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

Recovery and properties of oil from the copepod *Calanus finmarchicus*

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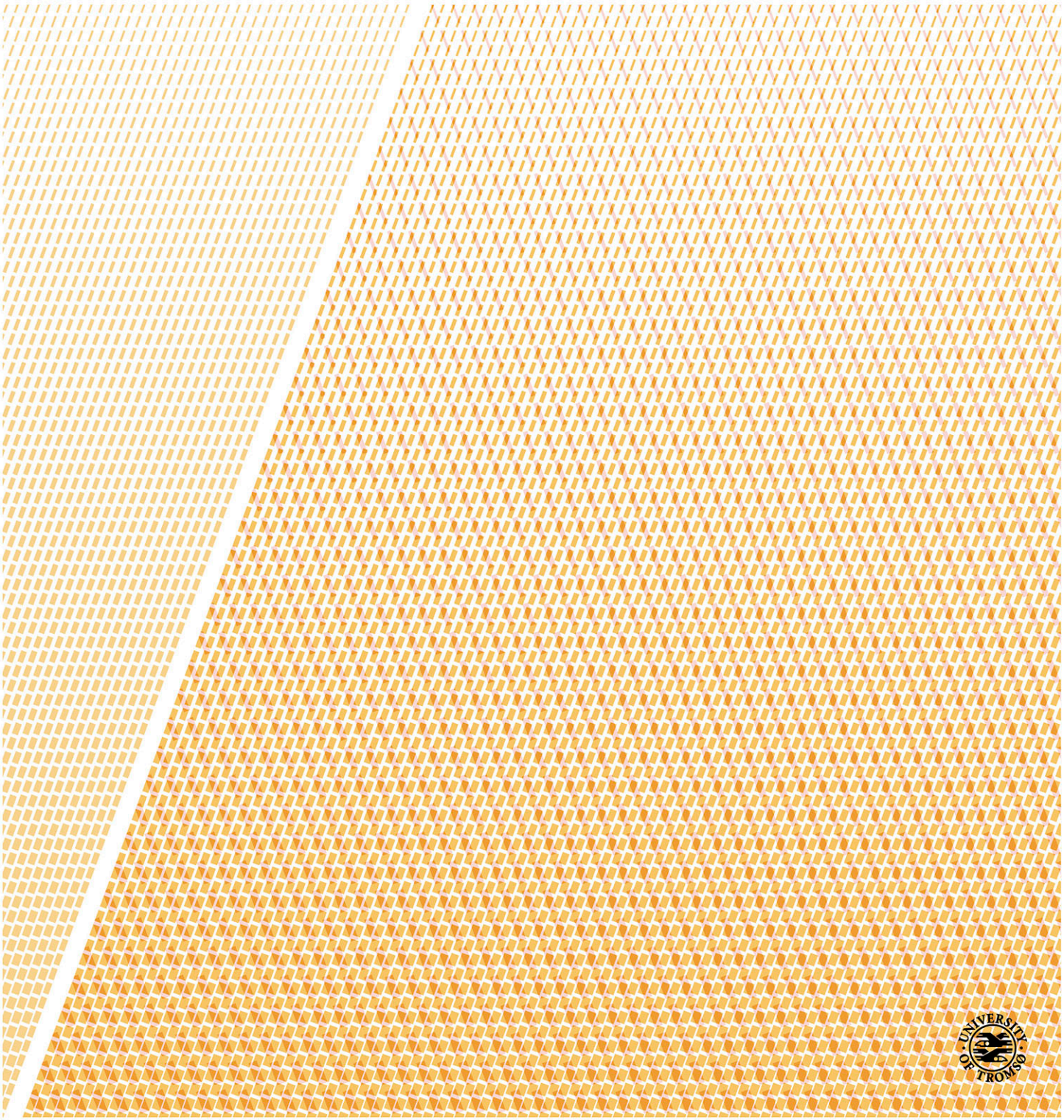

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Summary

The limited amount of fish oils available has led to extensive search for alternative sources of oils rich in long-chain polyunsaturated ω-3 fatty acids (n-3 LC-PUFA). The zooplankton *Calanus finmarchicus,* also known as redfeed, is present in large amounts in the North Atlantic and has lipid-rich stages that can be harvested. The valuable n-3 LC-PUFA eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are produced by phytoplankton in the marine food web. Zooplankton acts as an important link between phytoplankton and fish at higher trophic levels and may therefore be a potential source for these fatty acids. One of the main objectives of this study was to investigate if the use of food grade proteolytic enzymes, Alcalase® and Flavourzyme®, 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 oil from *C. finmarchicus* was rich in the powerful antioxidant astaxanthin and had a high content of n-3 PUFA mainly comprised of stearidonic acid (SDA, 18:4n-3), EPA and DHA. Wax esters consisting of mainly long-chain monounsaturated fatty alcohols esterified to saturated or unsaturated fatty acids were the dominating lipid class in the oil. One of the objects of this thesis was to evaluate the possible uses of Calanus oil. The wax ester rich oil is well utilized by fish, and recent reports indicate that oil from *C. finmarchicus* may have beneficial health effects beyond those which may be ascribed to intake of EPA and DHA alone.

Tropomyosin is known to be the main allergen in crustaceans and another objective of this study was to investigate if this protein could be detected in commercial crustacean oils from Antarctic krill (*Euphausia superba*) and *C. finmarchicus*. Western blotting showed that a commercial antibody against shrimp tropomyosin cross-reacted with a protein of similar size in Antarctic krill and *C. finmarchicus.* The protein tentatively identified as tropomyosin, was also detected in Krill oil products, but not in oils from *C. finmarchicus.* The acetone-heptane method used for extracting proteins in the oils is however not optimal, and other extraction methods should therefore be considered when investigating the presence of allergenic proteins in oils. The protein content in the crustacean oils were determined by direct amino acid analysis on the oils and showed a higher protein content in Krill than Calanus oils.

Sammendrag

Den begrensede mengden av tilgjengelige fiskeoljer har ført til omfattende søk etter alternative kilder til oljer med høyt innhold av langkjedede flerumettede ω-3-fettsyrer (n-3 LC-PUFA). *Calanus finmarchicus*, også kjent som raudåte, er et dyreplankton som fins i store mengder i Nord-Atlanteren og kan høstes når de når lipidrike utviklingsstadier. De verdifulle n-3 LC-PUFA eikosapentaensyre (EPA, 20:5 n-3) og dokosaheksaensyre (DHA, 22:6 n-3) produseres av planteplankton i den marine næringskjeden. Dyreplankton fungerer som et viktig bindeledd mellom planteplankton og dyr høyere i næringskjeden som fisk og hval, og kan derfor være en potensiell kilde for disse fettsyrene. Ett av hovedmålene i denne avhandlingen var å undersøke om bruk av proteolytiske enzymer, Alcalase® og Flavourzyme®, kunne øke oljeutvinningen fra *C. finmarchicus* i en industriell-lignende prosess, samt å karakterisere fettsyresammensetningen i den ekstraherte oljen. Resultatene viste et vesentlig høyere utbytte av olje ved bruk av proteolytiske enzymer sammenlignet med standard produksjonsteknologi for utvinning av fiskeolje.

Olje ekstrahert fra *C. finmarchicus* hadde et høyt innhold av den kraftige antioksidanten astaxanthin og n-3 PUFA, hovedsakelig stearidonsyre (SDA, 18:4 n-3), EPA og DHA. Voksester som består av langkjedede enumettede fettalkoholer forestret til mettede eller umettede fettsyrer var den dominerende lipidklassen i oljen. Ett av målene med avhandlingen var å vurdere mulige anvendelser av Calanusolje. Oljen, som har et høyt innhold av voksester, kan utnyttes av fisk. Det er også rapportert at olje fra C. *finmarchicus* kan ha gunstige helseeffekter utover de som kan tilskrives inntak av EPA og DHA alene.

Tropomyosin er et muskelprotein og kjent for å være det viktigste allergenet i krepsdyr. Ett annet mål i denne avhandlingen var å undersøke om dette proteinet kunne påvises i kommersielle krepsdyroljer fra Antarktisk krill (*Euphausia superba*) og *C. finmarchicus*. Immunoblottanalyser viste at et kommersielt antistoff mot reketropomyosin kryssreagerte med et protein av tilsvarende størrelse i Antarktisk krill og *C. finmarchicus*. Proteinet som vi antar er tropomyosin, ble også påvist i Krillolje, men ikke i olje fra *C. finmarchicus*.

Proteininnholdet i Krill og Calanusoljer ble bestemt ved bruk av aminosyreanalyse direkte på oljene. Det ble funnet et høyere proteininnhold i Krill enn i Calanusoljer. Det ble også gjennomført aminosyreanalyser på protein ekstrahert fra Krill og Calanusoljer der proteininnholdet ble funnet å være betraktelig mindre enn ved direkte analyse av oljene. Dette tyder på at aceton-heptan metoden brukt for å ekstrahere protein fra oljene ikke er optimal og andre metoder burde derfor vurderes for ekstraksjon og undersøkelse av allergifremkallende proteiner fra olje.

Contents

List of publications

Paper I

Vang, B., Pedersen, A. M., & 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 II

Vang, B., Mæhre, H. K., Jensen, I-J. & Olsen R. L. (2013) Detection of tropomyosin and determination of proteins in crustacean oils. *Food Chemistry* 141, 72–76.

Paper III

Pedersen, A. M., Vang, B., & Olsen, R. L. (2014) Oil from Calanus finmarchicus-Composition and Possible Use: A Review. *Journal of Aquatic Food Product Technology.* 23:6, 633-644.

Abbreviations

AH	Acetone-heptane
ALA	α-linolenic acid
ARA	Arachidonic acid
COX	Cyclooxygenase
C1 – 6	Copepodite stage 1 – 6
C5-D	Dormant copepodite stage 5
DGLA	Dihomo γ-linoleic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FAO	Food and Agricultural Organization of the United Nations
FFA	Free fatty acid
GLA	γ-linoleic acid
IFFO	The marine ingredients organisation
IgE	Immunoglobulin E
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acid
LOX	Lipoxygenase
N1 – 6	Nauplii stages 1 – 6
SDA	Stearidonic acid
SPE	Solid phase extraction
TAG	Triacylglycerol
TLC	Thin layer chromatography
TM	Tropomyosin

1. Introduction

Fish oils used to be a cheap commodity applied mainly for industrial purposes. In the 1960s approximately 80% of fish oil was hydrogenated to make margarine and shortening, while the remaining 20% was being used for industrial purposes like tanning of hides, emulsifiers and paint (Bimbo, 2000; Shepherd and Jackson, 2013). At the same time the marked for fish oil in the aquaculture industry and for direct human consumption, with the exception of cod liver oil, was virtually non-existent (**Figure 1**) (IFFO, 2013; Tocher, 2015).

