Faculty of Biosciences, Fisheries and Economics
The Norwegian College of Fishery Science

Calanus® Oil

Utilization, composition and digestion

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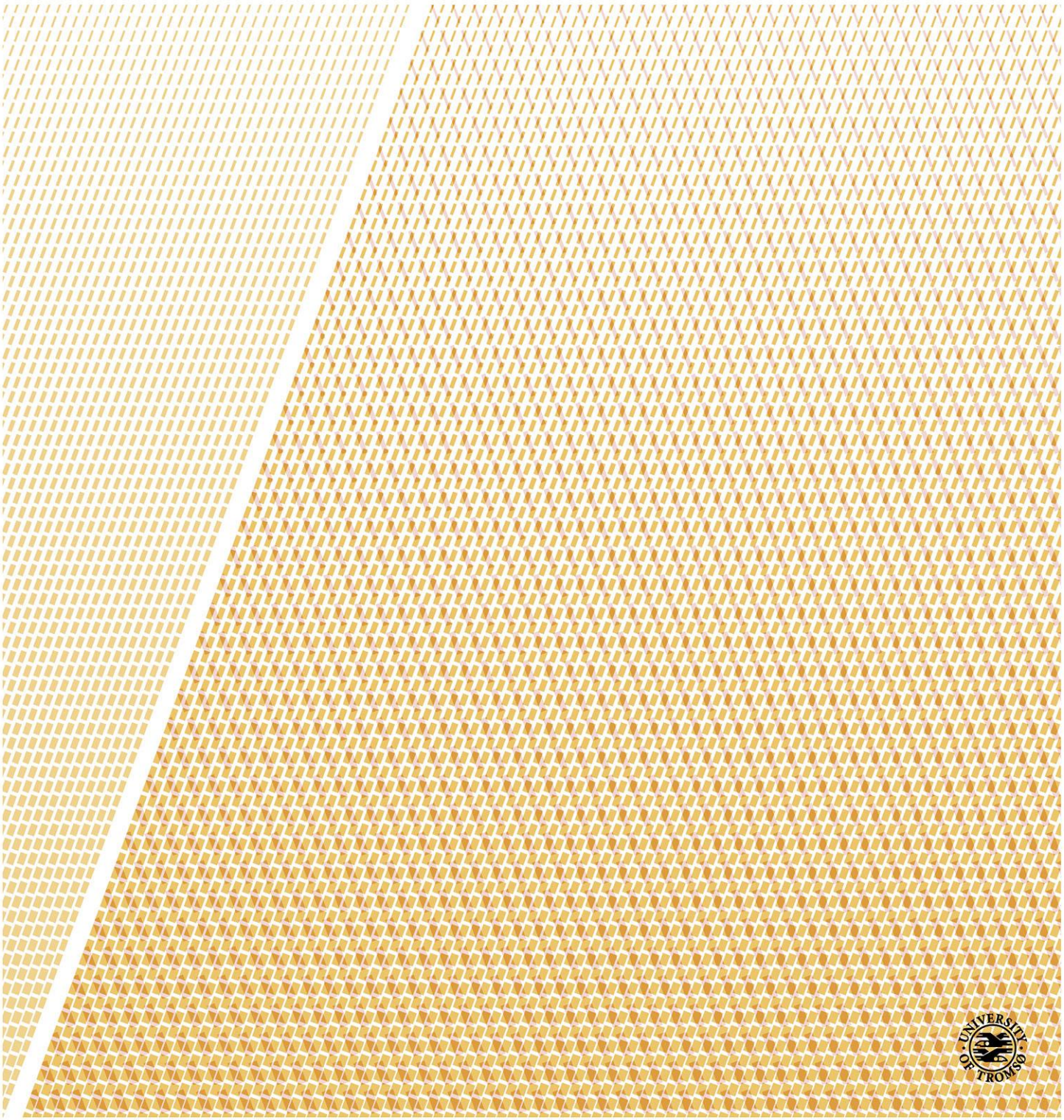
Alice Marie Pedersen

A dissertation for the degree of Philosophiae Doctor – February 2016



Calanus® Oil

Utilization, composition and digestion

By

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The Norwegian College of Fishery Science,

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and

Calanus AS

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Sammendrag

Et betydelig fokus på positive helseeffekter ved inntak av de langkjedede omega-3 fettsyrene eikosapentaensyre (EPA, 20:5n-3) og dokosaheksaensyre (DHA, 22:6n-3) har ført til økt etterspørsel av fiskeoljer. Tilgjengeligheten av marine oljer begrenses på grunn av strengere regulering av fiskeriene og en økt utnyttelse av pelagiske fiskearter direkte til human konsum. Det er ikke nok fiskeolje på verdensmarkedet for å dekke behovet til en økende befolkning og samtidig en økende akvakulturproduksjon. Dette har ført til et omfattende arbeid med å lete etter andre alternative, og ikke minst bærekraftige kilder som inneholder disse langkjedede omega-3 fettsyrene (n-3 LC-PUFA). Raudåte (*Calanus finmarchicus*) er det mest tallrike dyreplankton i Nord-atlanteren, og spiller en viktig rolle i energioverføringen oppover i næringskjeden. Raudåta er beskrevet som Norges største fornybare ressurs, med en årsproduksjon på mellom 100 og 200 millioner tonn. Nyutviklet industriell høstingsteknologi har gjort det mulig for bærekraftig utnyttelse av denne ressursen.

Hensikten med doktorgradsarbeidet var å framskaffe kunnskap som kunne bidra til den kommersielle utnyttelsen av raudåte. Studier har vist at olje fra raudåte kan benyttes til fiskefôr i oppdrettsindustrien, hvor fisken utnytter næringsstoffene effektivt og vokser tilfredsstillende. Calanusolje kan også benyttes til humant konsum og finnes i dag tilgengelig som et kosttilskudd. Nylige prekliniske studier peker mot positive helseeffekter av Calanusolje utover det som vanligvis kan forklares ved inntak av omega-3 fettsyrer alene. I dette doktorgradsarbeidet ble det undersøkt om bruk av proteolytiske enzymer i fremstillingen kunne bedre oljeutbyttet og sammensetningen i oljeproduktet ble karakterisert. Resultatene viser at bruk av enzymteknologi frembringer et atskillig høyere oljeutbytte sammenlignet med tradisjonell fiskeoljeproduksjon. Oljen som utvinnes fra raudåta består hovedsakelig av monoestere som er satt sammen av langkjedede fettsyrer og fettalkoholer, også kjent som voksestere. Calanusolje har et høyt innhold av omega-3 fettsyrene stearidonsyre (SDA, 18:4n-3), EPA og DHA, men også et betydelig innhold av enumettede fettsyrer, spesielt gadolensyre (20:1n-9) og cetolensyre (22:1n-11). Innholdet av den røde antioksidanten astaxanthin foreligger i all hovedsak som mono- og diestere, og det høye innholdet av astaxanthin bidrar formodentlig til den oksidative stabiliteten til Calanusoljen. Den siste delen av doktorgradsarbeidet bestod i å undersøke fordøyelse hos mus gitt en såkalt høyfettdiett tilsatt 2% Calanusolje. Fettsyresammensetning i fettvev og lever bekreftet at musene kunne fordøye og absorbere voksesterene tilført via fôret og det ble registrert en reduksjon i vektøkning hos dyrene som var i samsvar med det som er sett i tidligere arbeider.

Summary

The availability of the omega-3 fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) is limited due to tighter quotas, better fish management and more use of pelagic species directly as food. Furthermore, the production of fish oils cannot keep pace with the demands from the growing markets. This has led to an extensive search for alternative and sustainable sources of lipids containing omega-3 polyunsaturated fatty acids (n-3 LC-PUFA). In the pelagic system, phytoplankton are the main producers of n-3 LC-PUFA and make up the foundation of the oceanic food web. Zooplankton, such as copepods and krill, are the most numerous primary consumers in the marine environment and have a central role in the energy transfer to higher trophic levels. The copepod *Calanus finmarchicus* is present in large amounts in the North Atlantic and has lipid-rich stages that can be harvested in a sustainable manner.

The aim of this thesis was to provide knowledge which could contribute to the commercial utilization of *Calanus finmarchicus*. The wax ester-rich oil may be used as an alternative lipid source to fish oil in feeds for aquaculture, leading to good growth and efficient nutrient utilization. Moreover, the oil can be used as a health promoting nutraceutical as several recent publications indicate that oil from *C. finmarchicus* may have beneficial health effects beyond those which may be ascribed to the intake of EPA and DHA alone. In this work it was investigated if the use of commercial proteolytic enzymes could improve oil recovery from *C. finmarchicus* in an industrial-like process, and to characterize the oil obtained. The results showed a substantially higher oil yield with the use of proteolytic enzymes compared to standard fish oil production technology. The main components of the oil extracted from *C. finmarchicus* are monoesters of long-chain fatty acids and fatty alcohols, namely wax esters. In addition, the oil is rich in the deep red antioxidant astaxanthin present mostly as di- and monoesters. The fatty acid moiety of the wax esters consists of high quantities of stearidonic acid (SDA, 18:4n-3), EPA and DHA, but also a considerable amount of monounsaturated fatty acids, especially gondoic acid (20:1n-9) and cetoleic acid (22:1n-11). The final part of the thesis was to study the digestion of wax esters in mice fed a high fat diet supplemented with 2% Calanus® Oil. The findings confirmed that the mice were able to digest and absorb the Calanus® Oil, as the fatty acid composition of the adipose tissue and liver reflected the enrichment with the marine wax esters. Feeding mice a high fat diet supplemented with a small amount of wax ester-oil reduced the body weight gain, in line with recent published studies.

List of papers

Paper I Pedersen, A. M., Vang, B. and Olsen, R. L. (2014) Oil from *Calanus finmarchicus*—Composition and Possible Use: A Review, *Journal of Aquatic Food Product Technology, 23:6, 633-646*

Paper II Vang, B., Pedersen, A.M. and Olsen, R.L. (2013) Oil extraction From the Copepod Calanus finmarchicus Using Proteolytic Enzymes, *Journal of Aquatic Food Product Technology, 22:6, 619-628*

Paper III Pedersen, A. M., Salma, W., Höper, A. C., Larsen, T. S. and Olsen, R. L. (2014), Lipid profile of mice fed a high-fat diet supplemented with a wax ester-rich marine oil. *European Journal of Lipid Science and Technology, 116: 1718–1726*

The thesis was carried out as an industrial PhD financed by the biomarine company Calanus AS.

Error: In paper II (Table 3) the fatty acid 22:1n-11 (cetoleic acid) has wrongly been denoted 22:1n-9

Abbreviations:

AA	Arachidonic acid
ALA	α-linolenic acid
AV	Anisidine value
C1-C6	Copepodite stages 1-6
CE	Cholesteryl ester
CL /C	Cholesterol
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EE	Ethyl ester
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
eWat	Epididymal white adipose tissue
FAO	Food and Agricultural Organization of the United Nations
FAOH	Fatty alcohol
FFA	Free fatty acids
GC	Gas chromatography
HFD	High fat diet
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acids
LP	Lysophospholipids
MAG	Monoacylglycerol
MUFA	Monounsaturated fatty acid
N1-N6	Nauplii stages 1-6
PL	Phospholipids
PV	Peroxide value
pWat	Perirenal white adipose tissue
SDA	Stearidonic acid
SFA	Saturated fatty acid
spp	*species pluralis* , multiple species
TAG	Triacylglycerol
WE	Wax ester
WHO	World Health Organization

1. INTRODUCTION

A continuously growing body of evidence has shown positive health effects from consumption of seafood and marine lipids (Bang *et al.*, 1971; Bang *et al.*, 1976; Virtanen *et al.*, 2008; Schiepers *et al.*, 2010; Larsen *et al.*, 2011; Mozaffarian and Wu, 2011; Lund, 2013; Wójcik *et al.*, 2013). The positive effects on conditions like atherosclerosis, thrombosis, and embolic phenomenon, hypertriglyceridemia, hypertension, and autoimmune disease are generally related the long chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Uauy-Dagach and Valenzuela, 1996; Connor, 2000; Riediger *et al.*, 2009; Chang and Deckelbaum, 2013; Calder, 2015). Several health organizations such as FAO/WHO (2003), the American Heart Association (Lichtenstein *et al.*, 2006), the International Society for the Study of Fatty Acids and Lipids (Cunnane *et al.*, 2004) and several governmental agencies in France (Martin, 2001), United Kingdom (UK-SACN, 2004), USA and Canada (Kris-Etherton and Innis, 2007) have therefore made formal dietary recommendations for sufficient omega-3 fatty acid intake or to increase fish consumption. Even though the consumption of these fatty acids in foods now are strongly advised, the daily intake is generally far below the suggested quantities (Meyer *et al.*, 2003b; Ervin *et al.*, 2004; Calder, 2013). Dietary changes are often difficult to implement, and dietary supplements may for that reason function as an alternative source of these fatty acids. There are many different types of dietary supplements containing EPA and DHA available on the market, such as cod liver oils, whole fish body oils and products containing concentrated amounts of these fatty acids, primarily in the form of ethyl esters (EE) or as triacylglycerols (TAG).

The demand for marine lipids directly for human consumption and for fish oils for use in aquaculture feed have increased strongly in the last decade. The global use of fish oil during the past 50 years has changed considerably. From being mainly hydrogenated to margarines and used for industrial purposes, fish oil became the lipid source of choice for the growing aquaculture feed industry during the 1980's. By 2010, aquaculture had become the major consumer, using 71% of the global fish oil supply. However, the amount of fish oil refined for human consumption has also grown readily from 5% in 1990 to 24% by 2010 (Shepherd and Jackson, 2013). The traditional sources of the LC-PUFA through fish and fish oils are limited. The future sustainability of the global fisheries stocks is uncertain and there is evidence that many fisheries are already fully or over-exploited (FAO, 2014). Seafood supply

from aquaculture has risen over the past decades and depends on harvest of wild catch fisheries to meet the need for fish oil to be used in the feed (Naylor *et al.*, 2009). There is also an increasing competition between users of small pelagic forage fish as more are being used directly for human consumption instead of processing to fish oil and fishmeal (Olsen and Hasan, 2012). Worldwide capture fisheries have plateaued at about 85-95 million tonnes per year, even though fishing efforts have intensified (Naylor *et al.*, 2000). Consequently, the need for alternative sustainable LC-PUFA sources has led to extensive research in several fields. Emerging sources of these fatty acids include large-scale culturing of microalgae (Khozin-Goldberg *et al.*, 2011; Ratledge, 2011) and genetically modified oil seed plants (Venegas-Calerón *et al.*, 2010) and yeast (Xue *et al.*, 2013).

