.01	41.25	1.02	0.5
Free fatty acids	10.98	12.85	9.62	10.40	10.96	1.38	0.7
FFA -CHOL	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Cholesterol	3.02	3.68	3.10	2.97	3.19	0.33	0.2
Mono and diglyserides	1.66	1.44	3.78	2.95	2.46	1.10	0.6
Polar lipids	42.16	41.25	43.46	41.45	42.08	1.00	0.5
Total	100.00	100.00	100.00	99.78	99.95		

Table 8. Lipid composition of krill meal 2 extracts by Iatroscan

Sample	Krill meal, 2nd extraction						
Number	282	195	205	136	Average	STD	SEM
Lipid class (g/100g lipid)							
Cholesterolester	0.00	0.00	0.00	0.00	0.00	0.00	
Triglycerid	54.44	55.76	52.55	52.37	53.78	1.62	0.8
Free fatty acids	0.00	0.00	0.00	0.00	0.00	0.00	0.0
FFA -CHOL	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Cholesterol	2.89	2.59	2.10	2.26	2.46	0.35	0.2
Mono and diglyserides	2.07	4.42	3.78	3.92	3.55	1.02	0.5
Polar lipids	40.35	38.53	41.46	40.76	40.27	1.25	0.6
Total	99.75	101.28	99.89	99.30	100.06		

Table 9. Lipid composition of krill meal 3 extracts by Iatroscan

Sample	Krill meal, 3rd extraction						
Number	283	166	176	251	Average	STD	SEM
Lipid class (g/100g lipid)							
Cholesterolester	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Triglycerid	36.26	37.24	36.14	35.47	36.28	0.73	0.4
Free fatty acids	14.94	13.60	13.79	13.83	14.04	0.61	0.3
FFA -CHOL	0.00	0.00	0.00	0.00	0.00	0.00	0.0
Cholesterol	2.37	2.68	2.76	3.14	2.74	0.32	0.2
Mono and diglyserides	0.39	0.80	0.54	0.90	0.66	0.23	0.1
Polar lipids	46.04	45.17	46.77	45.87	45.96	0.66	0.3
Total	100.00	99.49	100.00	99.22	99.68		

Table 10. Lipid composition of fish meal extracts by Iatroscan

Sample	Fish meal						
Number	250	38	317	402	Average	STD	SEM
Lipid class (g/100g lipid)							
Cholesterolester	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Triglycerid	52.54	53.81	54.90	55.16	54.10	1.19	0.60
Free fatty acids	3.25	3.57	4.31	3.81	3.74	0.45	0.22
FFA -CHOL	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cholesterol	4.32	3.83	5.44	5.13	4.68	0.74	0.37
Mono and diglyserides	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Polar lipids	39.25	37.94	35.35	36.05	37.15	1.78	0.89
Total	99.36	99.15	100.00	100.15	99.66		

Table 11. PL concentration of all B&D extracts by Iatroscan

Sample	PL concentration(g/100g sample)						
Number	1	2	3	4	Average	STD	
Lipid class (g/100g lipid)							
Salmon meal	6.25	4.62	5.11	6.03	5.50	0.14	
Fish meal	4.27	4.13	3.85	3.92	4.04	0.05	
Herring meal	4.47	4.47	5.09	4.58	4.65	0.07	
Cod roe	2.45	2.61	2.50	2.55	2.53	0.03	
Herring roe, 1 extr	1.52	1.55	1.59	1.53	1.55	0.09	
Herring roe, 2 extr	0.77	0.78	0.74	0.78	0.77	0.34	
Herring roe, 3 extr	0.23	0.23	0.22	0.22	0.23	0.38	
Krillmeal, 1 extr	13.24	12.95	13.65	13.02	13.21	0.03	
Krillmeal, 2 extr	5.78	5.52	5.94	5.84	5.77	0.06	
Krillmeal, 3 extr	3.28	3.22	3.33	3.27	3.28	0.10	