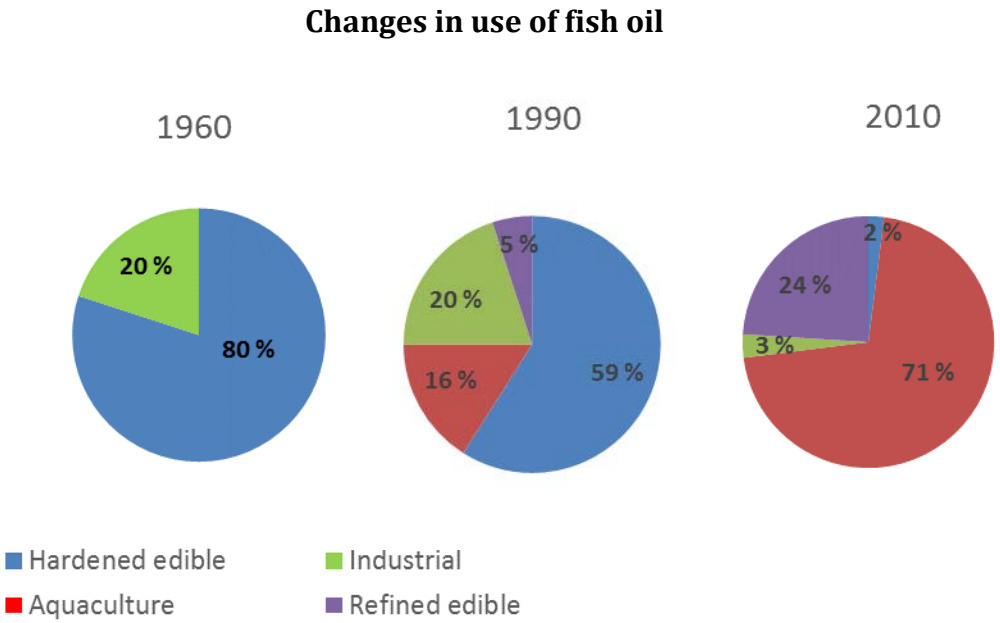

Figure 1. There have been massive changes in the use of fish oil. In 1960, 80% of fish oil was used to make hardened edible fat. By 2010, the use of fish oil in the aquaculture industry and for human consumption combined, made up 95% of the total fish oil consumption. Illustration from the marine ingredients organisation (IFFO), (2013).

During the 1980s and 1990s there was an increased demand for fish oil in aquaculture feeds due to the growth of marine aquaculture (**Figure 2**), especially farming of Atlantic salmon (*Salmo salar*) (Shepherd and Jackson, 2013). At the same time, there was increasing evidence for the many beneficial health effects connected with the consumption of the unique long-chain polyunsaturated ω-3 fatty acids (n-3 LC-PUFA) almost exclusively found in fish and other marine organisms (Bang, Dyerberg, and Nielsen, 1971; Herold and Kinsella, 1986; Tocher, 2015). The growing marked for n-3 LC-PUFA as a human nutritional supplement led

to further increase in the demand for fish oil (Food and Agricultural Organization of the United Nations (FAO), 2014). In addition, the adverse effects of industrial *trans* fatty acids created during partial hydrogenation of oils to make margarine began to be recognized (Booyens, Louwrens, and Katzeff, 1988; de Souza et al., 2015; Restrepo and Rieger, 2015). This also contributed to the dramatic shift in the use of fish oil since 1960 seen in **Figure 1**.

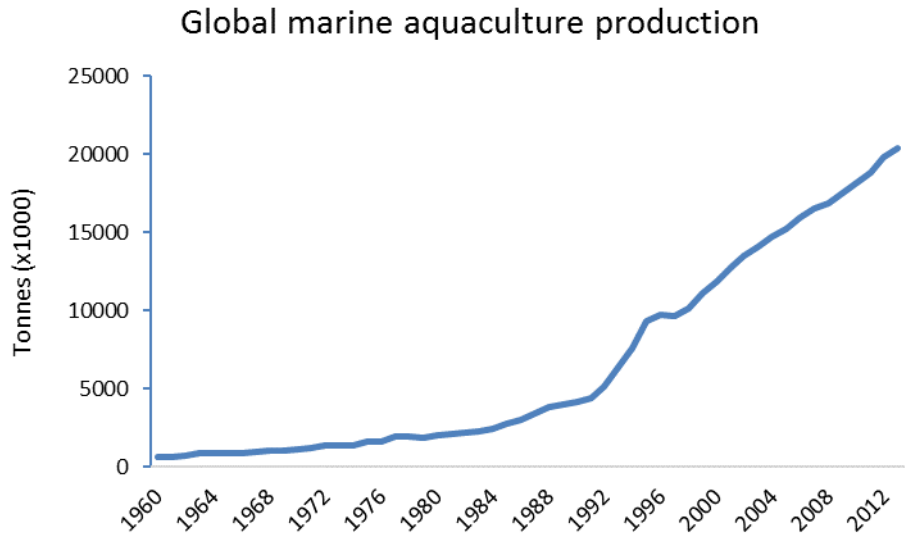

Figure 2. Global marine aquaculture production including fish, molluscs and crustaceans 1960-2013.

This development has continued and in the past decade both the global marine aquaculture production (**Figure 2**), including farming of salmonids, have had a massive growth (FAO, 2015). In 2010 the amount of fish oil used for aquaculture feed production accounted for 71 %, and of this more than ⅔ was used in salmon feed (Shepherd and Jackson, 2013). The same trend can be seen in the nutraceutical and functional food industries, which increased its market share of fish oil from modest 5 % in 1990 to 24 % in 2010 (**Figure 1**). The supply of fish oil is however limited and has remained relatively stable the past decade at approximately 1 million tonnes each year (**Figure 3**) (FAO, 1986; Hjaltason and Haraldsson, 2006; Shepherd and Bachis, 2014).

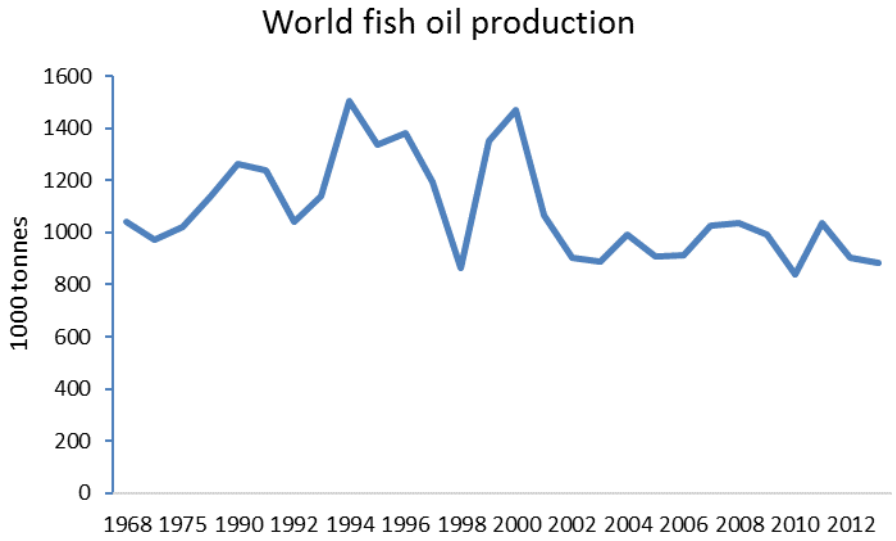

Figure 3. The annual global fish oil production has remained relatively stable at approximately 1 million tonnes in the past decade.

The competition for the limited supply of fish oil available is reflected in high prices particular during the last years (**Figure 4)** (FAO, 2014; Shepherd and Bachis, 2014) and has resulted in extensive search for alternative natural resources containing n-3 LC-PUFA. Possible sources of these fatty acids include microalgae cultured in large scale and genetically modified plants (Khozin-Goldberg, Iskandarov, and Cohen, 2011; Lenihan-Geels, Bishop, and Ferguson, 2013; Miller, Nichols, and Carter, 2010). Several studies have described the production of n-3 LC-PUFA in transgenic plants (Ruiz-Lopez, Haslam, Napier, and Sayanova, 2014; Venegas-Calerón, Sayanova, and Napier, 2010) and recently the first field trial of genetically engineered *Camelina sativa* capable of producing EPA and DHA was conducted (Usher, Haslam, Ruiz-Lopez, Sayanova, and Napier, 2015). Another possibility is to harvest on resources lower in the marine food web such as zooplankton (Miller, Nichols, and Carter, 2008). Zooplankton is a key component in the food web, and an important link between the primary producers of n-3 LC-PUFA (phytoplankton) and higher trophic levels species like fish and marine mammals. There has been increasing focus on the potential of the large amount of zooplankton biomass available at lower trophic levels in the oceans (Olsen, 2011; Olsen, Otterstad, and Duarte, 2008), as only a limited amount of the energy present is transferred to the next level. Therefore, assuming that harvesting of zooplankton is carried out in a precautionary and sustainable manner, these resources could be used for commercial products.

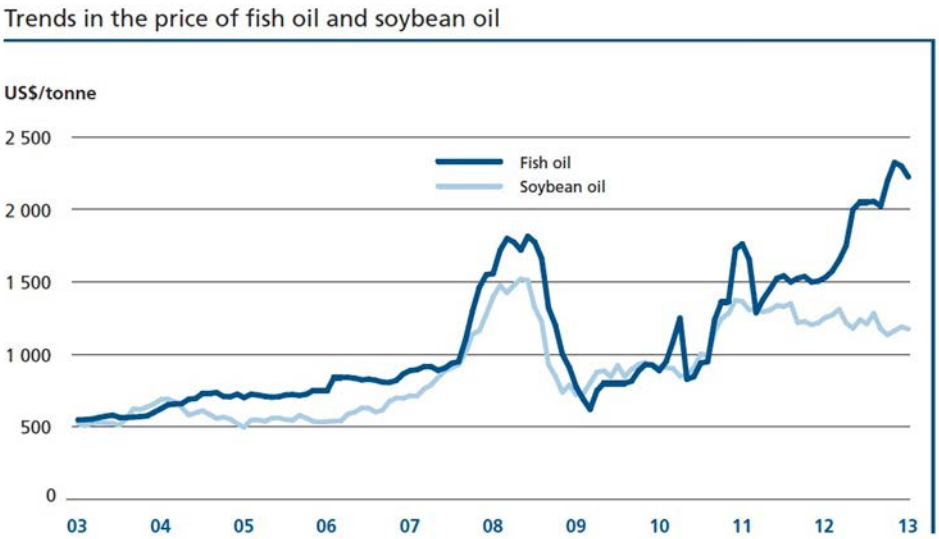

Figure 4. The development of fish oil and soybean oil prices from 2003-2013. The price of fish oil rose to new highs in 2013, while the price of soybean oil declined.