Primary production of LC-PUFA in the marine environment occurs in photosynthetic microalgae, heterotrophic protists, and bacteria (Monroig *et al.*, 2013). In the pelagic system, planktonic algae are the main producers of LC-PUFA (Dalsgaard *et al.*, 2003). Higher trophic organisms do not have the ability to efficiently synthesize these fatty acids and has adapted to obtaining them through their diet (Sargent *et al.*, 2002). Small crustaceans such as Antarctic krill (*Euphasia superba*) and copepods of the genus *Calanus* are the most numerous primary consumers in the marine environment and have a central role in the energy transfer to higher trophic levels (Garrison and Ellis, 2014). In the Nordic Seas, *C. finmarchicus* is the most important zooplankton by biomass, with an average annual standing stock of 70-80 million tonnes wet weight (Aksnes and Blindheim, 1996). This species accumulates large amounts of storage lipids in the form of wax esters, esters of long chain fatty acids and fatty alcohols, and is a central feed source for many of the commercially exploited fish species around the North Atlantic (Heath *et al.*, 2000; Melle *et al.*, 2004). However, only 10–20% of the energy is converted to biomass from one trophic level to the next (Parsons and Lalli, 1988). Therefore, harvest of such animals at lower trophic levels in a precautionary manner may provide a sustainable way to enhance marine supply of bioresources for fish feed, other industrial applications, as well as dietary supplements. Harvesting of zooplankton, *E. superba* and *C. finmarchicus,* is at present carried out predominantly for the production of oils rich in LC-PUFA and these are available on the nutraceutical market. While most studies on health effects so far have been published on krill oils (reviewed by Kwantes and Grundmann, 2015), several preclinical studies have recently demonstrated possible beneficial health effects from Calanus® Oil (Eilertsen *et al.*, 2012; Höper *et al.*, 2013; Höper *et al.*, 2014). According to these studies, the possible health effects may not only be related to the presence of LC-PUFA.

2. AIMS

The overall aim of this thesis was to provide knowledge which could contribute to commercial utilization of *Calanus finmarchicus*. The specific aims of the individual papers were:

Paper I: Elucidate the current knowledge of the lipids present in *Calanus finmarchicus* and assess the potential use of the oil extracted from this copepod and to study the oxidative stability of LC-PUFA and astaxanthin in the oil during a long storage period.

Paper II: Optimize the extraction process from the raw material by the use of proteolytic enzymes and further document the chemical composition of the oil produced.

Paper III: Study the digestion of wax esters by determining the lipid profile in liver, adipose tissue and feces of mice fed a high fat diet supplemented with 2% Calanus® Oil.

3. BACKGROUND

3.1 Biology of *Calanus finmarchicus*

Calanus finmarchicus is a central organism in the Norwegian Sea constituting the major fraction of the zooplankton biomass present (Planque and Batten, 2000). It is a relatively small herbivorous crustacean with a size of 3-4 mm, and a life span of one year in boreal waters (Diel and Tande, 1992). The copepod grazes on microplankton and is an important prey for fish larvae and fish such as herring and mackerel (Bauermeister and Sargent, 1979; Dalpadado *et al.*, 2000; Dommasnes *et al.*, 2004; Utne *et al.*, 2012) and also baleen whales and seabirds foraging at high latitudes (Place, 1992). During spring and summer *C. finmarchicus* spawns in the upper water layer where it feeds on the blooming phytoplankton. The new generation of copepods grow parallel to the blooming nutrients. They develop through twelve different stages, six naupliar (N1-N6), five copepodites (C1-C5) and finally a mature adult stage (C6) (Figure 1).

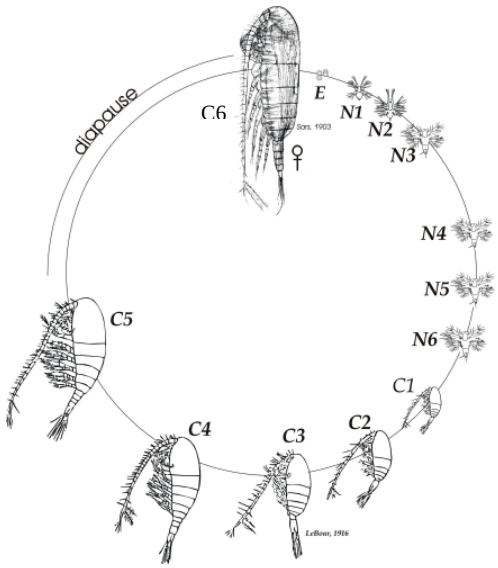

Figure 1: Life cycle of *Calanus finmarchicus*. The adult female spawns eggs that hatch and develop through six naupliar stages (N1-N6) and five copepodite stages (C1-C5), before reaching the final adult stage (C6) after winter hibernation, diapause. Adapted from Lebour (1916) and Sars (1903) by Baumgartner (2009).

By late summer and fall the copepods have reached C4-5 stages and have accumulated considerable lipid reserves in a membrane-bound oil sac that can extend the entire length of the prosome (Figure 2). These lipid reserves are composed entirely of wax esters (WE)

(Miller *et al.*, 1998). Wax esters have high calorific value, hence being an efficient energy store (Kattner and Hagen, 1995). The degree of unsaturation in the WE molecules can affect the physical properties, making vertical migration possible (Visser and Jónasdóttir, 1999), and it has now been recognized that WE phase transitions have an effect on buoyancy (Pond and Tarling, 2011). The lipid rich stages descends to the depths of 500-2000 m in mid to late summer and go in to hibernation, so called diapause, during the winter months (Lee *et al.*, 2006). In late winter and spring *Calanus finmarchicus* will mature, produce gonads and resurface, doing so completing its one year cycle.

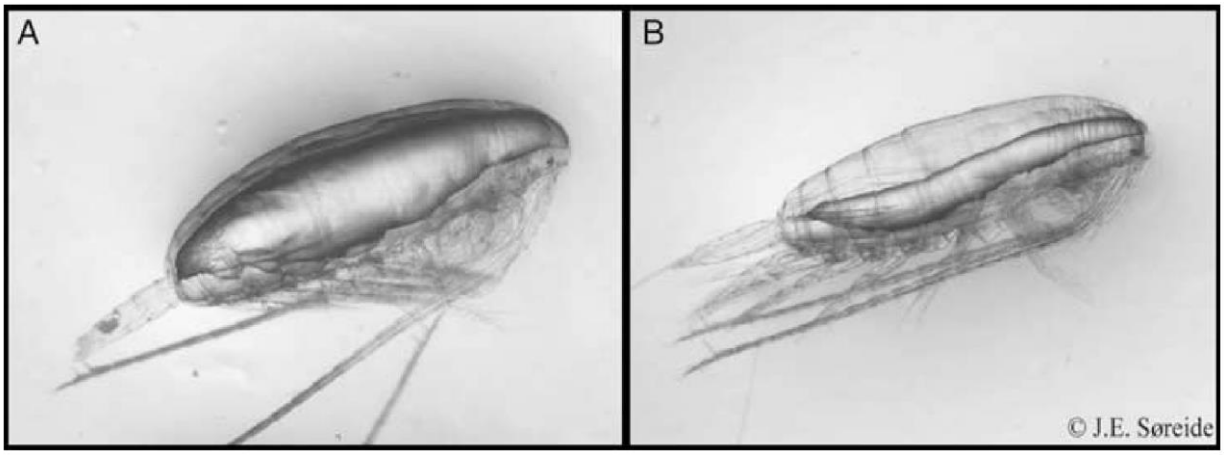

Figure 2: Photography of *Calanus* spp. specimens containing; (A) a well filled lipid sac, and (B) a thin and elongated lipid sac (Vogedes *et al.*, 2010).

3.2 Harvesting

The potential of zooplankton as marine resources in feed production, and for human consumption in general, is still largely untapped. However, plankton fisheries utilizing crustaceans have existed for many years in various parts of the world (Omori, 1978), though at relatively modest levels. In Norway as well as several other countries, there has been a growing interest for exploitation of marine zooplankton such as copepods and Antarctic krill (*Euphasia superba*). Furthermore, marine ingredients are also in demand by the functional food and dietary supplement industries. Krill fisheries, being the most prominent harvest of small crustaceans since the 1980's, are primarily taking place in the southern hemisphere around the Antarctic regions (Nicol and Endo, 1999) with annual landings of about 200.000 tonnes (Naylor *et al.*, 2009). Some commercial harvesting of *Calanus finmarchicus* has been conducted in Norwegian fjords since the late 1950's, with annual catches increasing from a few tonnes to more than 50 tonnes by the mid 1970's (Wiborg, 1976), limited by the lack of suitable harvesting technology and probably also market possibilities. However, sustainable harvesting technology has recently been developed and implemented for practical use (Angell *et al.*, 2010). In 2006, a general prohibition against harvesting of zooplankton was introduced as a precautionary measure. At present, the Norwegian Ministry of Fisheries and Coastal Affairs (FKD) is endorsing an experimental harvesting of copepods in Norwegian waters. Norwegian Ministry of Fisheries and Coastal Affairs has opened for a trial quota for several vessels in order to gain knowledge to build a management and regulatory system for harvesting this resource. Consequently, a consensus has been obtained between central authorities, funding bodies, R&D institutions, and industry that zooplankton *Calanus* spp. both can and should be exploited.

The harvesting areas are located along the Norwegian coast as well as in open waters off the coast of Norway. The zooplankton is harvested by trawling with fishing vessels using fine-meshed trawls in combination with so-called bubble flotation to vertically concentrate the copepods in the surface layer (Grimaldo *et al.*, 2011). By-catch is generally low or absent due to the harvesting technique as well as knowledge of the location of adult *Calanus finmarchicus* where there are no fish larvae as they usually graze on smaller stages of the copepod (Klungsøyr *et al.*, 1989; Heath and Lough, 2007).

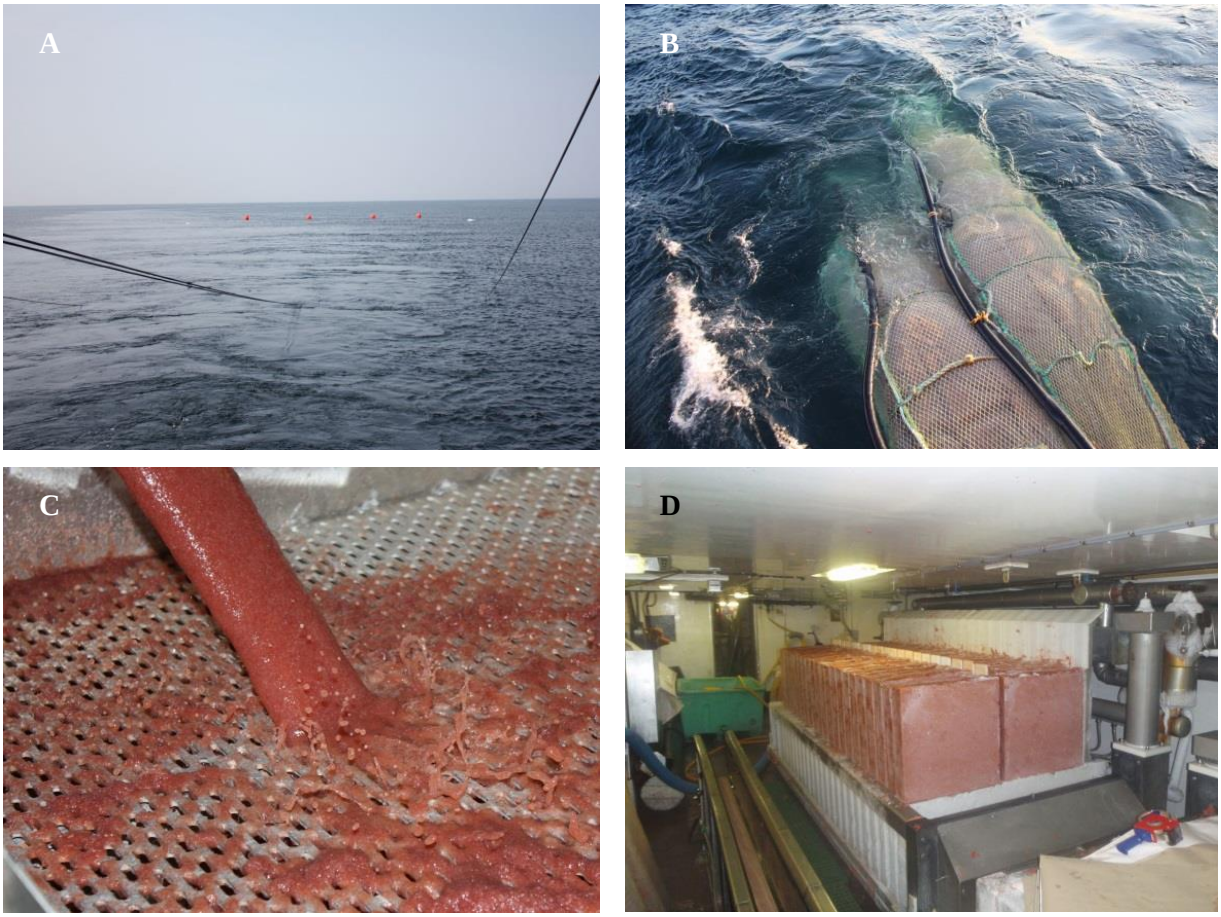

Figure 3: Photographs (A) and (B) show a towing system for harvesting *Calanus finmarchicus,* the catch is pumped on board (C) and immediately frozen (D) (photographs by Snorre Angell and Trond Larsen, Calanus AS).

The harvesting takes place in areas where the stocks of adult and juvenile planktivorous fish are low, and the scooping nets are being hauled at such low speed allowing adult fish and juveniles to escape. Immediately after each haul, the catch is frozen and stored in the standard freezing facilities on board (Figure 3). The raw material is brought ashore and freeze-stored until processing.

3.3 Processing

There are a variety of methods that can be used to produce meal and oil from marine biomass. These include wet rendering, hydrolysis, silage production (autolysis), dry rendering, supercritical fluid extraction and solvent extraction. The wet rendering process is used in the majority of factories that produce fish oil and fishmeal worldwide (Bimbo, 2012). The principal operations are cooking, pressing, separation of the liquid phase with recovery of the oil, and drying of the residual protein material (FAO, 1986) (Figure 4).