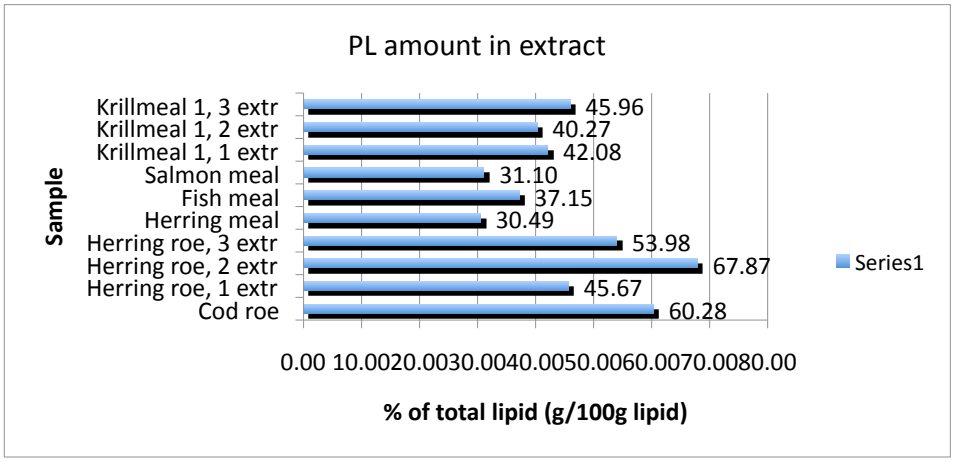


Table 12. Lipid class composition of all B&D extracts by Iatroscan (n=4)

	Cholesterolester		Triglycerid		Free fatty acids		FFA -CHOL		Cholesterol		Mono-diglyserides		Polar lipids	
	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD
Cod roe	0.83	0.26	9.24	1.44	4.80	0.54	0.00	0.00	15.42	1.28	8.67	0.95	60.28	1.66
Herring roe, 1st extraction	0.0	0.0	14.34	1.51	0.00	0.00	10.74	1.23	18.32	0.51	10.94	1.65	45.68	0.97
Herring roe, 2nd extraction	0.0	0.0	11.22	0.90	0.00	0.00	0.00	0.00	11.58	1.53	9.41	0.55	67.87	1.72
Herring roe, 3rd extraction	0.0	0.0	14.86	0.98	0.00	0.00	3.49	0.43	16.59	0.78	10.96	1.29	53.98	1.15
Krill meal 1, 1st extraction	0.0	0.0	41.26	1.02	10.96	1.38	0.00	0.00	3.19	0.33	2.46	1.10	42.08	1.00
Krill meal 1, 2nd extraction	0.0	0.0	53.78	1.62	0.00	0.00	0.00	0.00	2.46	0.35	3.55	1.02	40.28	1.25
Krill meal 1, 3rd extraction	0.0	0.0	36.28	0.73	14.04	0.61	0.00	0.00	2.74	0.32	0.66	0.23	45.96	0.66
Herring meal	0.0	0.0	62.84	1.51	0.00	0.00	0.00	0.00	4.39	0.88	2.06	0.37	30.49	1.95
Fish meal	0.0	0.0	54.10	1.19	3.74	0.45	0.00	0.00	4.68	0.74	0.00	0.00	37.15	1.78
Salmon meal	0.0	0.0	58.60	3.02	3.09	0.40	0.00	0.00	4.56	0.90	2.65	0.12	31.10	4.34

Appendix I

Washout Curve

Washout curve calculations:

A simple system of two immiscible phases, chloroform and water/methanol is governed by the distribution laws where K is the equilibrium constant. In this system the solute is lipids, x. The soluted lipids in the water/methanol phase are x_1 after the first extraction, x_3 after the second extraction and x_5 after the third. The main equation of K and the concentrations of lipids in the water/methanol phase are:

$$x_2 = x_1 K$$
$$x_0 = x_1 + x_2$$
$$x_3 = x_0 - x_2 - x_4$$
$$x_5 = x_0 - x_2 - x_4 - x_6$$

Where x_2 is the concentration of lipids in the first extraction, x_4 is the concentration of lipids in the second extraction and x_6 is the concentration in the third.

Rearranging and derivation of these equations give formulas that can be used in calculation of a washout curve. The derivation is:

$$\frac{x_2}{k} = x_0 - x_1$$
$$x_0 = \frac{x_2}{k} + x_2 = x_2\left(\frac{1}{k} + 1\right)$$
$$x_4 = x_2 + (1 + k) - x_2 k - x_4 k$$
$$x_4 = x_2 - k x_4$$
$$x_2 = x_4(1 + k)$$
$$k = \frac{x_2}{x_4} - 1$$

After two extractions are performed, their lipid concentrations are used to calculate k. The concentrations of x_1, x_3 and x_5 that is calculated are logarithmically (LN) estimated. The LN values are plotted as a function of extraction steps.

All washout curve values are calculated from these equations.

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