Antarctic krill (*Euphausia superba*) and *Calanus finmarchicus* are small shrimp-like crustaceans present in large amounts in the South and the North Atlantic Oceans, respectively (Greene et al., 2003; Nicol, Foster, and Kawaguchi, 2012). Krill and *C. finmarchicus* are currently being harvested primarily for the production of oils rich in n-3 LC-PUFA and the potent antioxidant astaxanthin (Guerin, Huntley, and Olaizola, 2003; Pashkow, Watumull, and Campbell, 2008). These oils are available mainly on the nutraceutical markets and so far most studies on health effects have been carried out with Krill oils (Grimstad et al., 2012; Konagai, Yanagimoto, Hayamizu, Han, Tsuji, and Koga, 2013; Piscitelli et al., 2011; Ramprasath, Eyal, Zchut, Shafat, and Jones, 2015; Tou, Jaczynski, and Chen, 2007; Yurko-Mauro, Kralovec, Bailey-Hall, Smeberg, Stark, and Salem, 2015). However, in recent years several articles regarding the health promoting effects of Calanus oil have been published as well. Small amounts of Calanus oil are found to reduce atherogenesis and body weight gain, in addition to improve glucose tolerance in mice feed Western type high fat diet (Eilertsen et al., 2012; Hoper et al., 2013; Hoper et al., 2014). Preliminary human trials regarding uptake and tolerability of Calanus oil have also been performed (www.calanus.com). Due to the novelty of Calanus oil further studies on properties of the oil as well as long-term effect of the oil are interesting.

2. Aims of the thesis

The overall aim of this thesis was to investigate oil recovery, properties and possible uses of oil derived from *C. finmarchicus.*

In **Paper I**, the objective was to investigate if enzymatic treatment of *C. finmarchicus* could improve oil recovery in a pilot scale production process, and to characterize the oil from *C. finmarchicus* with respect to lipid classes and fatty acid composition.

In **Paper II,** the aim was to investigate if the protein content in Calanus and Krill oils could be estimated using amino acid analysis. In addition, the possible presence of the main crustacean allergen, tropomyosin, in the commercial crustacean oil samples was investigated.

In **Paper III,** the aim was to review the composition and possible uses for Calanus oil.

3. Background

3.1. *Calanus finmarchicus*.

The herbivorous zooplankton *C. finmarchicus* (Gunnerus 1765) (**Figure 5**) is a calanoid copepod present in massive amounts in the North Atlantic Ocean (Aksnes and Blindheim, 1996) and have lipid-rich stages which can be harvested. The valuable n-3 LC-PUFA acids eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are produced by phytoplankton in the marine food web. Zooplankton acts as a link between phytoplankton and higher trophic levels organisms like marine fish and whales, and can therefore be a potential source of these fatty acids.

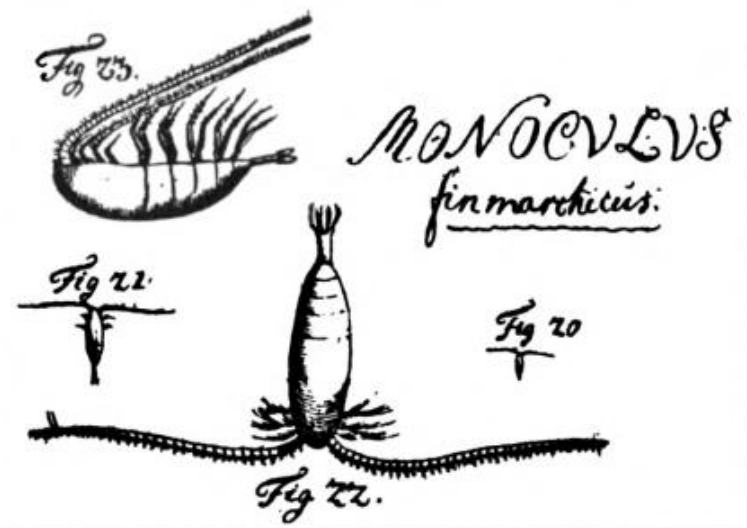

Figure 5. Sketches made in 1765 by the archbishop of Trondheim, Gunnerus, who was the first to describe and classify *Monoculus finmarchicus,* later changed to *Calanus finmarchicus*. Figure 20 in the sketch indicates the natural size of *C. finmarchicus.* Illustration from Damkaer, (2002).

C. finmarchicus spawns in early spring in the upper pelagic water layers (**Figure 6**). The eggs then hatch into nauplii, which develop through 6 moult stages (N1-N6) followed by 5 copepodite stages (C1-C5) before becoming adults (C6) while feeding on the blooming phytoplankton. The lipid-rich stages C4 and C5, migrate to depths of 500-2000 m in mid to late summer and go into hibernation during the winter months (Falk-Petersen, Mayzaud, Kattner, and Sargent, 2009; Lee, Hagen, and Kattner, 2006). The dormant over-wintering

stages (C5-D) awaken and develop into males and females and produce gonads in the first months of the year while ascending to the surface layers (Falk-Petersen et al., 2009). The lipid content and composition of *C. finmarchicus* at the different stages are well known from biological studies, and the copepodite stages C4 and C5, contain 15-64 % lipids on dry weight basis (Bergvik, Overrein, Bantle, Evjemo, and Rustad, 2012; Kattner and Krause, 1987; Lee et al., 2006; Scott, Kwasniewski, Falk-Petersen, and Sargent, 2000; Solgaard, Standal, and Draget, 2007).

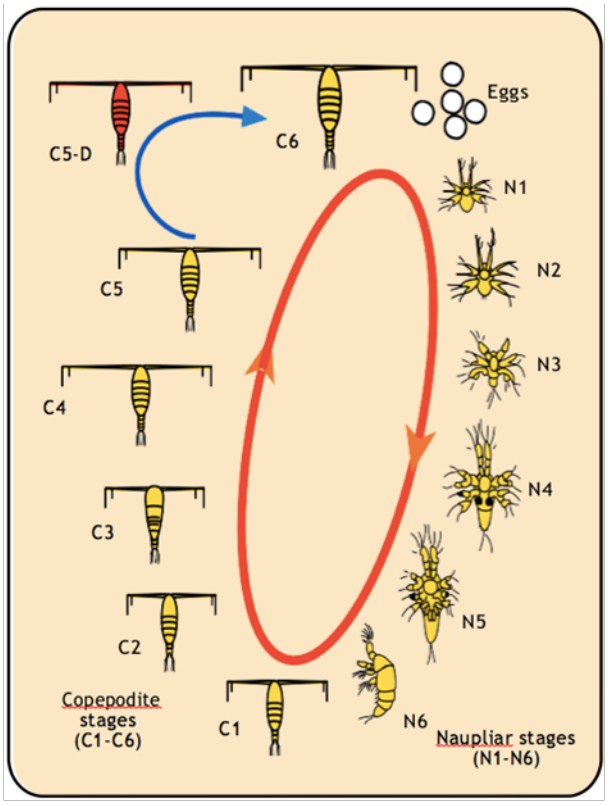

Figure 6. Schematic representation of the Calanus life cycle. The eggs hatch into nauplii, which develop through 6 moult stages (N1-N6) and then 5 copepodite stages (C1-C5). Stage C4 and C5 migrate to depths of 500-2000 m and enter a dormant stage (C5-D), before awakening and developing into adults (C6). Illustration from Fisheries Research service (FRS), (2004).

3.2. Lipid classes and fatty acid composition.

Calanus finmarchicus have a fatty acid composition largely reflecting its marine habitat and the phytoplankton it feeds on (Falk-Petersen et al., 2009; Kattner and Krause, 1987; Scott, Kwasniewski, Falk-Petersen, and Sargent, 2002). However, the lipid class composition of *C. finmarchicus* differs from most other marine oils. In fish- and krill oils, the main lipid classes

are triacylglycerol (TAG) and phospholipids, respectively. In *C. finmarchicus* however, the major lipid class is wax esters (Fraser, Sargent, and Gamble, 1989) (**Figure 7**).

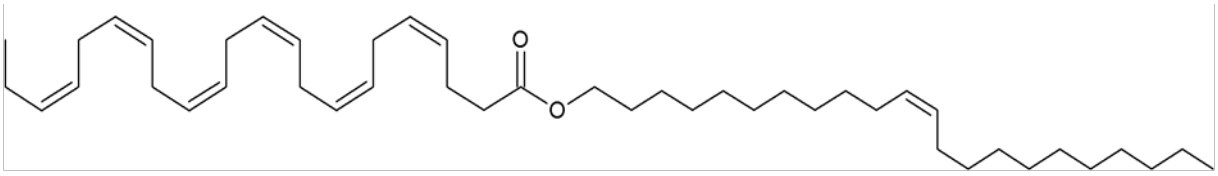

Figure 7. Wax ester consisting of DHA (22:6 n-3) esterified to a long chained monounsaturated alcohol (22:1 *n*-11)

Wax esters consist of simple esters of long-chain primary alcohols and long-chain fatty acids and are utilized as energy storage and to regulate buoyancy (Falk-Petersen et al., 2009; Lee et al., 2006; Visser and Jónasdóttir, 1999). In addition to approximately 85 % wax esters, the lipid class composition of *C. finmarchicus* also includes TAG, phospholipids, sterols and free fatty acids (FFA) (**Table 1)** (Falk-Petersen, Sargent, and Tande, 1987).

Table 1.