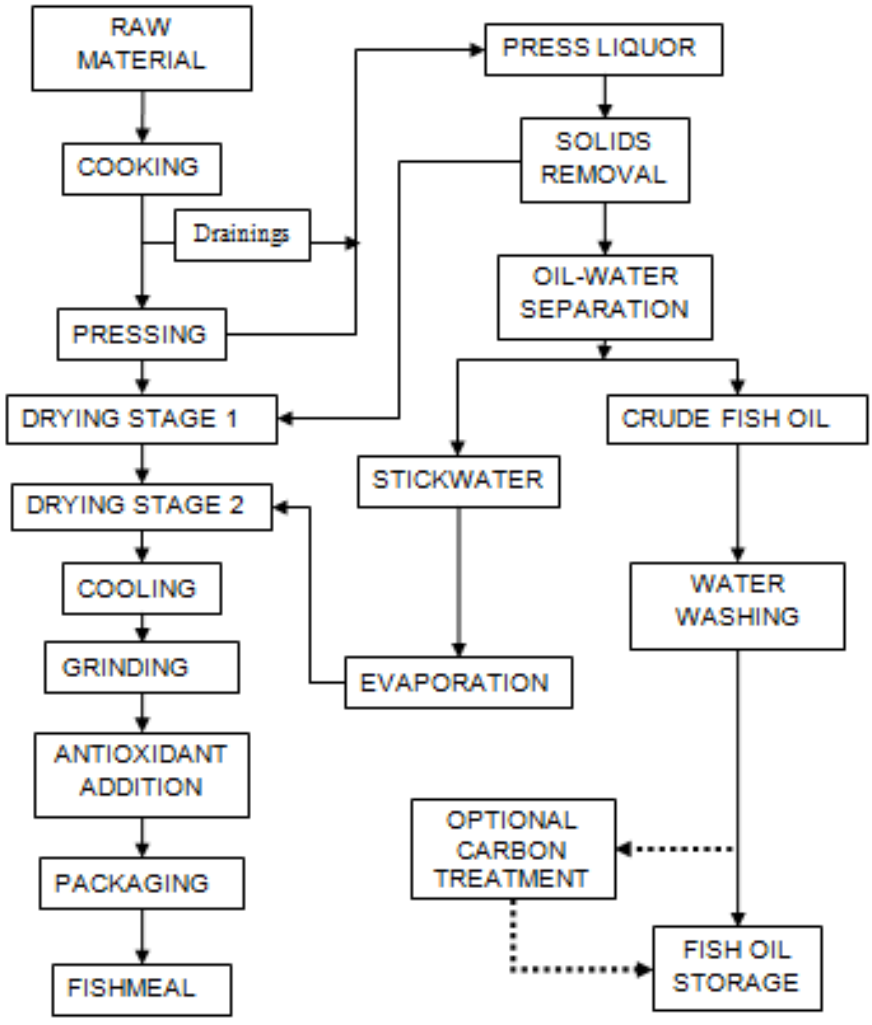

Figure 4: Simplified flow diagram of the wet rendering process for production of fish oil and fishmeal (Bimbo, 2012).

Cooking denatures the protein and makes it possible to extract the lipids by pressing. As the proteins coagulate to a firm mass, it is capable to withstand the pressure required to press out the liquid consisting of stickwater and oil. Cooking will also rupture the fat cells,

releasing the oil into a more fluid state. The temperature of this step will lower the oil viscosity, allowing it to flow more readily through the press. During coagulation, a high proportion of the bound water is liberated and deposits of lipids are released from the tissues. Pressing mechanically expresses free liquid from the solids producing a press juice (liquor) and a press cake (Drying stage 1). The separation process is made up by three steps; decanters will separate fine solids (sludge) from the press juice, separators split the liquid fraction into crude fish oil and stickwater. The third part of the process involves polishing (water washing) and the removal of the last traces of moisture and impurities from the oil. Simultaneously, sludge from the press juice, press cake and concentrated stickwater are most often mixed together, dried and grinded to a meal.

The use of enzymes in industrial processes may be utilized as a supplement to the traditional production methods in cases where the oil yield is low (Rubio-Rodríguez *et al.*, 2010). This extraction technology can be easily done and is less expensive regarding investment and energy cost as it requires neither organic solvents or high temperatures (Rolle, 1998). Enzyme-assisted hydrolysis in fish oil production uses proteases to degrade the tissue structure, as proteins are the main components that prevent the release of oil from fish tissue. Several commercial proteases are available and studies have shown that enzymatic degradation of fish by-products can replace the cooking stage or minimize the cooking time or temperature (Xu *et al.*, 2007) in addition rendering both a high quality oil and protein fraction (Rustad *et al.*, 2011; Carvajal *et al.*, 2015). It has also been claimed that gentle processing of salmon by-products using enzymatic hydrolysis may provide a better oil than thermal treatments and is comparable with solvent extraction when it comes to yield (Gbogouri *et al.*, 2006).

At this point, the final product of the extraction process is unrefined (crude) oil that often contains unwanted compounds. Some of these impurities may affect the quality of the oil, such as free fatty acids (FFA) and oxidation products, but also potentially harmful substances such as polychlorinated biphenyls (PCB) and dioxins may be present in the crude oil. These are resistant to environmental degradation, and have shown to accumulate in the fatty tissues of organisms and biomagnify across trophic levels (Borgå and Di Guardo, 2005). For that reason, it is often necessary to include a refining process before obtaining an edible oil (Bimbo, 2012). However, the concentration of such contaminants are significantly lower in the more primary sources of marine oils, namely algae and zooplankton (AMAP, 2002). The short lifespan of *C. finmarchicus* also contributes to the low levels of contaminants.

3.4 Lipids in *Calanus finmarchicus;* Composition and synthesis

In most fish oils, nearly all of the fatty acids are esterified in TAG. In krill oil (*E. superba*) they are esterified in phospholipids (PL) and TAG, reported at about 44% and 40%, respectively (Yurko-Mauro *et al.*, 2015). In contrast, the lipids from *C. finmarchicus* occur mostly as WE where fatty acids are esterified to long chain fatty alcohols (Figure 5).

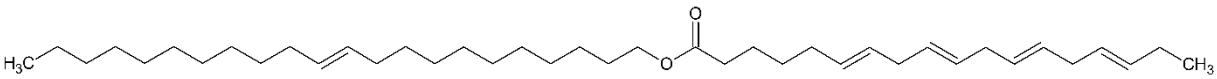

Figure 5: A typical wax ester present in lipids of *C. finmarchicus*, consisting of the long-chain fatty alcohol docosenol (22:1n-11), and the fatty acid stearidonic acid (18:4n-3).

The WE content has been found to be as high as 80-90% of the total lipids, while TAG, PL, sterols and FFA are minor constituents (Table 1). Cholesterol is by far the predominant sterol as it is an indispensable structural component of cell membranes. Still, phytosterols such as brassicasterol, campesterol, stigmasterol and β-sitosterol are present in lower proportions as dietary precursors of cholesterol (Martin-Creuzburg and von Elert, 2009). The total amount of lipids and wax esters in calanoid copepods are dependent on the latitude and the highest quantities are found in Arctic and Antarctic species. This is because the temperature in the ocean is low and the primary production occurs with a high intensity during a short period of time (Lee *et al.*, 2006). Typical WE-rich polar species exist both among copepods and krill species, such as *Calanus finmarchicus* (Diel and Tande, 1992*)*, *Calanus hyperboreus* (Lee, 1974) and *Euphausia crystallorophias* (Bottino, 1975).

Table 1: Lipid class composition of late copepodite stages and adult *C. finmarchicus* sampled in different periods and presented as % total lipids.

Lipid class	% of total lipid			
	June[1]	October[1]	March[1]	March[2]
Triacylglycerols	8,9	1,3	nd	3,1
Sterols	1,2	2,6	3,2	1,4
Free fatty acids	0,2	--	1,7	nd
Wax esters	85,4	88,1	84,9	73,8
Phospholipids	4,2	7,3	10,3	21

Source: [1] Falk-Petersen *et al*. (1987), [2] Fraser *et al.* (1989).

The structure of WE and the amount of unsaturated fatty acids and alcohols result in physical properties different from that of TAG containing similar fatty acids (Lee and Patton, 1989). Elevated levels of FFA have been reported in some publications to be present in zooplankton lipids, and based on the fatty acid composition, it has been suggested to be due to enzymatic breakdown of phospholipids (Sargent and Falk-Petersen, 1981; Parrish, 1988; Scott *et al.*, 2000; Marker *et al.*, 2003). This may occur *post mortem*, while others have suggested that certain development stages of zooplankton may have a high content of FFA due to intensive feeding activities (Scott *et al.*, 2000; Marker *et al.*, 2003).

The WE in *Calanus hyperboreus* have even numbered chain lengths mainly in the range of 30-44 carbon atoms, with C36-C42 as the dominating lengths (Sargent *et al.*, 1976). The WE in *Calanus finmarchicus* are of comparable size (F. Torres, personal communication, 2013). This is quite similar to the chain lengths found in the WE in fillets of orange roughy (*Hoplostethus atlanticus*) and slightly longer than the waxes present in mullet roe and sperm whale head oil (Table 2). Beeswax and WE from jojoba oil have more long-chained forms.

Table 2: Wax ester chain-length composition (wt %) in lipids from some marine and terrestrial sources

Chain length	26	28	30	32	34	36	38	40	42	44	46	48	50
C. hyperboreus[1]	--	--	3	7	7	20	21	17	20	4	--	--	--
Mullet roe[1]	--	--	5	23	18	12	12	2	--	--	--	--	--
Sperm whale[1]	2	6	11	18	25	20	8	--	--	--	--	--	--
Orange roughy[2]	--	--	--	2,1	11,4	16,7	24,8	23,4	14,8	5,5	1,1	--	--
Jojoba oil[2]	--	--	--	--	--	1,6	6,2	30,6	49,5	8,1	1	--	--
Bees wax[3]	--	--	--	--	--	--	--	15	11	14,4	32,2	48	6,3

Source: [1] Sargent *et al.*(1976) , [2] Buisson *et al*. (1982), [3] Hamilton (1995).

There is presumably an asymmetrical distribution of the fatty alcohols and fatty acids in calanoid WE (Sargent and Henderson, 1986). Long-chain monounsaturated fatty alcohols are esterified mostly to short-chain acids, and medium-chain saturated fatty alcohols are esterified mostly to PUFA, probably related to their phase transition temperatures (melting points). Fatty alcohols generally have higher transition temperatures than fatty acids, long-chain moieties have higher transition temperatures than short chain moieties, and transition temperature decrease in the order saturated > monounsaturated > polyunsaturated (Scrimgeour and Harwood, 2007). Coupling long-chain units with short-chain units or saturated medium-chain units with polyunsaturated medium-chain units are mechanisms by which those units that have intrinsically high phase-transition temperatures are maintained in

a liquid state at relatively low temperatures. In this way, *C. finmarchicus* is capable of accumulating saturated and long-chain monounsaturated fatty alcohols with high melting points at low ambient temperatures (about 2°C in high latitudes) by "fluidizing" high melting–point units such as 20:1 and 22:1 with low melting–point units such as polyunsaturated fatty acids (Sargent and Henderson, 1986).

The wax esters of *C. finmarchicus* contain fatty alcohols that are mainly monounsaturated. The fatty alcohols eicosenol (20:1n-9) and docosenol (22:1n-11) may constitute 62-82% of the total fatty alcohols, while the saturated alcohols tetradecanol (14:0) and hexadecanol (16:0), may make up from 8-24% (Table 3). The fatty alcohols derive preferentially from *de novo* biosynthesis of the corresponding fatty acids and subsequently the reduction of the fatty acids to fatty alcohols (Dalsgaard *et al.*, 2003; Graeve *et al.*, 2005). The concentration of the monounsaturated fatty acids may be as high as 50%, with palmitoleic acid (16:1n-7), oleic acid (18:1n-9), gondoic acid (20:1n-9), and cetoleic acid (22:1n-11) as the major contributors. Erucic acid (22:1n-9) is present only in minor quantities. The saturated fatty acids, primarily myristic acid (14:0) and palmitic acid (16:0), amount to 20-35% of the total fatty acids present in the wax esters. The content of polyunsaturated n-3 fatty acids in the wax ester may account for 20-30% of the fatty acids, with stearidonic acid (18:4n-3) and eicosapentaenoic acid (20:5n-3) as the dominating species (Table 3). Phytoplankton fatty acids are incorporated unmodified into zooplankton storage lipids, therefore the fatty acids found in the wax esters of *C. finmarchicus* are largely reflected by the fatty acid composition of the phytoplankton (Lee *et al.*, 1971; Kattner, 1989).

Table 3: Fatty acid and fatty alcohol composition (mass %) of wax esters in *Calanus finmarchicus*, late copepodite stages and adults.

Location:	Fram Strait	Balsfjord			Loch Thurnaig
Fatty acids:	June-August[1]	June[2]	October[2]	March[2]	March[3]
14:0	26,3	18,4	8,5	6,7	12,4
16:0	9,8	7,2	15,5	11,8	11,4
16:1 n-7	6,7	12,2	11,9	9,5	8,4
18:0	0,9	0,5	1,2	1,1	0,5
18:1 n-9	5,3	4	6,1	7,3	2,4
18:1 n-7	0,3	0,5	0,8	0,8	1,6
18:2 n-6	1,2	2,2	1,7	1,4	2,8
18:3 n-3	1,5	1,4	1,6	nd	2,7
18:4 n-3	13,7	22,5	4,8	1,2	13,7
20:1 n-9	7,8	8,9	9,6	14,2	8
20:5 n-3	11,4	6,4	10,7	7,1	6,3
22:1 n-11	7	11,8	13,6	19,4	15,1
22:1 n-9	0,2	0,8	0,9	1,4	nd
22:6 n-3	2,2	2,6	2,1	4,6	2,2
Others	5,7	0,6	11,0	13,5	12,5
∑ SFA	37.0	26,1	25,2	19,6	24,3
∑ MUFA	20,6	38,2	42,9	52,6	35,5
∑ n-3 PUFA	28,8	32,9	19,2	12,9	24,9
Fatty alcohols:					
14:0	3,9	6,4	1,3	0,5	1,2
16:0	14,6	17,8	8,9	7,9	9,8
16:1 n-7	3,4	2,5	2,6	1	1,5
18:0	nd	1,1	0,6	nd	0,7
18:1 n-9	nd	3,7	2,7	2,2	4,9
18:1 n-7	nd	1,6	1,7	1,1	nd
18:2 n-6	nd	4,7	1,4	0,9	nd
18:3 n-3	nd	2,6	1,3	1	nd
20:1 n-9	39,3	40,5	34,1	33,8	23,3
22:1 n-11	38,8	17,7	40,6	48,6	45,3
22:1 n-9	nd	1,2	1,4	1,1	nd
Others	nd	0,2	3,4	1,9	13,3

nd = not detected, SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids. *Source*: [1]Albers *et al*. (1996), [2]Falk-Petersen *et al.* (1987), [3] Fraser *et al.* (1989).

Only plants are capable of biosynthesising omega-3 and omega-6 *de novo* and consequently these fatty acids are essential nutrients for all higher species. Unlike animals, the primary producers possess the enzymes Δ12- and Δ15-desaturase, which enables them to introduce a double bond between the existing double bond in the Δ9 position and the terminal methyl group (Figure 6).