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

	% of total lipid			
Lipid class	*June*[1]	*October*[1]	*March*[1]	*March*[2]
Triacylglycerol	8.9	1.3	nd	3.1
Sterols	1.2	2.6	3.2	1.4
Free fatty acids	0.2	nd	1.7	nd
Wax esters	85.4	88.1	84.9	73.8
Phospholipids	4.2	7.3	10.3	21

nd = not detected. [1]: Falk-Petersen et al., 1987 [2]: Fraser et al., 1989

In biological and ecological studies were live *C. finmarchicus* are placed directly in liquid N_2 or organic solvents only minor amounts of FFA in have been reported (**Table 1**) (Falk-Petersen et al., 1987; Fraser et al., 1989; Ohman, 1996; Scott et al., 2000). However, other studies have found a high content of FFA in lipids extracted from *C. finmarchicus* and other zooplankton (Marker, Andreassen, Arashkewich, and Hansen, 2003; Parrish, 1988). The elevated content of FFA in certain development stages of zooplankton have been related to intensive feeding activities (Marker et al., 2003; Scott et al., 2000). In addition, *C.*

finmarchicus and other zooplankton species contain endogenous enzymes that can cause autolysis rapidly after death if they are not inactivated. Several studies have shown that the enzymes are active even at moderate freezing temperatures (-20 to -15ºC), leading to enzymatic degradation of phospholipids and formation of FFA during long time storage (Bergvik et al., 2012; Kolakowska, 1986; Ohman, 1996; Sasaki and Capuzzo, 1984). To avoid enzymatic hydrolysis of lipids in *Calanus* spp., Ohman (1996) recommended rapid freezing and storage below −70◦C when immediate lipid extraction is not possible. An alternative could be to inactivate the autolytic enzymes by heat treatment immediately after harvest (Bergvik et al., 2012; Kolakowski and Sikorski, 2000).

The n-3 PUFA may account for 40 – 45 % of total fatty acids in *C. finmarchicus* late copepodite stages C4 and C5 (Bergvik et al., 2012; Kattner and Krause, 1987). In addition to EPA and DHA, a substantial amount of stearidonic acid (SDA, 18:4 n-3) is also present. Stearidonic acid is an intermediate metabolite between α-linolenic acid (ALA) and EPA in the n-3 biosynthetic pathway (**Figure 8**) and have been reported to be a superior precursor to EPA compared to ALA (Brenna, Salem Jr, Sinclair, and Cunnane, 2009; Calder, 2012; James, Ursin, and Cleland, 2003). The reason for this is that SDA is not dependent on the rate-limiting enzyme Δ6 desaturase, in the conversion from ALA to the EPA (Calder, 2012; Lenihan-Geels et al., 2013). However, little or no effect have been seen on DHA blood levels by supplements with ALA, SDA or EPA, indicating that this part of the LC-PUFA biosynthesis pathway is not very active in humans (Arterburn, Hall, and Oken, 2006; Brenna et al., 2009; Calder, 2012).

Genetically, humans have evolved to consume a diet with a balanced ratio of 1:1 of n-3 and n-6 PUFA (Simopoulos, 2008). However, many modern "Western diets" have a high content of n-6 PUFA and a low content of n-3 PUFA. This creates an unbalance in the fatty acids and their role in lipid metabolism and in regulating inflammatory responses (Simopoulos, 2008). The conversion of C-18 n-3 and n-6 fatty acids to C-20 LC-PUFA and their respective eicosanoids are dependent on the same enzymes in the biosynthesis pathways (**Figure 8**) creating a competition between the two series (Arterburn et al., 2006; Brenna et al., 2009; Calder, 2014). The n-3 and n-6 LC-PUFA and their metabolites, are involved in many biochemical processes, and their actions and mechanics are complex (Calder, 2012). However, generally the n-3 LC-PUFA, EPA, DHA and their potent metabolites have been associated with beneficial health effects on cardiovascular disease, inflammatory diseases, neural development and some forms of cancer (reviewed by Calder, 2014; Mori, 2014; Tocher, 2015). Whereas n-6 derived metabolites are found to have more inflammatory, atherogenic

and prothrombotic effects (Schmitz and Ecker, 2008; Simopoulos, 2008), underlining the importance of increased intake of n-3 PUFA in the diet (Calder, 2014; Simopoulos, 2008).

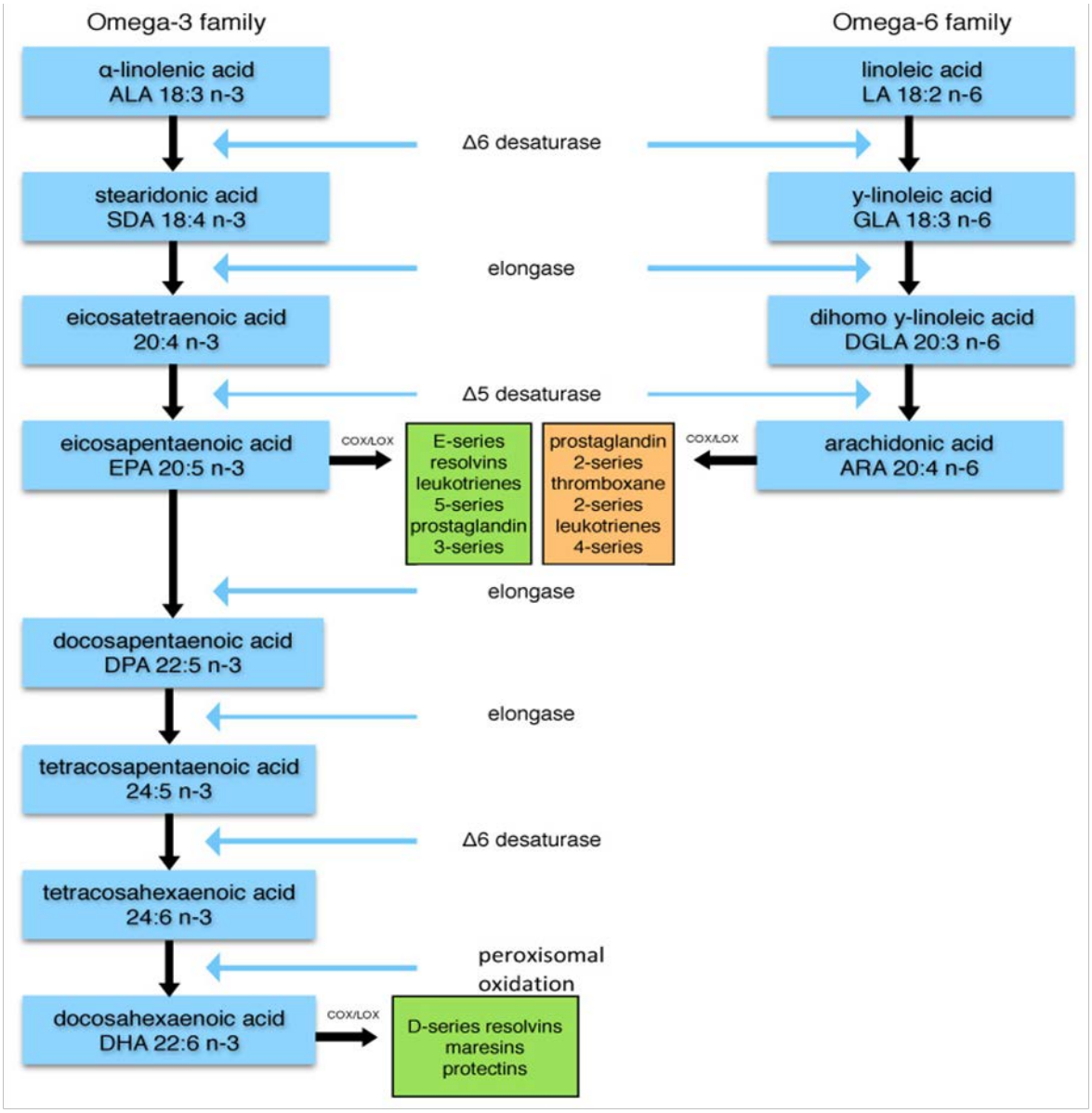

Figure 8. Simplified metabolic pathway with elongation and desaturation of n-3 and n-6 PUFA and conversion to their metabolites by cyclooxygenase (COX) and lipoxygenase (LOX). The same enzymes are involved in the n-3 and n-6 pathways, creating a competition between the two series, and the major limiting step in the conversion from ALA to the EPA and DHA is Δ6 desaturase. Metabolites derived from EPA and DHA have well documented health beneficial effects, while eicosanoids from n-6 family are more inflammatory, atherogenic and prothrombotic. Illustration by Peter Andre Jensen

3.3. Astaxanthin

Astaxanthin is a carotenoid fat-soluble pigment with an orange-red color found in a variety of plants and animals including salmonids, and crustacean species like shrimp and lobster

(Choubert, 2001; Hussein, Sankawa, Goto, Matsumoto, and Watanabe, 2006). The structure of astaxanthin (**Figure 9**) differs from other carotenoids due to the two hydroxyl groups on each ring, resulting in enhanced antioxidant properties (Fassett and Coombes, 2009; Terao, 1989). Astaxanthin exists as diester, monoester and in free form, (Choubert, 2001), and are commonly found bound to proteins as carotenoproteins (Matsuno, 2001). In *C. finmarchicus,* astaxanthin is the main carotenoid and is mostly present as mono and diesters (Foss, Renstrøm, and Liaaen-Jensen, 1987). It has been suggested that one of the central functions of astaxanthin in calanoid copepods is to protect the storage lipids against oxidation. In addition, astaxanthin also plays a part in lipid metabolism (Lotocka and Styczynska-Jurewicz, 2001; Sommer, Agurto, Henriksen, and Kiorboe, 2006). When lipids are extracted form *C. finmarchicus*, the lipophilic astaxanthin will accumulate in the oil, giving the oil a deep red color. Astaxanthin content in oil obtained from *C. finmarchicus* at relevant harvesting stages, have been reported to be 500 - 1600 ppm (Bergvik et al., 2012; Fisher, Kon, and Thompson, 1952; Hertrampf and Piedad-Pascual, 2000; Pedersen, 2007) with a high stability when stored with an inert atmosphere or at 4°C (Pedersen, 2007).

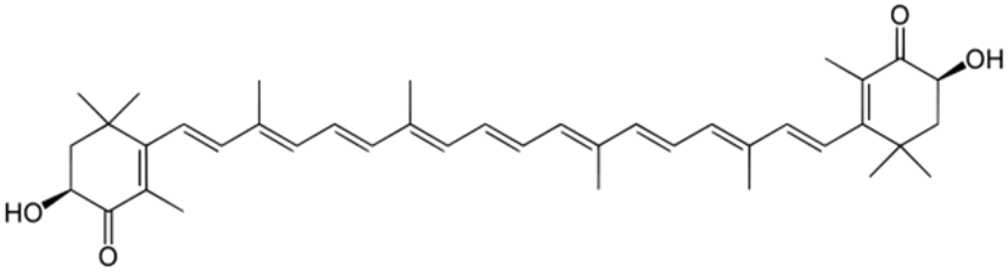

Figure 9. The carotenoid astaxanthin contains two hydroxyl groups on each ring structure. The powerful antioxidant occurs naturally in a variety of species including crustaceans and fish.