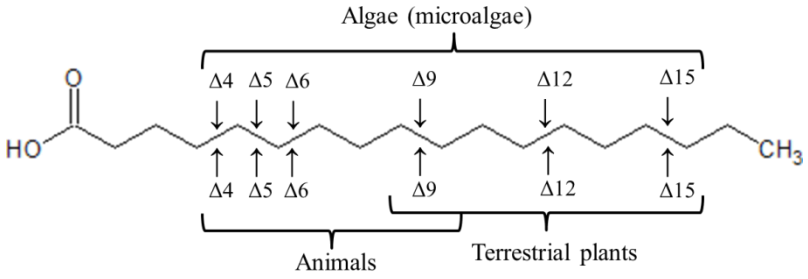

Figure 6: Positions of fatty acyl chain desaturation by enzymes of animals including fish, terrestrial plants and algae (microalgae) modified from Cook and McMaster (2002).

Double bonds are inserted to form linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), the parent molecules for the longer chain omega-6 and omega-3 fatty acids (Figure 7). Through the combined actions of Δ6- and Δ5- desaturase and 2-carbon unit chain elongations, LA may be converted further to arachidonic acid (AA, 20:4n-6) and ALA to SDA, EPA and DHA. Most carnivorous marine fish have lost the Δ6- desaturase activity, probably as an evolutionary adaptation due to the high accessibility of EPA and DHA through their diet (Tocher, 2003). In microalgae, the final steps to the formation of DHA is mainly via docosapentaenoic acid (DPA, 22:5n-3), and subsequently the introduction of the last double bond by a Δ4- desaturase (Meyer *et al.*, 2003a). In mammals and fish, the conversion to DHA via DPA is the result of a more complicated series of reactions that involve the elongation to a C24 fatty acid, a second Δ6-desaturation, and the final chain shortening (β-oxidation) in the peroxisomes, the so-called Sprecher pathway (Sprecher *et al.*, 1995; Tocher, 2003). However, the conversion of α-linolenic acid to LC-PUFA is rather inefficient in humans due to the rate-limiting Δ6- desaturase, with very limited conversion all the way to DHA (Arterburn *et al.*, 2006; Calder, 2013). Stearidonic acid (SDA, 18:3n-3), the first metabolite formed directly from ALA is present in considerable amounts in the oil from *C. finmarchicus*. This fatty acid is by far more efficiently converted to EPA than ALA and has been referred to as being a "pro-EPA" fatty acid (Whelan, 2009), therefore, the direct dietary intake of SDA has been proposed as another strategy to increase tissue EPA levels (Harris, 2012). Some plants, such

as the genus *Echium* and *Primula* have Δ6- desaturase activity, resulting in the presence of SDA in the seed oil (Sayanova *et al.*, 1999; Guil-Guerrero, 2007). SDA may also be produced in genetically modified canola and soybean plants (Whelan, 2009).

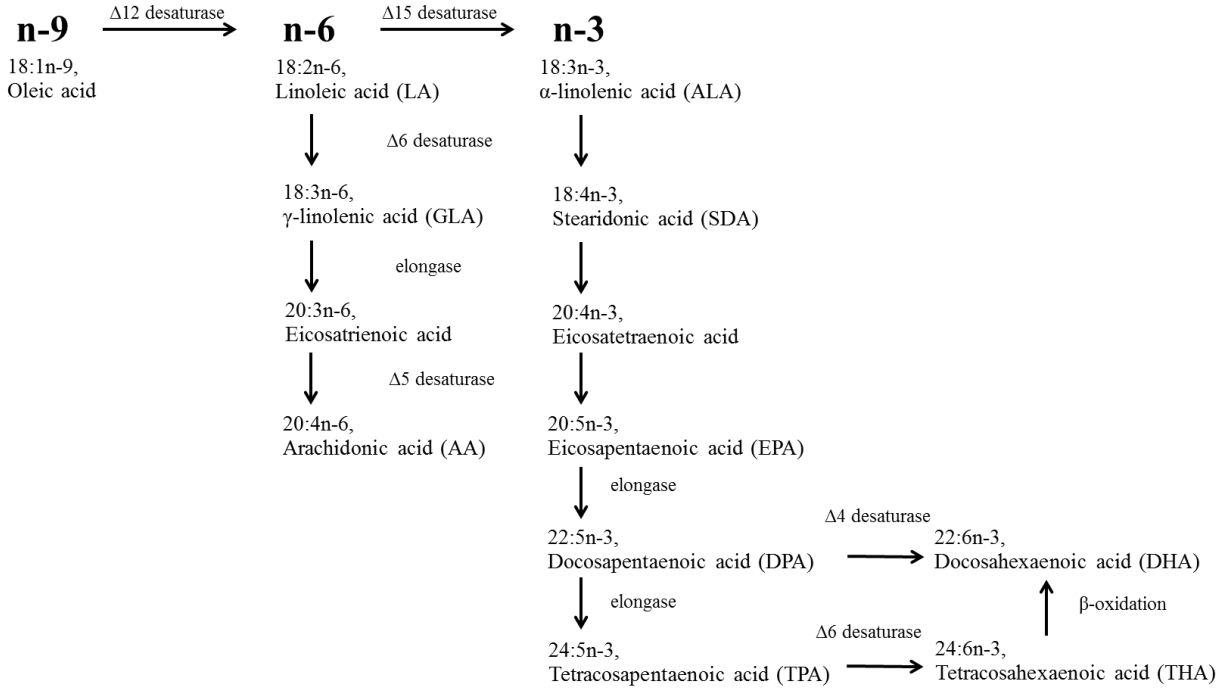

Figure 7: Simplified outline of the biosynthesis of omega-6 and omega-3 polyunsaturated fatty acids (Scrimgeour and Harwood, 2007).

The omega-6 and omega-3 families are metabolically and functionally distinct, and often have important opposing physiological functions. In addition they compete for the same enzymes in the synthesis of LC-PUFA (Figure 7). Excess of one fatty acid family can interfere with the metabolism of the other, significantly reducing its conversion and thereby the biological actions of the metabolites (Simopoulos, 2002). Consequently, the high amounts of omega-6 fatty acids consumed through the so-called "Western diet", the eicosanoid metabolic products from AA are formed in larger quantities than those formed from EPA (Simopoulos, 2006). In general, AA-derived eicosanoids are proinflammatory, but they have important homeostatic functions in regulating the promotion and resolution of inflammation in the immune response (Ricciotti and FitzGerald, 2011). In contrast, omega-3 PUFA and their long-chain derivatives mostly promote anti-inflammatory activities. The consumption of SDA, EPA and DHA may therefore be beneficial in different pathologies like cardiovascular

disease, rheumatoid arthritis, diabetes mellitus and neurological diseases, many of which are related to inflammation (Calder, 2006). Furthermore, resolvins, protectins and maresins are newly discovered families of highly potent mediators with inflammation-resolving properties derived from omega-3 PUFA, adding to the insights of the important, and diverse biological roles of LC-PUFA (Zhang and Spite, 2012).

The common name "red feed" reflects the red colour of *C. finmarchicus*, which is due to the large quantity of the lipophilic carotenoid astaxanthin (Figure 8). In zooplankton, astaxanthin is the most commonly occurring carotenoid and may contribute to as much as 85–90% of the total pigment (Funk and Hobson, 1991). Copepods utilize β-carotene, obtained from phytoplankton, as a precursor for astaxanthin synthesis (Andersson *et al.*, 2003). The specific structure of the astaxanthin molecule provides its ability be esterified, a higher antioxidative capacity and a more polar configuration than other carotenoids (Guerin *et al.*, 2003). Free astaxanthin is particularly sensitive to oxidation and as a result, in nature, it is found either conjugated to proteins; as carotenoproteins, or esterified with one or two fatty acids, which stabilize the molecule (Matsuno, 2001). It has been proposed that one of the central functions of astaxanthin (esters) in calanoid copepods is to improve antioxidant protection of storage lipids (Sommer *et al.*, 2006). Also, astaxanthin in copepods have been suggested to take part in lipid metabolism and serve as both photoprotection and/or camouflage (Hairston, 1976; Hansson, 2000). Lipids extracted from *C. finmarchicus* may contain as much as 500-1600 ppm astaxanthin (Pedersen, 2007; Bergvik *et al.*, 2012) and up to 90 % of the total pigment in the form of diesters and monoesters (Foss *et al.*, 1987). Astaxanthin is widely used in cosmetics, as food colorants and feed additives in aquaculture to colour the flesh of salmonid fish, or to enhance the colour of egg yolk in the poultry industry (Akiba *et al.*, 2000; Dufossé, 2006; Chimsung *et al.*, 2014). Additionally there has been a growing interest in the use of astaxanthin as a dietary supplement (Ambati *et al.*, 2014) owing to its possible health-promoting effects (reviewed by Hussein *et al.*, 2006; Yuan *et al.*, 2011).

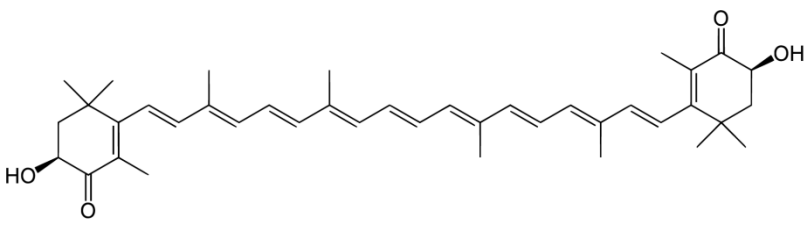

Figure 8: A general structure of unesterified astaxanthin 3S, 3'S molecule

3.5 Oxidation

Oxidation plays a fundamental role in the reduction of the quality of lipids. It deteriorates the sensory quality and nutritive value, and may ultimately lead to the production of toxic compounds (Min and Boff, 2002). Lipids may be oxidized during processing and storage via autooxidation or light induced oxidation (photooxidation), in which triplet oxygen ($^3O_2$) and singlet oxygen ($^1O_2$) react with the lipids, respectively (Choe and Min, 2006). Marine oils and products with a high content of long chain polyunsaturated fatty acids are particularly susceptible to oxidation (Van Dyck, 2007). Enzymatic oxidation is a third mechanism, where lipid oxidation is catalysed by enzymes (e.g. lipooxygenases) in the raw material, however, during oil processing, the high temperatures will efficiently inactivate any enzymes present (Oterhals and Vogt, 2013).

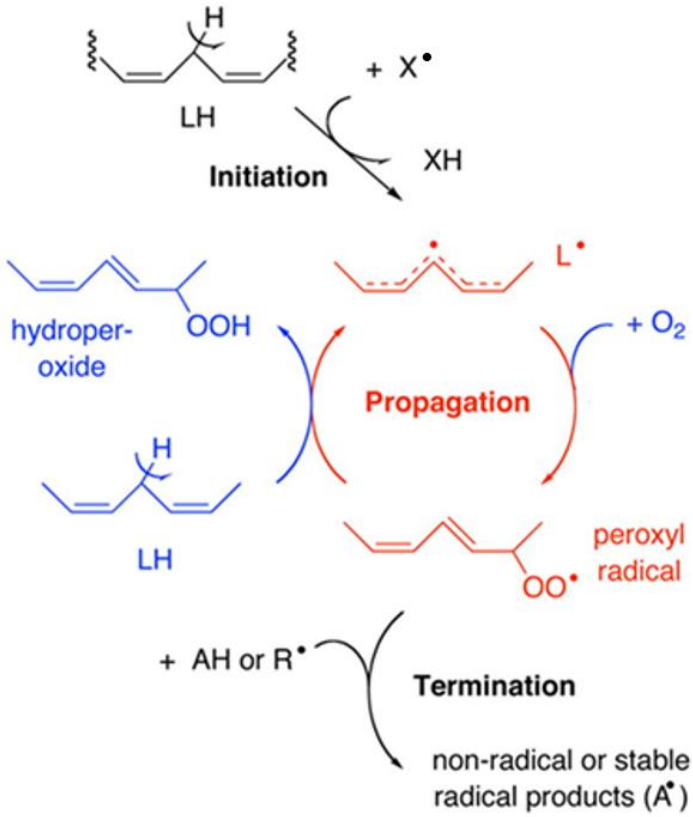

Figure 9: Overview of the autooxidation process; the initial removal of hydrogen **(Initiation)** and the formation of an alkyl radical (L•) via a radical initiator (X•). Oxygen and fatty acids are added to the cycle and give hydroperoxides (LOOH) as the product via electron donation from the peroxyl radical (LOO•) **(Propagation)**. The process ends **(Termination)** when either two radicals react and form a non-radical product, or an antioxidant (AH) reduces the peroxyl to hydroperoxide while being transformed to a stable radical (A•) without the formation of an alkyl radical (L•). Modified from Schneider (2009)

Autooxidation is the direct reaction of molecular oxygen with organic compounds (Frankel, 2005) and is considered the most important mechanism of the oxidation of omega-3 LC-PUFA (VKM, 2011). It involves a chain reaction consisting of three distinctive steps: initiation, propagation and termination (Figure 9). At the initiation step, a hydrogen atom is removed from the molecule LH by a radical initiator (X•) and a free radical of a fatty acid, alkyl radical (L•) is formed. A hydrogen bound to the carbon atom separating two non-conjugated C=C bonds is the easiest to remove, rendering the PUFA more vulnerable to oxidation compared to saturated and monounsaturated fatty acids. Alkyl radicals may also be formed by thermal cleavage or due to chemical oxidizers (reactive oxygen species, ROS), or by transition metals such as iron (Fe^{3+}/ Fe^{2+}) and copper (Cu^{2+}/Cu^{+}). These metals may influence the initiation by the direct reaction with a fatty acid (Equation 1).

$$Fe^{3+} + LH \rightarrow Fe^{2+} + L\bullet + H^{+} \quad \text{(eq.1)}$$

In the propagation step, the alkyl radical will react with molecular oxygen to form peroxyl radical (LOO•), which again is capable to remove hydrogen from another fatty acid and form lipid hydroperoxides (LOOH) and at the same time a new alkyl radical (Figure 9). In the presence of light and oxygen can photosensitizers (e.g. chlorophyll) convert triplet oxygen to singlet oxygen, which is highly reactive, and will bind directly to the fatty acid (Equation 2). The hydroperoxides formed by photooxidation may serve as initiators of the autooxidation process (Knothe *et al.*, 2007).

$$LH + {}^{1}O_2 \rightarrow LOOH \quad \text{(eq. 2)}$$

Some oxidation will occur during processing of fats and oils, and consequently, lipid hydroperoxides are present in essentially all lipid-containing foods to a certain degree. With the presence of transition metals, LOOH will decompose, and give rise to a group of alkoxyl (LO•) and peroxyl (LOO•) radicals (Equations 3 and 4). Subsequently, these will be capable of re-initiating lipid oxidation by redox-cycling of the metal ions (McClements and Decker, 2008).