3.4. Extraction of oil

The traditional method for extraction of fish oils is cooking of the raw material followed by centrifugations, whereas plant oils are often extracted using organic solvents (Gunstone, 2004). Industrial experience has shown that the yield is often low when a traditional fish oil production method is employed for extraction of oil from *C. finmarchicus*. It has been reported that organic solvents can be used for efficient extraction of oil from Antarctic krill (Gigliotti, Davenport, Beamer, Tou, and Jaczynski, 2011). However, solvent extraction in large scale oil production has several disadvantages, including emissions of volatile organic compounds, recycling costs (Rosenthal, Pyle, and Niranjan, 1996). An enzymatic hydrolysis

process utilising enzymes that disrupt tissue, resulting in release of lipids is however a more environmentally friendly and safe method for efficient release of oils. This method has been shown to function well on fish raw material (Dumay, Donnay-Moreno, Barnathan, Jaouen, and Bergé, 2006) as well as plant seeds (Rosenthal et al., 1996).

3.5. Allergy

Food allergy is defined as an specific immune response that occurs reproducibly on exposure to a given food, resulting in adverse health effects (National Institute of Allergy and Infectious Diseases, 2010) ranging from skin rash, oral allergy syndrome and digestive symptoms to severe asthma and life-threatening anaphylaxis (Lehrer, Ayuso, and Reese, 2003; Pedrosa, Boyano-Martínez, García-Ara, and Quirce, 2015; Waring, Daul, deShazo, McCants, and Lehrer, 1985). Components that cause allergy are termed allergens, and almost all allergens are proteins (Bannon, 2004). Most allergens themselves are harmless, and it is the immuneresposes to the allergen that cause damage (Widmaier, Raff, and Strang, 2004a).

Extraction of oil from a vegetable or animal source may result in proteins and peptides passing in to the oil, and several studies have shown that allergenic proteins may be found in oils extracted from peanuts, soybeans, sunflower seeds, and gourmet nuts (Errahali et al., 2002; Ramazzotti et al., 2008; Teuber, Brown, and Haapanen, 1997; Zitouni et al., 2000). Proteins and peptides can be found in both crude and refined oils (Hidalgo and Zamora, 2006; Rigby et al., 2011), however, traditional refining steps like degumming can greatly reduce the amount of protein present (Rigby et al., 2011). Oils extracted from Antarctic krill and *C. finmarchicus* are usually not extensively refined since they contain desirable components like phospholipids and/or astaxanthin which are lost during traditional refining steps (Gunstone, 2004). In addition, these crustacean oils have a low content of environmental pollutants due to their short life span (Diel and Tande, 1992; Siegel, 1987) and position at the base of the food web (Borga, Gabrielsen, and Skaare, 2001; Fisk, Stern, Hobson, Strachan, Loewen, and Norstrom, 2001), and therefor they are not in need of the same degree of refining that is required by fish oils (Maehre, Jensen, Elvevoll, and Eilertsen, 2015). Crustacean oils may therefore contain proteins that could cause reactions in people with crustacean allergies. Very few studies have been carried out on the allergenicity of fish oils (Mark, Beaty, and Slavin, 2008) and to the best of our knowledge, the presence of allergens in crustacean oils have not been reported previously.

Crustaceans are one of eight foods or food groups that are thought to account for more than 90% of all immunoglobulin E (IgE) food allergies world-wide (Hidalgo and Zamora, 2006).

The occurrence of shellfish allergy varies between countries and regions, and is usually higher in areas were the consumption of shellfish is high, and apparently about 2 % of the adult US population is affected (Kamath, Abdel Rahman, Komoda, and Lopata, 2013; Lopata, O'Hehir, and Lehrer, 2010; Pedrosa et al., 2015; Sicherer, Munoz-Furlong, and Sampson, 2004). Several allergens including sarcoplasmatic calcium-binding protein, myosin light chain, arginine kinase and troponin C among others, have been found in crustacean species (Ayuso et al., 2008; Bauermeister et al., 2011; García-Orozco, Aispuro-Hernández, Yepiz-Plascencia, Calderón-de-la-Barca, and Sotelo-Mundo, 2007; Shiomi, Sato, Hamamoto, Mita, and Shimakura, 2008). However, the myofibrillar protein tropomyosin (TM) (**Figure 10a**) have been documented to be the main allergen in species like shrimp, lobster, crab, and Antarctic krill (Leung, Chen, Gershwin, Wong, Kwan, and Chu, 1998; Leung, Chen, Mykles, Chow, Li, and Chu, 1998; Motoyama et al., 2008; Nakano, Yoshinuma, and Yamada, 2008; Reese, Ayuso, and Lehrer, 1999). Tropomyosin is present in much higher quantity than other shellfish allergens due to its role in muscle function (**Figure 10b**) (Kamath et al., 2013) and is a commonly used biomarker for detection of shellfish allergens (Fuller, Goodwin, and Morris, 2006; Kamath et al., 2013; Shibahara et al., 2007).

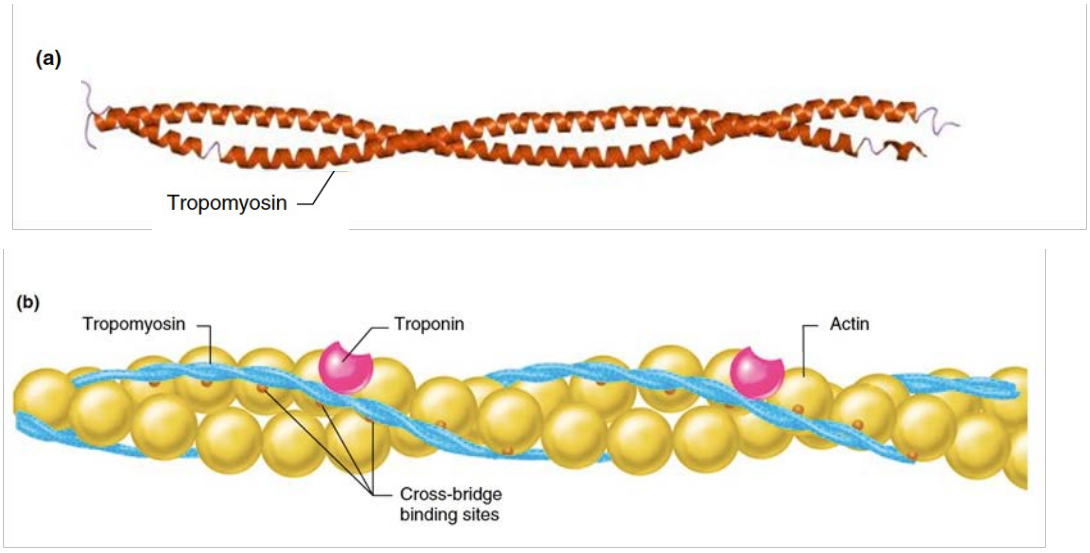

Figure 10 (**a**) Tropomyosin is a myofibrillar protein with an α-helix coiled-coil structure. Illustration from Brown et al., (2005). (**b**) Two chains of tropomyosin, held in place by troponin, are wrapped around actin thin filament to regulate the access of cross-bridges to binding sites on actin. Attachment of myosin heavy chain cross-bridges to the actin filament leads to the sliding-filament mechanisms of muscle contraction. Illustration from Widmaier, Raff, and Strang, (2004b).

Allergenicity of proteins is caused by the presence of allergenic sites (epitopes) that bind to IgE (Damodaran, 2008). The IgE-binding epitope is defined as the smallest sequence of amino acids that yields maximal IgE binding (Lehrer et al., 2003), and cross-reactivity between species occurs due to the presence of similar or identical IgE-binding epitopes (Reese et al., 1999). It has been documented that allergic epitopes on TM are conserved among invertebrates including not only shellfish, but also arthropods, which can result in cross-reactivity between crustaceans species like shrimp, lobster, crab, crawfish and krill, as well as molluscs, dust mite and cockroach (Ayuso, Reese, Leong-Kee, Plante, and Lehrer, 2002; Besler, Daul, and Leung, 2001; Leung et al., 2014; Leung, Chow, Duffey, Kwan, Gershwin, and Chu, 1996; Nakano et al., 2008; Pedrosa et al., 2015; Reese et al., 1999). However, in general crustacean allergic individuals do not react to TM from vertebrates like poultry and mammalians (Besler et al., 2001). Tropomyosin from several different shrimp species have been identified and classified (Fernandes, Costa, Oliveira, and Mafra, 2015; Leung et al., 2014) including Pen a 1 from Northern brown shrimp (*Penaeus aztecus*) were eight different IgE-binding epitopes have been identified in five different regions. The epitopes in Pen a 1 varied from 8-15 amino acids depending on region and individual (Lehrer et al., 2003).

Different processing methods like heat-treatment, enzymatic hydrolysis, Maillard reaction and physical processing can influence the allergenicity of proteins, resulting in either increased or reduced allergenic properties (Nowak-Wegrzyn and Fiocchi, 2009; Thomas et al., 2007). Increased allergenicity can be a result of the unmasking of epitopes while reduced allergenicity can be a result of altered conformational protein structure, cleavage of epitopes or removal of allergenic protein (Jiménez-Saiz, Benedé, Molina, and López-Expósito, 2014). Tropomyosin is, however, able to withstand heat-treatment as well as other forms of food processing (Pedrosa et al., 2015), and studies have reported that this is attributed to the stable α-helix coiled-coil structure of TM (Kamath et al., 2013). However, an enzymatic hydrolysis of peptide bonds in proteins (proteolysis), shortening the proteins into peptides and amino acids could lead to cleavage of the allergenic epitopes. This may result in a decrease or even elimination of allergenicity depending on the type of protease used and the degree of hydrolysis (Damodaran, 2008).

4. Methodological considerations

4.1. Raw material

Lipid content and composition of *C. finmarchicus* vary throughout the different developmental stages and harvesting locations. *C. finmarchicus* used in both **paper I** and **paper II** had been harvested off the coast of Norway during spring and summer months. According to the producer, Calanus AS, the catch was drained free of excess water and frozen in blocks of 25 kg. After approximately 3 hours in the vessel's freezing facilities, the blocks reached a core temperature of -20ºC. The raw material was kept at -20ºC until being processed to oil and meal (Pedersen, Vang, and Olsen, 2014). Krill meal and Superba™ Krill Oil was produced from Antarctic krill (*E. superba)* harvested in South Atlantic Ocean. The krill harvest was cooked and dried on the vessel to prepare krill meal, and Krill oil was produced by subjecting the krill meal to ethanol extraction (Aker Biomarine Antarctic AS, 2011).