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO\bullet + OH^{-} \quad \text{(eq. 3)}$$

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO\bullet + H^{+} \quad \text{(eq. 4)}$$

These transition metals may also catalyse the oxidation of hydrogen peroxide (HOOH) and form the highly reactive hydroxyl radical (OH•). The hydroxyl radical will immediately remove electrons from any molecule in its path, turning that molecule into a radical and so

propagating the chain reaction or act as an initiator forming a new alkyl radical. The termination phase occurs when two radicals react and form a non-radical, usually high molecular weight products such as dimeric and trimeric triacylglycerols (i.e. polymeric compounds) (Frankel, 2005). The reaction can also be inhibited if a peroxyl radical react with an antioxidant (AH) to form an unreactive free radical (A•), which does not remove hydrogen from another fatty acid (Figure 9).

The primary products of the autooxidation are taste- and odourless lipid hydroperoxides (LOOH), traditionally quantified by measuring the peroxide value (PV). These molecules will decompose further, giving rise to secondary oxidation products, such as aldehydes, ketones, alcohols, keto acids, hydroxyl acids, epidioxides, and other volatile compounds (Bartosz and Kolakowska, 2011). The decomposition of hydroperoxides to alkoxyl radicals (LO•) such as shown in equation 3, is generally followed by the β-scission reaction (Figure 10). This reaction breaks down the aliphatic chain of the fatty acid to produce shorter chain aldehydes and alkyl radicals. The alkyl radical can then react with a hydrogen radical to form a hydrocarbon, a hydroxyl radical to form an alcohol or oxygen to form a new hydroperoxide (McClements and Decker, 2008). Due to the several double bonds in the omega-3 LC-PUFAs, the decomposition of the omega-3 fatty acid hydroperoxides will lead to a highly complex mixture of secondary oxidation products (Jackobsen and Nielsen, 2007) and subsequently a decrease in the content of LC-PUFA. However, it is only the secondary, volatile oxidation products which are responsible for the changes in sensory properties causing the unpleasant odours and flavours from lipid oxidation (Jacobsen, 1999). The content of secondary oxidation products is traditionally expressed by the anisidine value (AV), which is given without any unit, but can give an impression of the oxidation status of oil at the time of analysis (VKM, 2011). The complexity of lipid oxidation is reflected by the array of oxidation products formed throughout the different stages of the oxidation process and furthermore, the variety of analytical methods developed to study lipid oxidation (reviewed by Barriuso *et al.*, 2012).

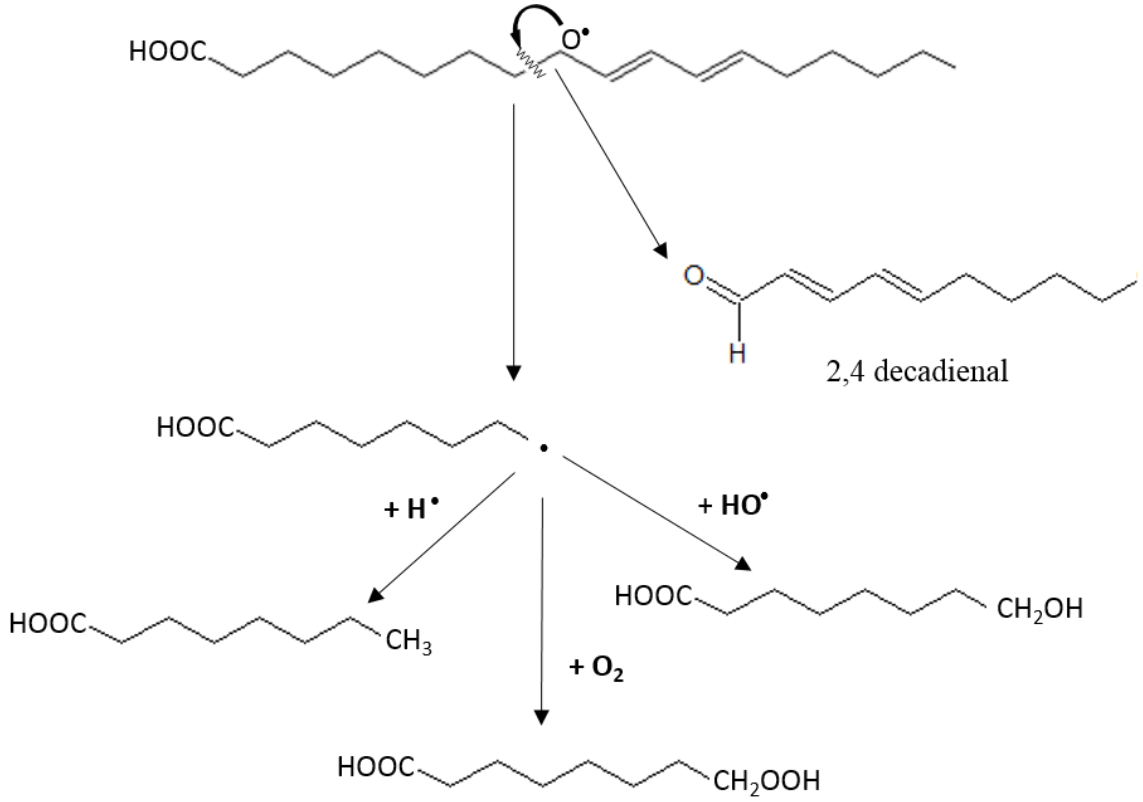

Figure 10: Possible reaction pathways for the decomposition of an alkoxyl radical by β-scission, forming an aldehyde and either a new hydrocarbon, alcohol or hydroperoxide. Modified from Frankel (2005) and McClements and Decker (2008).

Access to oxygen and light, surface area, heating, and irradiation will affect the rate of lipid oxidation. Thus, oxidation can be inhibited to a certain extent by several actions; the removal of oxygen during storage, low storage temperatures, avoidance of light and contact with metals such as iron and copper. Secondly, to improve oxidative stability, natural or synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) or vitamin E (tocopherols and tocotrienols) may be added to crude and refined oils (Knothe *et al.*, 2007). As mentioned previously, lipids extracted from *C. finmarchicus* contain high amounts of astaxanthin esters. Astaxanthin and similar carotenoids are known to possess several antioxidative abilities, such as lipid peroxyl radical-trapping, quenching of singlet oxygen and neutralizing of photo-sensitizers (Higuera-Ciapara *et al.*, 2006). When exerting these effects, the carotenoids themselves will eventually oxidize, and for example concentrated astaxanthin will lose its ruby colour (Halliwell and Gutteridge, 2007).

3.6 Lipid digestion

Lipids are a major source of metabolic energy, a source of essential fatty acids and lipid-soluble vitamins, and are vital components of biological membranes (Vance and Vance, 2008). The lipids provided in the human diet consist primarily of TAG (90-95 %), with smaller contributions from phospholipids and cholesterol (Gurr *et al.*, 2002). The complex mixture of dietary lipids needs to be broken down before absorption, and the human digestive system is very efficient, utilizing more than 95 % of the lipids provided (Carey *et al.*, 1983; Mu and Høy, 2004). The average consumption rate of TAG is about 100-150 g daily, while the consumption of exogenous cholesterol and phospholipids are estimated to 300-600 mg and 2-8 g per day, respectively (Gropper *et al.*, 2009; Cohn *et al.*, 2010). There is no exact data on the amount of wax esters in the human diet; however, WE are present in several foods such as cereal grains, bran, and germ, along with leaves, seeds, nuts and unrefined oils (Hargrove *et al.*, 2004). Wax esters in seafood are found in a number of caviar and fish roe products (Bledsoe *et al.*, 2003; Kalogeropoulos *et al.*, 2008), as well as in the fillets from the commercial fish species orange roughy and deep-sea oreo (Bakes *et al.*, 1995). In addition beeswax, candelilla and carnauba wax are extensively used as food additives; such as glazing agents, surface treatment on fruit and as flavour and colour carriers (EFSA, 2007; 2012a; 2012b). Outbreaks of so-called keriorrhea upon ingestion of large servings of WE-rich fish, have unfortunately led to the notion that WE is a poor substrate for digestion or even indigestible (Ling *et al.*, 2009). Conversely, several publications demonstrate that mammals are capable of wax ester digestion, at least, when consumed in relatively moderate amounts (Hansen and Mead, 1965; Yaron *et al.*, 1982; Gorreta *et al.*, 2002).

The digestive and absorptive processes primarily takes place in the small intestine, but lipid digestion is initiated by lingual (mouth) and gastric (stomach) lipases. Dietary lipids are dispersed to lipid droplets by the mechanical actions of chewing, and subsequently churning and peristaltic movements throughout the gastrointestinal system (Sherwood, 2006). Digestion occurs within an aqueous environment, and as most lipids are hydrophobic, it will require some facilitation to make them available for hydrolysis and transportable for absorption. Bile salts are amphipathic molecules, produced from cholesterol in the liver and are released from the gallbladder into the small intestine upon lipid digestion. They will exert a detergent action on fat droplets and emulsify them into smaller units. This provides a greater surface for the action of lipolytic enzymes (Figure 11). Wax esters are more hydrophobic than

TAG and therefore more difficult to emulsify, as a result, WE may exhibit a longer retention time to facilitate hydrolysis and absorption (Cowey and Sargent, 1977; Verschuren and Nugteren, 1989). The main digestive enzymes involved in breaking down dietary lipids are esterases that cleave the ester bonds in TAG (pancreatic lipase, EC 3.1.1.3), PL (phospholipase A_2, EC 3.1.1.4) and cholesteryl esters (pancreatic cholesterol esterase; CES, EC 3.1.1.13/ carboxylesterase EC 3.1.1.1)(Enzyme Nomenclature,1992; Gropper *et al.*, 2009). Human pancreatic cholesterol esterase is a non-specific lipase with activity against a variety of substrates. It acts on all *sn*-positions of TAG, as well as cleaving PL, ceramides, vitamin esters, WE and galactolipids (Hargrove *et al.*, 2004; Whitcomb and Lowe, 2007).

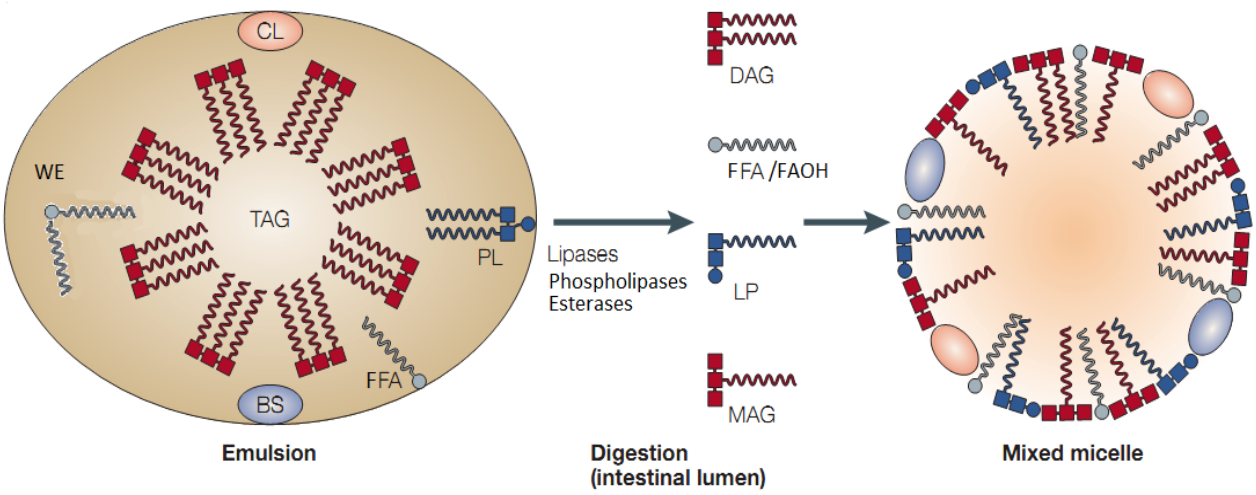

Figure 11: The process of dietary lipid digestion in the intestinal lumen. Bile salt (BS) emulsifies lipid droplets consisting of a TAG core and cholesterol (CL), PL, WE and FFA. The lipid droplets are exposed to the various lipases for hydrolysis. Monoacylglycerol (MAG), diacylglycerol (DAG), lysophosphospholipid (LP), FFA and fatty alcohols (FAOH) that are released by lipid hydrolysis join BS, CL and lipid-soluble vitamins to form micelles. Modified from Shi and Burn (2004).

The cleaved lipid products are also hydrophobic, and have to be transported in a form that shields them from the aqueous content of the intestinal lumen. Monoacylglycerols (MAG), hydrolysed PL, cholesterol, FFA, and presumably also fatty alcohols (FAOH) combine with bile salts to form negatively charged aggregates called micelles (Figure11). The micelles are sufficiently water soluble to interact with the absorptive cells of the small intestine, the enterocytes. When a micelle reaches the epithelial surface, the lipolytic products will be absorbed across the brush border membrane of the enterocytes (Figure 12). The

molecular mechanisms of lipid absorption is not fully understood, however they include both passive diffusion and active transport, mediated by transporters such as intestinal FA-binding protein (IFABP), CD36 and FA-transport protein-4 (FATP4) (Werner *et al.*, 2003; Schwenk *et al.*, 2010). Bile salts are absorbed in the last segment of the small intestine and are returned to the liver, known as the enterohepatic circulation. After passing across the membrane of the enterocyte, the lipids migrate to the endoplasmic reticulum where the re-esterification of fatty acids into TAG takes place. There are different pathways involved in TAG resynthesis, of which the MAG-pathway is the most significant as long as dietary TAG is in excess (Porter *et al.*, 2007). Via a series of acyltransferase enzymes, fatty acids are reattached to MAG to form diacylglycerols (DAG), and subsequently to TAG again. Lysophospholipids and cholesterol are re-esterified to fatty acids to generate PL and cholesteryl esters (CE), respectively (Brody, 1994). The absorbed FAOHs are oxidized to the corresponding fatty acids by a NAD-dependent process which in turn is coupled to a NADPH-dependent production of glycerol-phosphate, the G3P-pathway , resulting in TAG (Bauermeister and Sargent, 1979; Hargrove *et al.*, 2004).