4.2. Lipid extraction by enzymatic hydrolysis

In **paper I,** three experiments were carried out as outlined in **Figure 11**, each using 350-400 kg of partially thawed and grinded *C. finmarchicus.* The experiments were carried out in a pilot scale study to better simulate an industrial extraction of oils. Several laboratory experiments were conducted in advance to determine optimal pH, holding time and adequate enzyme dosage.

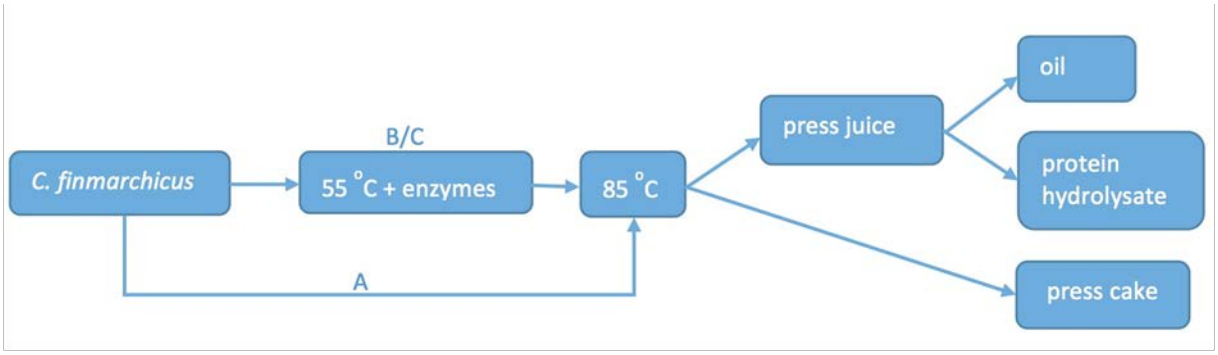

Figure 11. Flow chart of the lipid extraction process using traditional fish oil technology (A) and enzymatic hydrolysis (B and C). After heating to 85ºC the raw material was separated using a centrifugal decanter to press juice and press cake. An oil separator was then used to obtain oil and protein hydrolysate from the press juice.

Experiment A was used as a control simulating a traditional fish oil production, while experiments B and C implemented the use of enzymatic hydrolysis to improve the oil recovery. Alcalase®, a non-specific endoprotease was applied in experiment B. While in experiment C, Flavourzyme® containing both endoproteases and exopeptidases, was used in combination with Alcalase®. All three batches were heated to 85ºC to inactivate enzymatic activities, and subsequently separated to press juice and press cake using a centrifugal decanter. An oil separator was then used to obtain oil and protein hydrolysate from the press juice.

Alcalase® and Flavourzyme® have optimal pH working conditions of 6.5 – 8.5 and 5.5 – 7.5 respectively, depending on substrate. The pH of minced *C. finmarchicus* was determined to 7.5, and no pH adjustment was therefore needed. Optimal temperature conditions for Flavourzyme® is approximately 50 – 55ºC and between 55 – 70ºC for Alcalase®, a temperature of approximately 55ºC and a holding time of one hour was chosen in the experiment. The recommended dosage for Alcalase® at these conditions are between 1 - 1.5g/kg raw material. Slightly higher concentrations of the commercial enzymes were used in the experiments.

4.3. Extraction of proteins from oils

There is apparently no established good method for extracting proteins present in oils (Crevel, Kerkhoff, and Koning, 2000; Martín-Hernández, Bénet, and Obert, 2008). Proteins are commonly extracted with different aqueous buffers (Crevel et al., 2000; Ramazzotti et al., 2008; Rigby et al., 2011) or precipitated using acetone or a combination of acetone and hexane (Hidalgo, Alaiz, and Zamora, 2001; Martín-Hernández et al., 2008). After testing several different methods by spiking the oil with protein (bovine serum albumine) we found that a combination of acetone-heptane (AH), based on the acetone/hexane method described by (Martín-Hernández et al., 2008), worked best with Calanus- and Krill oils. However, due to the high amounts of phospholipids in Krill oil and wax esters in Calanus oil, a combination of different solvents were used to remove lipids from the extracted protein samples.

4.4. Determination of protein content by amino acid analysis

When determining the protein content in oils, amino acid analysis of extracted proteins is often recommended instead of using colorimetric methods (Hidalgo et al., 2001; Martín-Hernández et al., 2008; Ramazzotti et al., 2008). However, extracting protein from oils

require several washing steps that may lead to loss of peptides and proteins. To obtain a more accurate determination of the proteins present, the amino acid analysis was also executed directly on the oils without prior precipitation of the proteins. Protein content in the samples was calculated as the sums of the individual molecular weight of the amino acid residues after the deduction of the molecular weight of water according to recommendations by FAO (2003). To our knowledge, amino acid analysis has not previously been used directly on oils to determine protein content

4.5. Detection of tropomyosin

IgE cross-reactivity between shrimp TM and other crustacean TM have been well documented (Leung et al., 2014; Leung et al., 1996; Nakano et al., 2008). During preliminary Western blot analysis, a commercial polyclonal TM antibody from South American shrimp produced in mouse (Biorbyt Limited, Cambridge, UK) gave a strong reaction to both krill meal and *C. finmarchicus.* This antibody was therefore chosen to analyze for TM in protein extracted from Calanus- and Krill oils.

5. Main results and general discussion

5.1. Oil recovery with enzymatic hydrolysis

The results from our experiments in **paper I** demonstrated that enzymatic hydrolysis greatly improves the lipid recovery (**Figure 12**). When using standard fish oil technology, the lipids in *C. finmarchicus* were not efficiently extracted. Only 4.5% of the lipids present were recovered as oil, thus leaving large amounts of lipids in the press cake and the protein hydrolysate fractions. With the enzymatic treatments, 76 and 83% of the lipids in *C. finmarchicus* were recovered as oil and the amount of press cakes was greatly reduced. The high lipid recovery was also reflected by low lipid content in the protein hydrolysates and press cakes acquired.

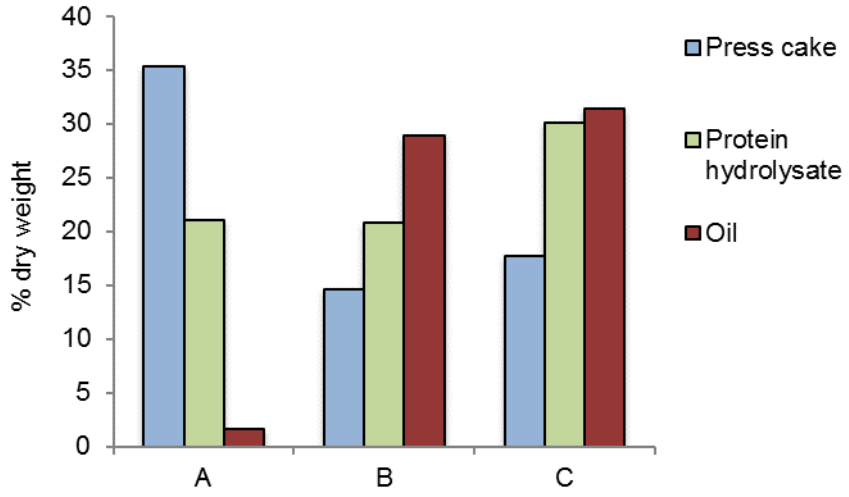

Figure 12. The amount of press cake, protein hydrolysate and oil obtained after traditional fish oil process (A) and after enzymatic hydrolysis (B and C). The enzymatic hydrolysis greatly improved the lipid recovery while simultaneously decreasing the amount of press cake.

Most of the lipids in the relevant development stages (C4 and C5) of *C. finmarchicus* are stored in an oil sac surrounded by a single layer of epithelial cells in the body cavity (Falk-Petersen et al., 1987; Lee et al., 2006). The enzymatic treatment resulted hydrolysis of proteins to peptides and amino acids, and the disruption of tissue led to release of lipids from the tissue structure. Increased lipid release followed by tissue disruption by enzymatic hydrolysis has been reported for sardines (*Sardina pilchardus*), cuttlefish (*Sepia officinalis*)

and oilseeds among others (Dumay et al., 2006; Kechaou et al., 2009; Rosenthal et al., 1996). According to Damodaran (2008). The combined use of Alcalase® and Flavourzyme® (experiment C) led to a slightly higher oil yield, increased amount of protein hydrolysate and less lipids in the protein hydrolysates than use of Alcalase® alone. This was probably a result of a higher degree of hydrolysis due to the combination of endoproteases (Alcalase®) and exopeptidase (Flavourzyme®). The higher all-round output in experiment C might also be related to this, as a more soluble solution will result in less loss of raw material during production.

5.2. Lipid analysis

In **paper I**, oil from *C. finmarchicus* was subjected to solid phase extraction (SPE), and the lipid fractions obtained were analyzed by thin layer chromatography (TLC) (**Figure 13**). The results showed a large amount of wax ester in the oil confirming results from biological studies (Falk-Petersen et al., 1987; Fraser et al., 1989; Scott et al., 2000). Free fatty acids could also clearly be detected, but only trace amounts of phospholipids were found.

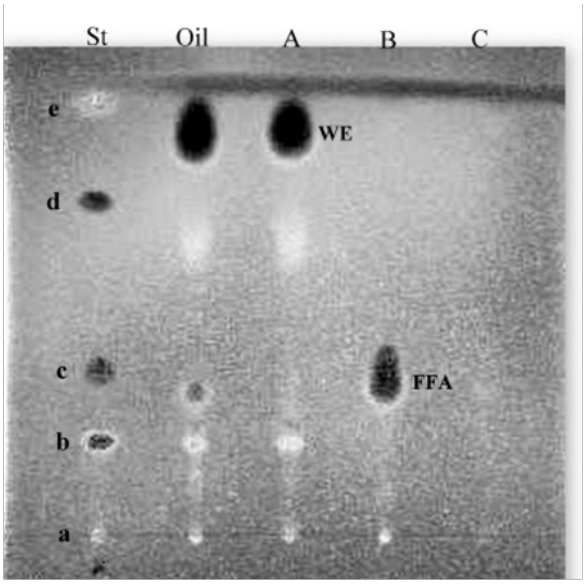

Figure 13. TLC of lipids separated by SPE. St.: fatty acid standard 18-5 A contained lecithin (a); cholesterol (b); oleic acid (c); TAG, triolein (d); and cholesteryl oleate (e). Oil: oil from *C. finmarchicus*. Lane A: neutral lipids, including a large amount of was ester (WE). Lane B: a substantional amount FFA. Lane C: only trace amounts of phospholipids were detected.

The fatty acid composition of *C. finmarchicus* oil and of the FFA and wax ester fractions were determined by gas chromatography (**Table 2**). Substantial amounts of n-3

PUFA including the SDA, EPA and DHA were present in the oil as well as in the FFA and wax ester fractions, however only small amounts of linoleic acid (18:2 n-6) were found. Demonstrating that oil from *C. finmarchicus* is a good source for n-3 LC-PUFA since EPA and DHA constituted about 22% of the total fatty acids.

The composition of the FFA fraction was dominated by the fatty acids 16:0, EPA, and DHA, which are also the major fatty acids in the phospholipid fraction from *C. finmarchicus* (Fraser et al., 1989; Scott et al., 2002). Results from studies on *C. finmarchicus* and other zooplankton species clearly suggest that FFA originate in particular from hydrolysis of phospholipids (Bergvik et al., 2012; Ohman, 1996; Saether, Ellingsen, and Mohr, 1986), while the wax ester fraction apparently is not influenced (Overrein, 2010; Saether et al., 1986). This may be the reason for the difficulties detecting any phospholipids during our TLC analysis (**Figure 13**).

The wax ester fraction contained relatively large amount of esterified SDA and EPA but lower levels of DHA compared to previously reported results from biological studies (Fraser et al., 1989; Lee et al., 2006).

Table 2. Fatty acid composition (%) in oil derived from *C. finmarchicus* as well as the FFA and wax ester fractions obtained by SPE.

Fatty acid	Oil	FFA	Wax ester
14:0	14.7	4.7	15.4
16:0	9.3	21.2	7.6
16:1 n-7	6.7	4.1	6.4
16:2 n-4	1.0	0.6	0.9
16:3 n-4	2.5	1.7	2.4
18:0	1.0	7.9	0.8
18:1 n-9	4.1	2.9	4.0
18:1 n-7	2.5	1.8	2.4
18:2 n-6	0.9	1.1	0.8
18:3 n-3	1.3	1.2	1.1
18:4 n-3	12.4	8.0	11.4
20:1 n-9	5.9	1.3	6.0
22:1 n-9	9.0	1.7	8.8
20:5 n-3	14.4	17.0	11.6
24:0	0.7	0.5	0.6
24:1 n-9	0.8	0.6	0.6
22:6 n-3	7.7	18.3	4.1
Σ SAT	25.7	34.3	24.4
Σ MUFA	44.1	30.0	40.4
Σ PUFA	40.2	48.0	32.3
n-3	35.8	44.6	28.1
n-6	0.9	1.1	0.8

5.3. Protein extraction and protein content in oils

In **paper II,** the proteins present in the oil samples were extracted using a modified version of the AH method described by Martín-Hernández (2008). Analysis of the extracted proteins indicated that the Krill oil contained 0.19 mg protein/g oil, while the Calanus oil contained 0.02 mg/g. However, the extensive washing procedure may have resulted in loss of amino acids, peptides and proteins from the precipitate. To investigate possible loss during the extraction procedure, the protein content was determined by direct amino acid analysis of the oils. The results showed that Krill- and Calanus oils contained 5.68 and 0.18 mg protein/g oil, respectively (**Table 3**). These results show that only 3.3% of proteins in Krill oil and 12% of the proteins in Calanus oil were recovered in the precipitates. In addition to loss of proteins during the washing steps, inefficient precipitation may also have contributed to the low yield. The AH method used for extraction of proteins from oil samples was therefore further investigated by spiking the Calanus oil with different amounts of protein (bovine serum albumin). The results suggests that approximately 1/3 of the protein added to the oil was recovered by the AH extraction method. These results clearly demonstrate the inefficiency of the AH method to recover proteins from Calanus and Krill oils.

To avoid inaccurate results due to low recovery of intact and degraded proteins during extraction, the possibility of quantitative determination of proteins by direct amino acid analysis on the oils should be investigated further. The protein content should then be adjusted for the possible presence of free amino acids in the oils.

Table 3. Protein content in the Superba^{TM} Krill Oil and the Calanus® Oil and in the protein pellet extracted from the oils by the acetone-heptane method. The protein content was based on amino acid analysis and expressed as mg/g oil.

	Protein pellet	Oil
Superba^{TM} Krill Oil	0.19 mg/g ± 0.01 mg/g	5.68 ± 0.20 mg/g
Calanus® Oil	0.02 mg/g ± 0.01 mg/g	0.18 ± 0.00 mg/g

5.4. Allergens present in Calanus and Krill oils

In **paper II**, Western blotting analysis (**Figure 14 B**) of proteins extracted from *C. finmarchicus* (lane 2), krill meal (lane 5), Krill oil (lanes 6 and 7) and shrimp muscle (lane 8) reacted with the commercial primary antibody produced against shrimp TM. A protein band with a molecular weight of about 38 kDa, corresponding to that of TM (Nakano et al., 2008;

Reese et al., 1999) can be seen in the samples, and based on the known allergenic cross-reactivity of TM in different crustacean species, it is reasonable to assume that this protein band is TM. In the shrimp muscle sample (lane 8), the TM antibody also showed weak cross-reactivity with protein bands at approximately 40 and 45 kDa. The immunoreactive 45 kDa band can also be seen in the *C. finmarchicus* (lane 2), krill meal (lane 5) and Krill oil samples (lanes 6 and 7). The identities of these bands are uncertain, however Kamath et al., (2013) observed the presence of higher molecular weight TM bands from shellfish, and suggested that it may be caused by Maillard reaction due to high amount of lysine residues reacting with sugar moieties. Other studies have also reported occurrence of band smearing and formation of high molecular weight bands of TM from scallop, squid and shrimp extracts (Nakamura, Sasaki, Watanabe, Ojima, Ahn, and Saeki, 2006; Nakamura, Watanabe, Ojima, Ahn, and Saeki, 2005; Shriver, Yang, Chung, and Percival, 2011). It is possible that a heating process can generate smaller and larger IgE-binding fragments or aggregates (Lopata et al., 2010), and according to some reports, the Maillard reaction can for shellfish result in the formation of new epitopes (Nowak-Wegrzyn and Fiocchi, 2009; Thomas et al., 2007).

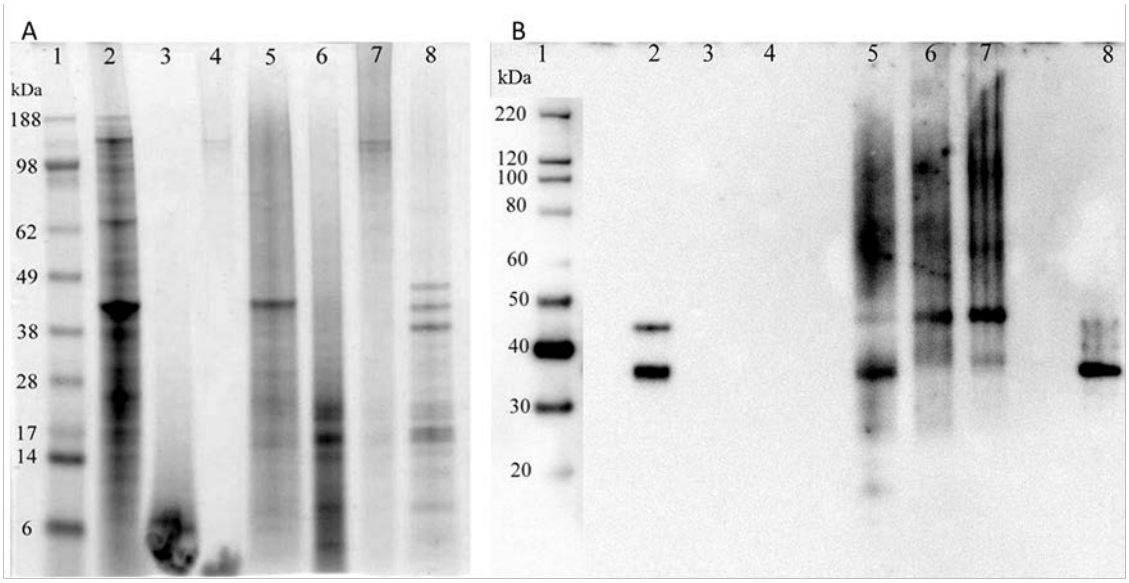

Figure 14 Gel electrophoresis analysis (**A**) and Western blot analysis (**B**) of proteins extracted from *C. finmarchicus*, Calanus® Oil and Calanus® Oil capsules are shown in lanes 2, 3, and 4 respectively. Proteins extracted from Antarctic krill meal, Superba^{TM} Krill Oil and Superba^{TM} Krill Oil capsules are shown in lanes 5, 6 and 7. Lane 8: Proteins extracted from shrimp muscle. SeeBlue® Plus2 Pre-Stained Protein Standard (**A**) and MagicMarker^{TM} XP Western Protein Standard (**B**) were used as molecular weight markers.

In **figure 14 B** (lanes 3 and 4), no cross-reactivity between the TM antibody and proteins extracted from the Calanus oil samples could be detected. The reason for this could be that the proteins in the Calanus oil samples are more degraded compared to the proteins in

the Krill oil samples as seen in **Figure 14 A**. In the Calanus oil samples, the Coomassie staining indicated the presence of only poorly resolved low molecular weight proteins (<10 kDa), and apparently the ability to cause IgE responses declines with lower molecular masses and peptides below 3.5 kDa lack this capacity (Thomas et al., 2007). The poor resolution of proteins extracted from the Calanus oil samples (**Figure 14 A**, lanes 3 and 4) may be due to residual lipids present in the samples (Rigby et al., 2011).

It is well known that both Antarctic krill and *C. finmarchicus* are very susceptible to autolytic degradation unless endogenous enzymes are rapidly inactivated post mortem (Bergvik et al., 2012; Overrein, 2010; Saether et al., 1986; Tou et al., 2007). Antarctic krill harvested by Aker Biomarine AS, is immediately processed onboard the vessels by boiling and drying to krill meal to avoid autolysis. The krill meal is then subjected to ethanol extraction to produce Krill oil (Aker Biomarine Antarctic AS, 2011). The heat-stability qualities of TM and the production methods used when extracting Krill oil could be the cause of the relatively high protein content compared to Calanus oil, and the presence of allergens in Krill oil products.