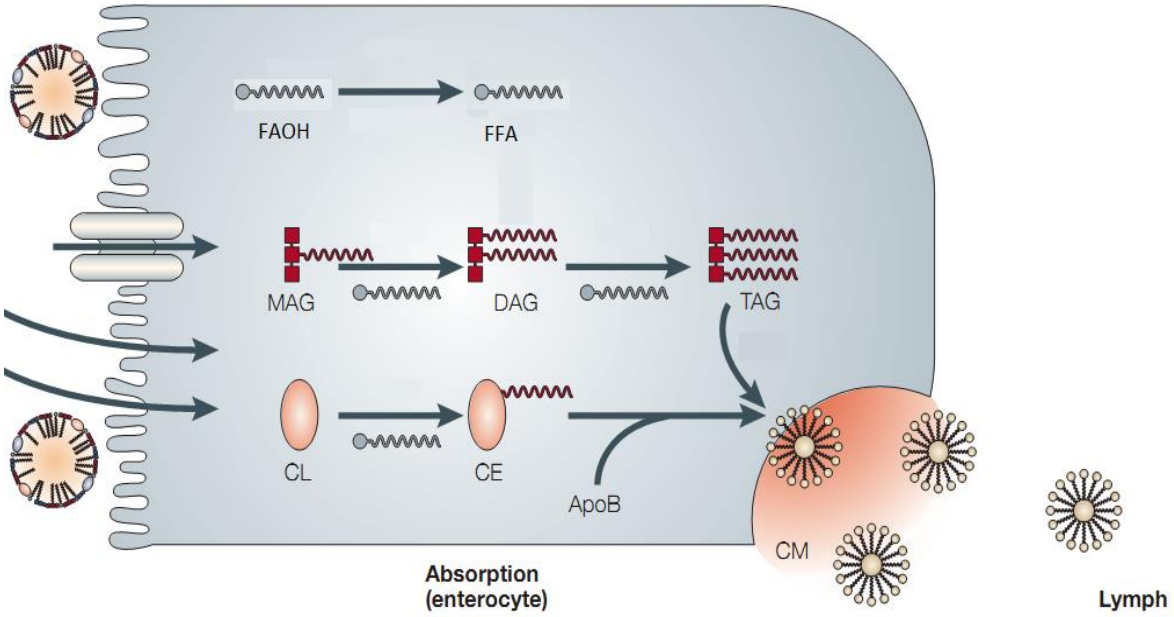

Figure 12: The micelles present the digested lipid products for absorption at the brush border membranes of the enterocytes. The hydrolysed lipids may enter the cell by passive diffusion or active transportation. The re-esterification of MAG to DAG and subsequently to TAG again, occurs inside the endoplasmatic reticulum. Lysophospholipids and dietary CL are esterified to fatty acids to form PL and cholesteryl esters (CE), respectively. The newly formed lipid products come together with apolipoprotein B (ApoB) to form chylomicrons that enter circulation through the lymph. Modified from Shi and Burn (2004).

The lipid products are further processed in the Golgi apparatus in which TAG molecules are combined with CE and PL, and coated with lipoproteins (ApoB) to form water soluble chylomicrons (Phan and Tso, 2001). Short chain fatty acids (< 12C) may to some extent remain unesterified, only bound to albumin, and can pass directly into the portal blood and be metabolized (Gropper *et al.*, 2009). However, chylomicrons are the main route for the transport of dietary long-chain fatty acids and these large lipoproteins are released by the enterocyte through exocytosis (Tso and Balint, 1986). The chylomicrons will not enter the blood stream directly; instead they are secreted into the lymph vessels outside the enterocytes. From there they will move in to the main branch of the lymphatic system and enter the blood circulation for distribution around the body. The role of chylomicrons is to deliver dietary lipids mostly to peripheral tissues other than the liver, such as muscles and adipose tissue, for energy and storage (Vance and Vance, 2008; Gropper *et al.*, 2009).

While transported by the blood throughout the body, the chylomicrons undergo hydrolysis at different tissue sites and the lipolytic products are quickly absorbed by the endothelial cells. As much as 85 % of the lipids in the chylomicrons are delivered before reaching the liver in the form of chylomicron remnants and are rapidly removed from the blood stream by liver cell endocytosis (Cooper, 1997). The remnant lipids can be metabolized for energy, or modified by chain elongation and resynthesized along with endogenous fatty acids to new lipid molecules, such as PL, TAG and CE. These are subsequently released in to circulation again as very low density lipoproteins (VLDL) and high density lipoproteins (HDL). The circulating VLDL will release TAG molecules in the same manner as chylomicrons. Additionally, when the VLDL donates TAG, they will rapidly convert to intermediate-density lipoprotein (IDL) and subsequently to low density lipoprotein (LDL). The LDL molecules in turn, bind cholesterol from the serum and transport it to the various tissues to be utilized for membrane construction, or conversion to cholesterol derived molecules, such as steroid hormones (Nelson and Cox, 2000). In contrast, HDL has an opposite function; it removes cholesterol from cells and returns it to the liver. The cholesterol from the liver may be secreted in bile again, either converted to bile salts or as a neutral sterol (Gropper *et al.*, 2009).

4. METHODOLOGICAL CONSIDERATIONS

4.1 Raw material and oil

Harvest and storing of the raw material used in paper II were conducted as described in chapter 3.2. Calanus® Oil used in experiments in paper I and III was commercially produced and provided by Calanus AS.

4.2 Production of oil from *Calanus finmarchicus*

Prior to the pilot scale production presented in paper II, optimization of the different process requirements was conducted at laboratory scale. The type of enzymes applied, sufficient enzyme concentrations, optimal pH and hydrolysis time were determined. The enzymes chosen, Alcalase® and Flavourzyme®, are widely used and commercially available (www.novozyme.com). Alcalase® is a non-specific endoprotease responsible for the overall protein disruption, while Flavourzyme® contains both endo – and exopeptidases responsible for hydrolysing bitter peptides into smaller fragments to reduce the bitterness of the hydrolysate produced.

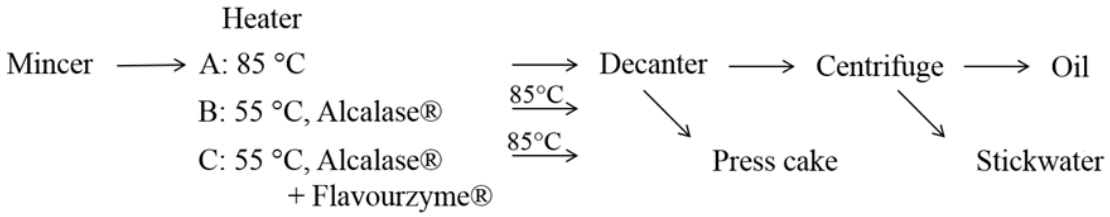

Figure 13: Production of oil following the general steps of mincing, heating, removal of solids via a decanter and finally separating oil and stickwater by a centrifuge. A: traditional thermal treatment or B and C enzymatic hydrolysis at 55°C, and subsequently heating to 85 °C for enzyme inactivation.

Three experiments were carried out as outlined in Figure 13, each using 350-400 kg of partially thawed and grinded *C. finmarchicus.* Experiment A was carried out as a control, similar to that of traditional fish oil production. For experiments B and C, enzymatic hydrolysis was applied after the initial grinding step. In experiment B, only Alcalase® was added for the hydrolysis step. In experiment C the enzymes were applied to function sequentially, adding Flavourzyme® to the reaction mixture after an initial hydrolysis (15 min)

with Alcalase®. To ensure inactivation of the enzymes, the reaction mixtures were subsequently heated to 85 °C. The heated materials were transferred to a decanter separating the solids (press cake), and lastly separating the oil from the stickwater by a centrifuge. Samples from all the fractions were collected and frozen prior to analysis.

4.3 Oxidative stability

The oxidative stability of Calanus® Oil presented in paper I was obtained from a long term storage study; Calanus® Oil was distributed in glass bottles (50 ml), both airtight and flushed with N_2, or perforated caps exposing the oil to air. The bottles were divided to three storage areas; refrigerated (dark, 4 °C), and at room temperature (20-22 °C), in darkness or exposed to light. Samples were taken at the beginning and then every three months until 425 days of storage. Each sample was flushed with N_2 and stored at – 55 °C until analysis.

Sensory analyses are the most closely related methods to evaluate the quality of food lipids; however, the usefulness is limited due to high costs and requirement of a properly trained sensory panel (Frankel, 2005). Peroxide value, as previously mentioned, is the most commonly used measurement of the extent of primary oxidation in oils. It can be performed using iodometric titration methods or spectrophotometric ferrothiocyanate methods (VKM, 2011). Additionally, the AV-value is obtained by spectrophotometric analysis of a colour reaction between aldehydes and *p*-anisidine. Other methods for measuring oxidation products, such as conjugated dienes and trienes and carbonyl compounds also depend on spectrophotometrically methods. In summary, most methods commonly used to evaluate the oxidation in edible oils rely either on the visual inspection of colour change (e.g. titration) or absorbance measured spectrophotometrically in UV-areas. The oil extracted from *Calanus finmarchicus* has such a high content of astaxanthin leading to colour interferences with all previously mentioned methods. The content of astaxanthin in itself was therefore used as an indirect measurement of the degree of oxidation of Calanus® Oil, as the astaxanthin molecules oxidize when exerting its antioxidative effects upon the oil product. The content of astaxanthin was measured directly by dissolving oil in acetone and determining absorbance spectrophotometrically at 470 nm as described by Foss *et al.* (1984).

Non-colorimetric measuring of oxidation products can also be accomplished. A common method is the measurement of the volatile secondary oxidation products by gas chromatography (GC) head-space and subsequent identification by mass spectrometry

(Jackobsen and Nielsen, 2007). Measuring the apparent loss of LC-PUFA during storage is an alternative GC-method applicable for evaluating oxidative stability (Dulavik *et al.*, 1998). It is however a relatively insensitive method, as formation of primary oxidation products can occur before a decrease in LC-PUFA can be detected. Nonetheless, the latter was chosen for this study.

4.4 Digestion of Calanus® Oil in mice

The use of animals in science is of great ethical concern. Moreover, standardization is often difficult to achieve. Ethical guidelines are established and regulations are implemented to control animal experiments. The most important principles were introduced by Russell and Burch (1959) to ensure animal welfare without comprising the research. These principles are known as the three R's: replacing animal experiments with non-animal alternatives whenever possible, reducing the number of animals to a minimum and refining the experimental protocol, making sure animals suffers as little as possible. The animals used in paper III were part of a larger experiment (Salma, 2014), as such in compliance with the principles stated above. However, this led to some limitations of this study, such as the possibility to include indigestible markers or radiolabelling of the dietary lipids to measure efficiency of digestion and the absorption of the wax ester constituents. The quantification of lipid classes, together with analysis of fatty alcohols presented in paper III was performed by a commercial laboratory.

5. MAIN RESULTS AND GENERAL DISCUSSION

Based on the aims presented in chapter 2, the work was carried out in three parts; I) The compilation of current knowledge on the lipids from *Calanus finmarchicus*, together with an assessment of the possible use of Calanus® Oil. In addition the study of the oxidative stability of LC-PUFA and astaxanthin present in the oil. II) Optimizing the industrial process requirements for the extraction of Calanus® Oil with the intention of increasing yield, and extend the current work on characterizing the oil obtained. III) Study lipid digestion and absorption in mice models relevant for human physiology, carried out by a feeding trial where the mice were give a high fat diet with 2% Calanus® Oil. Each part was presented in **papers I, II** and **III**, respectively.

5.1. Review and possible use

Based on the large number of biological studies published, *Calanus finmarchicus* might be one of the most thoroughly investigated marine planktonic animals. The copepod has a life strategy adapted to low temperatures and short periods of feeding; taking full advantage of the concentrated spring and summer algal blooms. The accumulation of large lipid stores allows them to migrate to great depths, survive during the winter months in the dormant phase, and resurface to mature and spawn in early spring. As such, the lipid content and composition of *C. finmarchicus* depends greatly on development stages, season, diet and location of harvest, as compiled in **paper I**.

Over the last years, the share of fishmeal and oil in aquaculture feed are restricted and are to a large degree replaced with products of vegetable origin (Naylor *et al.*, 2009; Ytrestøyl *et al.*, 2015). The vegetable sources will probably be the major component in feed due to accessibility and low cost. The relevant plant oils are often rich in monounsaturated FA and C18 PUFAs, but lack the LC-PUFA (Turchini and Francis, 2009). As a result, several investigations have focused on the inclusion of alternative omega-3 containing lipids to farmed fish. Studies aiming at utilizing oil from *Calanus finmarchicus* in aquaculture feeds have been performed, and results show that Atlantic salmon (*Salmo salar*) tolerate well a 30% substitution, while higher inclusion results in reduced digestibility and growth (Reviewed by Bogevik, 2011). The salmon is apparently able to adapt to increased dietary intake of WE by increasing bile production and lipolytic activity (Bogevik *et al.*, 2009). Similar adaptions have

also been reported in Atlantic halibut (*Hippoglossus hippoglossus*)(Colombo-Hixson *et al.*, 2011). The cost of obtaining zooplankton lipids is currently much higher than producing fish oils and most plant oils. Therefore, it is more realistic to use Calanus® Oil as an ingredient, rather than as the bulk lipid in aquaculture feed. This may also be applicable where other components, such as astaxanthin, in addition to the fatty acids are in demand. Astaxanthin is the most effective carotenoid used for salmonid pigmentation, and is also proposed to be of importance to maintain the health of the fish (Nakano *et al.*, 1995; Bjerkeng, 2008). In this respect Calanus® Oil may be valuable as a natural source of flesh pigmentation in Atlantic salmon (Hynes *et al.*, 2009).

The high content of n-3 LC-PUFA and astaxanthin makes Calanus® Oil very interesting as a nutraceutical. Studies on rodents indicate that dietary inclusion of low levels of Calanus® Oil might have beneficial effects beyond those which may be ascribed to intake of EPA and DHA alone. The reported effects are reduced abdominal obesity and adipose tissue inflammation, improvement in systemic glucose and attenuation of atherosclerotic lesions (Eilertsen *et al.*, 2012; Höper *et al.*, 2013; Höper *et al.*, 2014). In addition, a recent study also report anti-hypertensive action of Calanus® Oil in a mouse model of obesity (Salma, 2014).

Wax esters have gained a rather negative reputation due to occurrences of so-called keriorrhea following ingestion of WE-rich fish (Ling et al., 2009). This has led to the assumption that mammals cannot digest wax esters. However, there is good evidence that mammals are capable of WE digestion when consumed in relatively moderate amounts (Hansen and Mead, 1965; Yaron *et al*., 1982; Gorreta *et al*., 2002). This is also supported by the presence of WE in several recommended foods such as cereal grains, seeds, nuts and unrefined oils (Hargrove et al., 2004). In addition to the natural content of WE in foods, terrestrial waxes are used as food additives; for example adding wax esters to edible oils to change viscosity, as such producing healthier spreads without trans fatty acids (Yılmaz and Öğütcü, 2014)

5.2. Oxidative stability of astaxanthin and LC-PUFA

In **paper I**, the content of astaxanthin and LC-PUFA in Calanus® Oil was monitored during a long term storage period and depicted in Figure 14 and Table 4, respectively. The results in Figure 14 indicated that exposure to light in the presence of air at room temperature did not have any major negative effects on the oxidative stability of the astaxanthin content compared to dark storage under the same conditions. This might suggest that any singlet oxygen formed due to light exposure is not quenched by astaxanthin. The exposure to air in combination with ambient storage temperature (22 °C) led to a rapid decline of astaxanthin during the first months of storage and continued until total depletion of the pigments present. The best protective effect was seen in the samples stored at inert atmosphere, and especially in combination with low storage temperature (4 °C), where the content of astaxanthin was almost unchanged throughout the entire storage period. A second oil sample stored in darkness at 4 °C was exposed to air. This sample exhibited the same stability of astaxanthin content as the samples protected by N_2. However, after 300 days of storage, a steep fall of the astaxanthin content was seen, and an almost complete consumption of astaxanthin was seen at the end of the study.