The harvested *C. finmarchicus* are immediately frozen on board and freeze-stored in standard freezing facilities (-20^{o}C) before being processed to oil and meal (Pedersen, Vang, et al., 2014). Other details about the production process of Calanus oil are not available. It has been reported that autolytic enzymes in *C. finmarchicus* are active even during frozen storage (Bergvik et al., 2012). However, it is apparent from the gel electrophoresis (**Figure 14 A**) that the proteins recovered from the Calanus oil are more degraded than the protein extracted from the frozen *C. finmarchicus,* which might indicate the use of proteolytic enzymes during industrial processing of *C. finmarchicus*. Enzymatic hydrolysis is reportedly the most efficient process for disrupting epitopes and the degradation of allergenic proteins can result in either complete or almost complete loss of allergenicity (Shimakura, Tonomura, Hamada, Nagashima, and Shiomi, 2005; Thomas et al., 2007). However proteolytic treatments are not always able to destroy all epitopes due to incomplete hydrolysis, and peptides may still contain allergenic epitopes (Shimakura et al., 2005; Thomas et al., 2007). The threshold dose for inducing allergic symptoms varies greatly between individuals (Thomas et al., 2007) and doses as low as 11 mg have been reported to cause symptoms in individuals highly allergic to shrimp (Pedrosa et al., 2015). In 2011, The Allergen Bureau of Australia & New Zealand established reference doses for 11 allergenic foods based on parametric modeling of minimal eliciting doses for use on food labels, and for shrimp protein the reference dose was set to 10 mg (Taylor et al., 2014).

5.5. Possible use of Calanus oil

In **paper III**, known properties of Calanus oil were reviewed, and in addition, results concerning stability of astaxanthin were presented.

The limited amount of fish oil available has led to investigations about the possibility of partially replacing fish oil with Calanus oil in feed for farmed fish, and used for other purposes as well. Studies have shown that Atlantic salmon tolerate well a 30 % inclusion in the feed. A higher amount of Calanus oil however, resulted in reduced lipid digestibility and growth (Bogevik, 2011). The salmon was apparently able to increase production of bile and lipolytic activity to compensate for the heavily digestible wax ester in the feed (Bogevik, Tocher, Langmyhr, Waagbo, and Olsen, 2009). Similar adaptations have also been observed in Atlantic halibut (*Hippoglossus hippoglossus*) (Colombo-Hixson, Olsen, Milley, and Lall, 2011). However, currently the cost of Calanus oil is much higher than fish and most vegetable oil used in feed for farmed fish. It is therefore more likely that Calanus oil could be used as a component in the feed at early development stages or as a natural source of astaxanthin.

The high content of n-3 LC-PUFA and astaxanthin makes Calanus oil interesting as a nutraceutical, yet, the high amounts of wax ester in the oil have been a concern as mammals have a limited ability to digest wax esters. However, small amounts of wax esters seem to be tolerated in humans as they are found in common foods like cereal grains, in addition to some deep-sea fish like orange roughy (*Hoplostethus atlanticus*), and traditional fish products like bottarga (Hargrove, Greenspan, and Hartle, 2004; Ling, Nichols, and But, 2009; Scano, Rosa, Mereu, Piras, Atzeri, and Dessi, 2010). A recent study also confirm that rodents absorb wax ester to some degree (Pedersen, Salma, Höper, Larsen, and Olsen, 2014). Recent reports also indicates that Calanus oil may have beneficial health effects beyond those that may be ascribed to intake of n-3 LC-PUFA alone. Small amounts of Calanus oil are found to reduce atherogenesis and body weight gain, in addition to improve glucose tolerance in mice fed Western type high fat diet (Eilertsen et al., 2012; Hoper et al., 2013; Hoper et al., 2014).

A high amount of the antioxidant astaxanthin is present in Calanus oil, and analysis have shown that the stability of astaxanthin in oil derived from *C. finmarchicus* (**Figure 15**) stored for 425 days is very high when stored with an inert atmosphere (■/●). When the oil was stored dark and exposed to air at 4ºC (□), the astaxanthin levels remained relatively stable for 300 days. However, when the oil was exposed to air at room temperature, at both light (o) and dark (Δ) conditions, a reduction of astaxanthin could clearly be seen after 3 months.

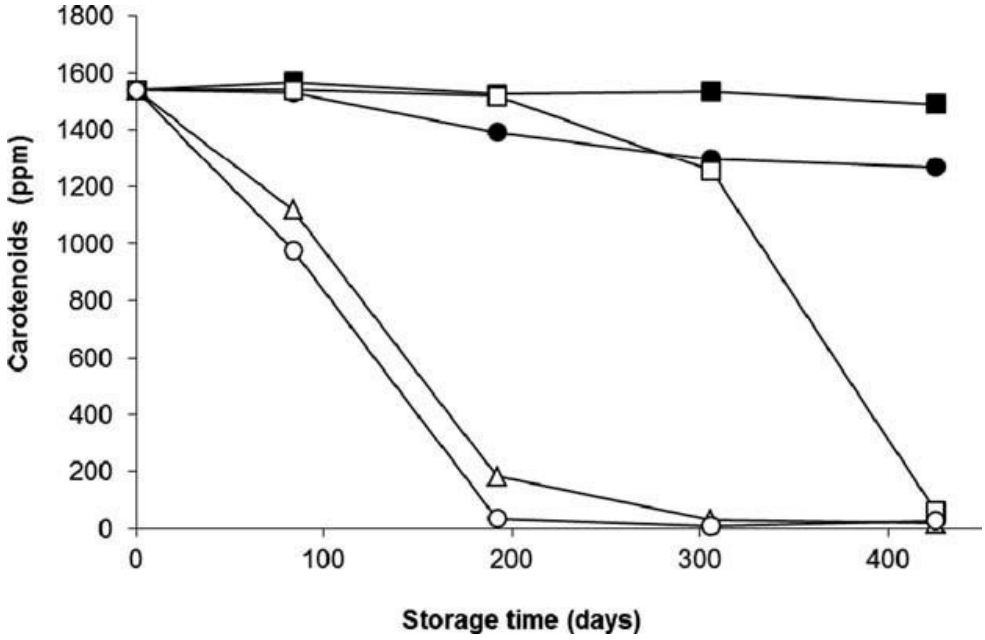

Figure 15. Astaxanthin stability in oil extracted from *C. finmarchicus*. Oil samples stored under nitrogen (N_2), in the dark at 4ºC (■) or in light conditions at 22ºC (●) both had very high stability during the 425 days experiment. The oil that was stored dark and exposed to air at 4ºC (□), had a high stability for more 300 days before being extensively deteriorated. When the oil was exposed to air at room temperature (22ºC), at both light (o) and dark (Δ) conditions, a reduction of astaxanthin content could clearly be seen after 3 months.

In **paper III**, the changes in the relative amounts of the highly unsaturated fatty acids; SDA, EPA and DHA after the long storage period were also presented. Not surprisingly, the results showed that these unsaturated fatty acids were most profoundly oxidized when the oil was stored without nitrogen at room temperature. This was similar to results found for the astaxanthin content.

6. Conclusions

Large amounts of biomasses are present at lower trophic levels in the oceans, and only a limited amount of the energy present is transferred to the next level. Zooplankton acts as an important link between phytoplankton and higher trophic levels such as fish. As long as harvesting of zooplankton is carried out in a sustainable manner without competing with fish, seabirds, and marine mammals, these resources may be used for commercial products. The copepod *Calanus finmarchicus* is a zooplankton that can be harvested with modern technology and processed to astaxanthin-rich protein meal and oil.

The results from **paper I** demonstrated that enzymatic hydrolysis using food grade proteolytic enzymes significantly improves the oil recovery from industrially harvested *C. finmarchicus*. In addition, a hydrolysed protein fraction is obtained. Fatty acid analysis confirmed previous findings from biologicals studies, that oil from *C. finmarchicus* is rich in wax ester and has a high content of total n-3 PUFA. The elevated content of free fatty acids in the oil could be a result of enzymatic degradation du to non-optimal on-board storage technology and processing procedures.

The work in **paper II** showed that the protein content in Calanus oil was significantly lower than the protein content in Krill oil and this may be due to different processing methods. A commercial antibody directed against shrimp muscle tropomyosin (TM) cross-reacts with a protein assumed to be TM in *C. finmarchicus*, Antarctic krill and in proteins extracted from commercial Krill oil, but not in Calanus oil products. The aceton-heptan method used for extracting proteins from these oils is however not optimal, given that direct amino acid analysis of the oils gave a much higher protein content. This was not unexpected, as the protein extraction method requires several washing steps prior to protein estimation, and other extraction methods should therefore be considered. Even though TM seems to be hydrolyzed to such a degree that the allergenic epitopes have apparently been eliminated, studies have shown that people with shellfish allergy can have allergic reactions to other shellfish proteins. In addition to TM, antibodies against other known crustacean allergens should be included when investigating the possible presence of allergenic proteins in Calanus oil. It is also well recognized that the threshold dose for inducing allergic symptoms may vary greatly between individuals, and this issue should be considered before Calanus oil can be safely recommended to people with crustacean allergies.

In **paper III**, possible uses of Calanus oil were reviewed in addition to investigations regarding the astaxanthin content and stability of Calanus oil. The oil is well utilized by

farmed fish and could provide a natural source of astaxanthin and n-3 PUFA for farmed salmonids. Another possible use for Calanus oil is as a health promoting nutraceutical as the oil has a high content of n-3 PUFA and the strong antioxidant astaxanthin, with high stability when stored at the right conditions.

7. Future work

- It would be interesting to use direct amino acid analysis on oils to determine the protein content in other edible oils. There is at present, no established method for investigation the protein content in oils, and this method could be used as a helpful tool when investigating the protein content and presence of possible allergens in edible oils like soy, peanut and rapeseed.

- So far the main product from *Calanus finmarchicus* has been the oil. However to optimize the use of the harvested biomass, more knowledge on the composition and properties of the two side streams should be acquired. Such knowledge is of course a prerequisite for a possible commercial use of both the press cake and the protein hydrolysate.

- Another important future work could be to understand the mechanisms behind digestion of wax ester and the subsequent absorption of the individual parts.

- Health effects of Calanus oil should be further investigated.

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