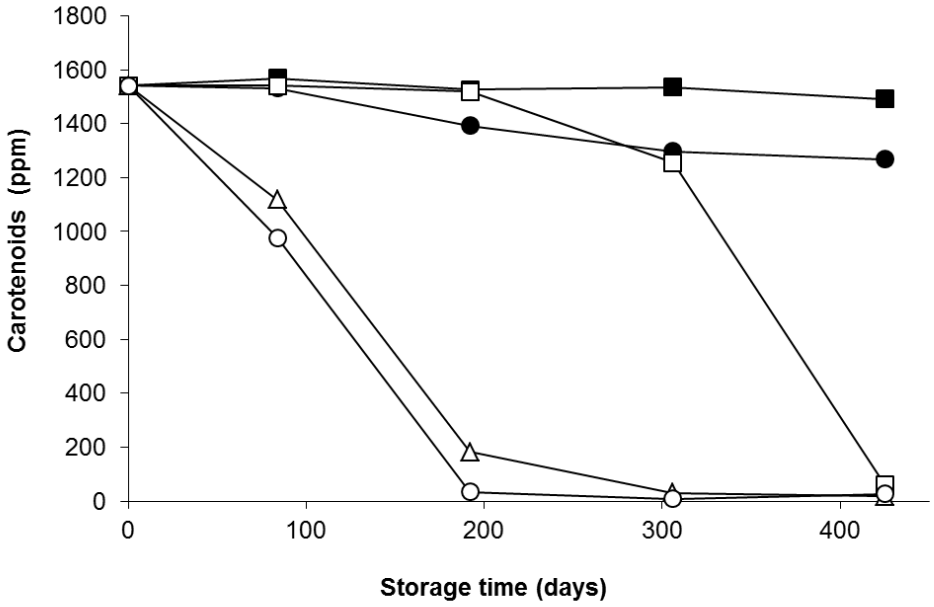

Figure 14: Stability of Calanus® Oil stored for a period of 425 days. The oil was stored with an inert atmosphere (N_2; closed symbols) or exposed to air (open symbols), and in the dark at 4°C (-■-/-□-) or at room temperature, 22°C, exposed to light (-●-/-○-) or in darkness (-Δ-)

While chain breaking antioxidants, such as tocopherols, trap radicals by donating a hydrogen atom, the carotenoids can exert its antioxidative effect by a mechanism in which the peroxyl radical (LOO•), or alkoxyl radical (LO•), is added to the conjugated polyene system of the carotenoid molecule (Equation 5). An important factor for the antioxidative effect of carotenoids is the relative stability of the carotenoid radical formed (LOO-Car•). This is known as resonance stabilization, which is the delocalization of the unpaired electron over the conjugated backbone of the astaxanthin molecule. Addition of a second peroxyl radical to the carotenoid radical will produce a non-radical product, resulting in an overall trapping of two peroxyl radicals per carotenoid consumed (Jørgensen and Skibsted, 1993).

$$LOO\bullet + Car \rightarrow LOO\text{-}Car\bullet \quad \text{(eq 5)}$$

Conversely, in the presence of O_2, the carotenoid radical can add oxygen, which gives an unstable peroxyl radical (Equation 6). This may lead to further degradation, and form new radicals with no net inhibition of oxidation (Jørgensen and Skibsted, 1993). The rapid fall of astaxanthin content for the oxygen exposed oils may be explained by this mechanism.

$$LOO\text{-}Car\bullet + O_2 \rightarrow LOO\text{-}Car\text{-}OO\bullet \quad \text{(eq 6)}$$

At low O_2 concentrations, the radical trapping mechanism predominates over the formation of carotenoid peroxyl radical (Eq 6), and the overall stability of the samples stored at inert atmosphere might have been supported by this. Likewise, others have reported a pronounced antioxidative effect of carotenoids at low O_2 concentrations, reducing the amount and rate of oxidation (Burton and Ingold, 1984; Kennedy and Liebler, 1992)

The fatty acid composition of the oil samples was only measured at start and endpoint of the storage period (Table 4). The content of SDA, EPA and DHA, followed the same overall pattern as for the astaxanthin measurements. The samples stored in air at room temperature showed a substantial decrease of SDA, EPA and DHA. This is to be expected when stored at less optimal conditions and may also have been amplified by the rapid oxidation of astaxanthin and subsequently the formation of astaxanthin oxidation products. The samples stored at inert atmosphere, on the other hand, exhibited only a modest loss of the LC-PUFA. A similar stability of LC-PUFA content was observed for the refrigerated oil exposed to oxygen. This might be due to the physiochemical properties of the wax ester molecules; at low temperatures the wax esters are solid or semisolid, reducing the susceptibility to oxidation, since oxygen reactions are confined to the surface (Gorreta *et al.*,

2002). Moreover, all biological reactions have a slower rate at low temperatures, thus such storage will provide a certain delay in oxidation reactions.

Table 4: Concentration (% of total) of the major n-3 fatty acids in oil from *C. finmarchicus* at time 0 of storage and after 425 days.

	Time 0	4 ºC (dark)		22 ºC (light)		22 ºC (dark)
	-	N_2	air	N_2	air	air
SDA, 18:4 n-3	15,8	11,9	12,9	13,1	8,9	7,7
EPA, 20:5 n-3	12,3	8,4	7,9	9,5	5	4,5
DHA, 22:6 n-3	6,9	4	4	4,6	2,3	1,8

The fatty acid composition from each time point as analysed of astaxanthin contents should clearly have been conducted, in the attempt to provide a more comprehensive overview to the oxidative stability of Calanus® Oil. The long term storage of Calanus® Oil indicates an overall high stability of both astaxanthin and LC-PUFA content when stored at optimal conditions that is at low temperature and inert atmosphere.

5.3. Pilot scale production of oil from *Calanus finmarchicus*

In **paper II**, it was investigated if the use of commercial proteolytic enzyme could improve oil recovery from *Calanus finmarchicus* in an industrial-like process. The outcome of the productions of oil by experiments A, B and C is given in Figure 15. The results showed that the oil recovery increased profoundly with the employment of proteolytic enzymes compared to the traditional fish oil processing. The dried *C. finmarchicus* contained 32,5 % lipids. By the means of thermal treatment alone, only 4,5% of the lipids present in the raw material were recovered as oil, whereas most of the lipids were retained in the press cake and the protein concentrate. When adding the enzymatic hydrolysis step to the oil processing, 76% lipid was recovered after the treatment with Alcalase®, and 83% lipid was recovered after the sequential treatment with Alcalase® and thereafter Flavourzyme®. As described in the background section, *Calanus finmarchicus* accumulates most of the lipids in a separate membrane bound sack. A sufficient disruption of the membrane sack is accordingly necessary for the release of lipids during oil processing, and a likely explanation for the high oil recovery in experiments B and C. An increase in oil yield by the use of similar enzymatic hydrolysis have been demonstrated by several studies on marine raw materials, reviewed by Rubio-Rodríguez *et al.* (2010).

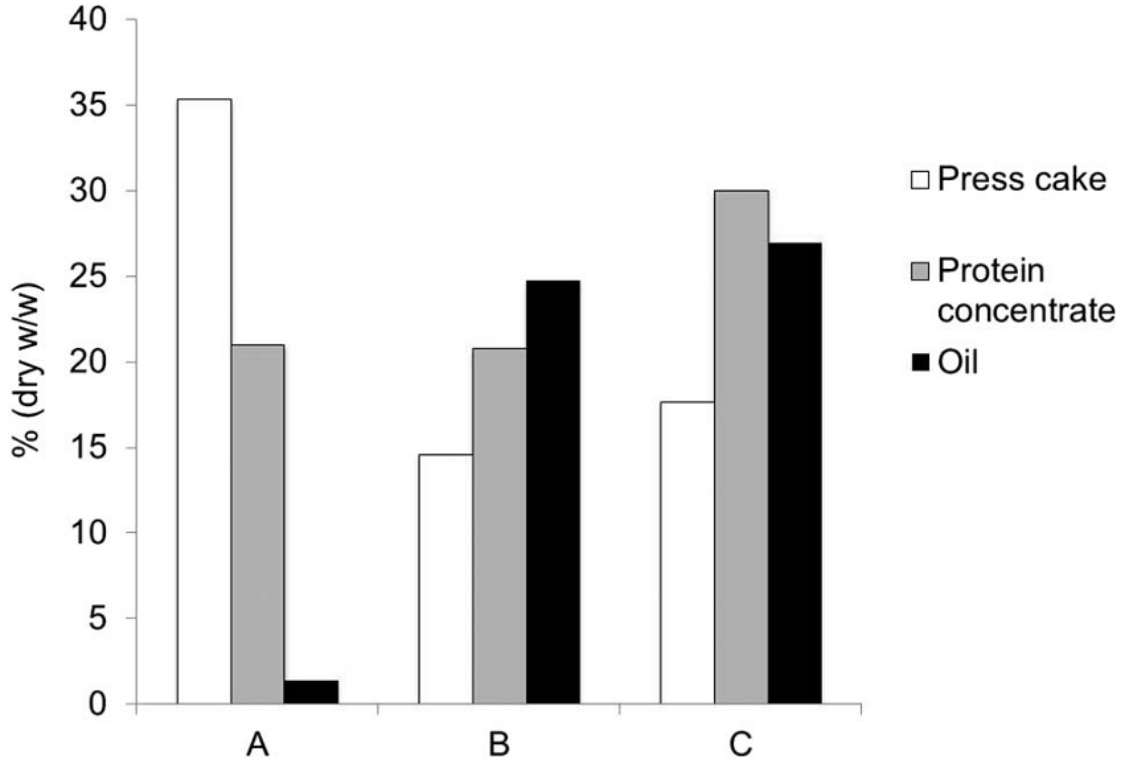

Figure 15: Press cake, protein concentrate (hydrolysate) and oil recovered in experiment A (control) and in the experiments using proteolytic enzymes; experiment B (Alcalase®) and experiment C (Alcalase® and Flavourzyme®). Expressed as % of dry weight starting material.

The employment of enzyme hydrolysis also affected the output and proximate composition of the press cakes and protein concentrates (hydrolysate) as given in **paper II**. The press cakes obtained after experiment B and C accounted for 15% and 18% of the dry weight, respectively, compared with the standard fish oil process, where the press cake amounted to 35%. The combined use of Alcalase® and Flavourzyme® in experiment C increased the amount of protein concentrate, and at the same time less lipids were retained in the protein concentrate, compared to the use of Alcalase® alone.

Using the titration method described in **paper II**, the oil from experiment A was estimated to contain 7,5% FFA, while the values for the oils from experiment B and C both were 8,5% FFA. Only the oil obtained by traditional fish oil processing was analysed further in regard to composition. The oil was fractionated and assessed qualitatively by thin layer chromatography (TLC). These results showed a substantial amount of WE present in the crude oil, and while not quantified, this was in line with results from biological studies (reviewed by Lee et al., 2006). Free fatty acids could also be seen clearly, but only trace amounts of phospholipids were observed. Subsequently, the fatty acid composition was

determined for the FFA and WE fractions, and as well as the crude oil (Table 5). Considerable quantities of the n-3 fatty acids SDA, EPA and DHA were present, constituting as much as 34,5% of the fatty acid moiety of the oil. The overall composition of the fatty acids in the WE fraction was similar to the composition of the oil in total. The composition of the FFA fraction differed from the oil sample and the WE fraction, and was dominated by the fatty acids 16:0, EPA and DHA, resembling the composition of the fatty acids in the phospholipids of *Calanus finmarchicus*, as compiled in **paper I**.

Table 5: Concentration (% of total) of relevant fatty acids in oil extracted from *C.finmarchicus*, FFA and WE fractions obtained by solid phase extraction.

	Oil	WE	FFA
16:0	9,3	7,6	21,2
18:0	1,0	0,8	7,9
20:1 n-9	5,9	6,0	1,3
22:1 n-11	9,0	8,8	1,7
18:4 n-3	12,4	11,4	8,0
20:5 n-3	14,4	11,6	17,0
22:6 n-3	7,7	4,1	18,3
Σ SFA	10,3	8,4	29,1
Σ MUFA	14,9	14,8	3,0
Σ n-3 PUFA	34,5	27,1	43,3

WE: wax esters, FFA: free fatty acids.

Increased levels of FFA has been reported in other studies regarding zooplankton lipids (Overrein, 2010; Bergvik *et al.*, 2012), and it has been suggested that the rapid degradation of phospholipids compared to neutral lipids is a result of a higher phospholipase activity compared to other lipolytic activities (Sikorski and Kolakowski, 2000). The raw material used in **paper II** was harvested in May, and according to biological studies, the lipids from *C. finmarchicus* may contain as much as 10-20 % phospholipids in early spring (Table 1, chapter 3.4). High phospholipase activity may therefore be a reasonable explanation to the high content of FFA, as well as the minor amounts of phospholipids detected when assessing the lipid classes in the oil produced.

5.4. Digestion and absorption of Calanus® Oil

In **paper III**, a more extensive characterization of Calanus® Oil was undertaken prior to the feeding trial, where Calanus® Oil was given as a 2% supplement in a high fat diet (HFD) to mice. The Calanus® Oil was quantitatively analysed to determine lipid class distribution, as well as both fatty acid, and fatty alcohol composition. The analysis of fatty acid composition of Calanus® Oil showed that the oil contained 180 mg n-3 fatty acids/g lipid with SDA, EPA and DHA, contributing with 70, 55 and 39 mg/g lipid, respectively (Table 6). The major fatty alcohols present in Calanus® Oil were gondoic acid (20:1n-9) and cetoleic acid (22:1n-11). The results presented in Table 7 showed that WE was the dominating lipid class present in Calanus® Oil, with minor contributions from TAG, FFA and sterols (cholesterol, C).

Lipids extracted from the experimental diets were also analysed with regard to fatty acid composition (Table 6). The overall fatty acid composition of the experimental diets were similar, with palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n-9) and linoleic acid (18:2n-6) as the major fatty acids present in both diets. The inclusion of Calanus® Oil to the diet (HFD+Cal) was verified by the presence of SDA, EPA and DHA, as well as the fatty alcohols 20:1n-9 and 22:1n-11, neither detected in the HFD. The n-6/n-3 ratio for the HFD was calculated to 10,94 and the ratio for HFD+Cal was 5,36. Triacylglycerol was the only lipid class detected in the lipids from the HFD, whereas the inclusion of Calanus® Oil to the diet were confirmed by the detection of 63 mg WE /g lipid and TAG constituting the remaining part of the lipids (Table 7).

As shown in **paper III**, the supplementation of HFD with 2% Calanus® Oil resulted in reduced body weight gain compared to the HFD group during the 11 week feeding trial. A reduced body weight gain has also been reported by other studies when including marine oils in diets for rodents (Ruzickova *et al.*, 2004; Arai *et al.*, 2009; Sato *et al.*, 2010), this have also been reported when small amounts of wax esters from *Calanus finmarchicus* have been included (Höper *et al.*, 2014). No adverse effects were observed, and no other significant differences in the recorded biometric data were seen between the two diet groups.

The lipid class analysis showed that FFA was a major lipid class in feces from both groups (Table7). The feces from the HFD+Cal group contained 115 mg cholesterol (or sterols)/g extracted lipids, while for the HFD group were 240 mg cholesterol (or sterols)/g lipids. The discrepancy of excreted cholesterol between the diet groups may be explained by

Table 6. Fatty acid and fatty alcohol content (mg/g lipid) of Calanus® Oil and experimental diets

Fatty acids	Calanus® Oil	High fat diet	High fat diet + Cal
14:0	64,42	10,41	11,95
16:0	45,05	173,65	149,83
18:0	2,42	106,63	92,16
20:0	0,40	1,70	1,37
Σ SFA	112,29	292,39	255,31
16:1*n-7*	17,17	10,95	9,98
18:1*n-7*	1,53	15,84	13,52
18:1*n-9*	15,54	243,82	208,42
20:1*n-9*	24,01	4,74	5,64
20:1*n-11*	3,90	nd	nd
22:1*n-9*	2,63	nd	nd
22:1*n-11*	43,33	nd	2,20
24:1*n-9*	2,81	nd	nd
Σ MUFA	110,92	275,36	239,76
18:2*n-6*	6,64	133,04	116,06
18:3*n-3*	13,72	12,49	11,67
18:4*n-3*	69,58	nd	4,54
20:2*n-6*	0,71	3,15	2,69
20:4*n-6*	1,39	0,48	1,15
20:5*n-3*	54,73	nd	3,35
22:5*n-3*	2,96	nd	nd
22:6*n-3*	39,35	nd	2,81
Σ PUFA	189,08	149,16	142,27
Σ *n-6*	8,74	136,67	119,90
Σ *n-3*	180,34	12,49	22,37
Σ *n-6/n-3*	0,05	10,94	5,36
Σ Fatty acids	412,29	716,91	637,34

Fatty alcohols	Calanus® Oil	High fat diet	High fat diet + Cal
14:0	4,50	nd	nd
16:1*n-7*	5,80	nd	nd
18:1*n-9*	10,40	nd	nd
20:1*n-9*	128,80	nd	9,65
22:1*n-9*	10,40	nd	nd
22:1*n-11*	188,10	nd	9,93
Σ Fatty alcohols	348,00	-	19,58

the argument that high-fat feeding may increase cholesterol synthesis in mice, whereas the enrichment of n-3 LC-PUFA to HFD has shown to reduce the rate of cholesterol synthesis (Oosterveer *et al.*, 2009). Neutral lipid classes such as TAG and wax ester/cholesteryl ester (WE/CE) could also be detected in the feces from both diet groups, unfortunately, it was not possible to quantify WE separately from CE. In evaluating the results it should be remembered that the fecal lipids will include a fraction of the secreted bile lipids as well as bacterial lipids, and lipids from excreted intestinal cells. Thus, it is likely to assume that the presence of cardiolipid (CL), cholesterol and, at least in part, WE/CE in the feces were of endogenous origin. Fatty alcohols were present in the feces lipids from the HFD+Cal group, but were not detected in the HFD group (Table 7). The analysis of fatty alcohol composition presented in **paper III** showed that 20:1n-9 and 22:1n-11 was the major fatty alcohols in the feces from the HFD+Cal fed mice. The relative amount of free fatty alcohols and FFA in the feces suggests that the absorption process may be the limiting step when fed a high fat diet.

Table 7. Lipid class composition (mg/g lipid) of Calanus® Oil, diets and feces.

	Calanus® Oil	Diet		Feces	
		HF	HF + Cal	HFD	HFD + Cal
WE/ CE*	857,70	nd	63,06	33,59	53,93
TAG	16,96	970,33	936,04	105,95	42,88
FAOH	nd	nd	nd	nd	126,90
C	41,91	nd	nd	240,36	115,65
FFA	16,59	nd	nd	223,79	332,55
CL	nd	nd	nd	19,42	18,02
PC	nd	nd	nd	24,61	nd
∑ lipid class	933,16	970,33	999,10	647,72	689,92

nd: not detected, WE: wax ester, CE: cholesteryl ester, TAG: triacylglycerol, FAOH: fatty alcohol, C (sterols, cholesterol), FFA: free fatty acids, CL: cardiolipid, PC: phophatidylcholine. * WE and CE coelute.

The fatty acid composition of adipose tissue has been considered a gold standard for the representation of dietary fatty acids, due to the slow turnover time in (weight stable) individuals (Hodson *et al.*, 2008). Therefore, within the limitations of this study, the compositional data of the adipose tissues was a valuable tool for assessing the absorption of Calanus® Oil. Phospholipids are the major class of lipids in animal membranes and small lipoproteins. The majority of PL synthesis occurs in the endoplasmic reticulum of the liver,

Table 8: Fatty acid content (mg/g lipid) extracted from pWat, eWat and liver.

	pWat		eWat		Liver	
Fatty acids	HFD	HFD + Cal	HFD	HFD + Cal	HFD	HFD + Cal
14:0	8,55±0,89	10,77±1,34*	9,41±0,79	11,55±1,45*	3,15±0,73	2,68±0,83
16:0	178,40±18,60	177,05±18,23	193,85±12,88	189,02±9,23	192,82±19,45	182,02±13,49
18:0	30,42±3,00	33,35±7,43	35,15±15,83	26,77±11,14	75,97±14,94	92,29±14,58*
20:0	1,07±0,06	1,13±0,26*	0,22±0,44	1,02±0,16	2,82±0,40	2,77±0,49
22:0	nd	nd	nd	nd	1,71±0,53	2,20±0,50
Σ SFA	217,54±21,98	221,88±24,47	238,64±25,37	227,78±17,15	276,49±16,90	281,95±20,85
16:1*n-7*	45,53±6,00	38,06±3,34*	53,45±10,43	49,15±9,15*	15,34±4,62	9,88±2,16*
18:1*n-7*	24,58±2,27	21,81±2,86*	25,52±1,15	23,48±2,03	16,45±2,93	10,50±0,94*
18:1*n-9*	440,60±36,86	411,44±57,35	446,43±26,46	428,10±38,77	216,08±44,77	154,85±28,66*
20:1*n-9*	6,73±0,72	11,25±2,39*	5,59±0,75	8,80±0,89*	5,30±0,83	5,07±0,71
20:1*n-11*	nd	nd	nd	nd	nd	0,60±0,17
22:1*n-9*	nd	2,24±0,53	nd	1,66±0,12	0,55±0,10	0,55±0,10
22:1*n-11*	nd	nd	nd	nd	0,55±0,13	1,85±0,31*
Σ MUFA	515,44±44,13	484,80±60,35	530,99±34,55	511,21±48,00	254,22±52,47	183,30±32,31*
18:2*n-6*	157,88±14,41	152,74±18,26	174,20±15,31	175,32±19,63	129,05±6,46	123,73±4,76
18:3*n-3*	6,66±0,78	7,11±0,82	8,54±0,94	9,06±1,09	3,89±0,65	3,87±0,67
18:4*n-3*	nd	1,60±0,38	nd	1,12±0,11	0,43±0,12	0,77±0,19*
20:2*n-6*	3,62±0,33	3,39±0,63	3,39±0,19	3,15±0,29	2,97±0,23	2,44±0,10*
20:4*n-6*	2,16±0,30	1,46±0,26*	2,97±0,82	1,77±0,33*	81,03±16,49	70,18±8,75
20:5*n-3*	nd	nd	nd	1,01±0,08	2,12±0,27	13,50±1,74*
22:5*n-3*	nd	1,68±0,57	0,10±0,30	1,55±0,17*	4,04±0,66	7,90±0,80*
22:6*n-3*	0,15±0,33	3,33±0,58*	0,99±0,78	3,77±0,47*	47,23±8,46	89,98±6,98*
Σ PUFA	170,46±15,47	170,74±19,03	190,18±16,29	196,02±20,82	270,67±21,33	312,38±16,88*
EPA+DPA+DHA/ SDA	-	3,13	-	5,65	124,16	144,65
Σ Fatty acids	903,43±79,33	877,42±109,95	935,00±71,75	959,78±41,46	801,37±49,72	777,63±42,66

Values are mg FA/g lipids (mean±SD of 8 mice/group) * HFD + Cal group significantly different from HFD using One-Way ANOVA test ($P < 0,05$). Abbreviations: nd - not detected; SFA - saturated fatty acids; MUFA - monounsaturated fatty acid; PUFA - polyunsaturated fatty acid; pWat - perirenal white adipose tissue; eWat - epididymal white adipose tissue

where PL associates with other lipids and proteins as lipoproteins released into the bloodstream. Hence, the fatty acid composition of the liver lipids from the diet groups was important information to obtain in this study. The fatty acid composition of the adipose tissues (pWat and eWat) and liver lipids reflected to a large extent the enrichment of HFD with Calanus® Oil (Table 8). The content of the n-3 fatty acids in pWat and eWat was generally related, with the exception of EPA in pWat, to the amount present in the feed. Probably, some elongation and desaturation have occurred, resulting in the presence of docosapentaenoic acid (DPA) and an increased amount of DHA compared to the fatty acid composition in the HFD+Cal feed. It was also noted that the amount of the more pro-inflammatory AA (20:4n-6) was lower in the liver tissue, and significantly reduced in the adipose tissues of the mice fed HFD+Cal. This may be due to the competition in interconversion of C18 n-6 and n-3 fatty acids to the longer chain acids.

The results in our study demonstrated a significantly higher content of the C16-18 monounsaturated fatty acids in the liver lipids from the HFD mice, compared to the HFD+Cal group. Oosterveer *et al.* (2009) showed that high-fat feeding induced hepatic fatty acid synthesis and chain elongation in mice, and led to an increase of medium chain monounsaturated fatty acids in the liver lipids. This was shown to be efficiently counteracted by the inclusion of fish oil in the high fat diet. The effect was explained by an adaptive remodelling of the hepatic fatty acids in the HFD + fish oil fed mice, in contrast to *de novo* fatty acid synthesis and elongation in the high-fat fed mice. The analysis of the liver lipids showed a pronounced elongation and desaturation of the C18 n-3 fatty acids from the feed and accumulation of the longer chain n-3 fatty acids. A significant higher amount of EPA, DPA and DHA were found in the livers of the HFD+Cal fed mice, and probably not only reflecting the content of these fatty acids in the feed, but also the conversion of dietary SDA to EPA (James *et al.*, 2003; Arterburn *et al.*, 2006). Most of the n-3 fatty acids in Calanus® Oil are esterified to long chain alcohols, and the results demonstrate that the wax esters provided in the feed were hydrolysed and the fatty acids absorbed, reesterified and transported to the tissues examined.

6. CONCLUSIONS

The role of wax esters in the marine food web is considerable, as zooplankton is the major step between phytoplankton and the higher consumers. It has been claimed that because of this step, that nearly half of the earth's photosynthetic production is for a time converted to wax esters. *Calanus finmarchicus* is a zooplankton which can be harvested with modern technology and processed to astaxanthin-rich oil.

As elucidated in **paper I** the oil extracted from *Calanus finmarchicus* has an important feature distinguishing it from other marine oils; it contains mainly wax esters, consisting of equal portions of fatty acids and fatty alcohols. The oil may be used as an alternative lipid source to fish oil in feeds for aquaculture, leading to good growth and efficient nutrient utilization. Moreover, Calanus® Oil can be used as a health promoting nutraceutical, as the oil provides the highly sought after EPA and DHA, and additionally the n-3 fatty acid SDA. The Global Organisation for EPA and DHA n-3s (GOED) has recognized oil from *Calanus finmarchicus* as a so-called third generation n-3 product, that is new sources or n-3 formulations with high potential market opportunity. Additionally, in **paper I**, the oxidative stability of the oil was investigated. The long term storage of Calanus® Oil indicated an overall high stability of both astaxanthin and LC-PUFA content when stored at optimal conditions, that is, at low temperature and inert atmosphere.

The results in **paper II** showed that including an enzyme hydrolysis step to conventional oil processing greatly improved oil recovery compared to thermal treatment as a control. Analysis of the lipids obtained through traditional oil production was in line with that reported of biological studies, that oil extracted from *Calanus finmarchicus* has a high content of wax esters and the fatty acid moiety contributes with a high amount of medium and long chain n-3 fatty acids. The fatty acid composition of the free fatty acid-fraction of the oil produced, revealed a close similarity to that reported of phospholipids from *C. finmarchicus*. The content of FFA in the oil was probably a result of high phospholipase activity in the raw material.

The study presented in **paper III** demonstrates that wax esters are far from metabolically inert. When feeding mice a high fat diet supplemented with a small amount (2%) of Calanus® Oil, the wax esters were hydrolysed during digestion and the fatty acids influenced the lipid profile of the animals. The medium chain n-3 fatty acids present in

Calanus® Oil, were efficiently absorbed and metabolized further to long chain n-3 fatty acids in the liver. The study confirmed that feeding mice a high fat diet supplemented with a small amount of wax esters reduced the body weight gain. Elevated levels of free fatty acids and alcohols in the feces suggest that the absorption process, not the hydrolysis, could be a rate limiting step in utilizing small amounts of wax esters included in high fat diet to mice.

7. FUTURE PERSPECTIVES

On the basis of existing literature and the results obtained throughout these studies, there are several aspects which should be further investigated.

To elucidate the mechanisms of the beneficial health effects of the supplementation of Calanus® Oil, as reported from biomedical studies. Additionally, a more thorough explanation of the fate of the dietary wax esters, herein to study the possible differential utilization of lipids assimilated through the MAG- or G3P-pathway from the enterocytes, and the "hot topic" of GPR 120 activation by long chain fatty acids in the distal part of the small intestine.

During the production of Calanus® Oil, two side streams are obtained; a protein hydrolysate and a sludge (press cake). These need to be further characterized to help the commercialization process.

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