Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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How can proteins, lipids and astaxanthin be extracted from snow crab (*Chionoecetes opilio*) rest raw material in a sustainable manner?

Master's thesis in Biotechnology
Supervisor: Turid Rustad
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NTNU
Kunnskap for en bedre verden

Nofima

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Forord

Denne masteroppgaven er avslutningen på 5 års studie på masterprogrammet i bioteknologi ved Institutt for bioteknologi og matvitenskap (IBT), NTNU i Trondheim. Oppgaven er skrevet i samarbeid med gruppa for marin bioteknologi på Nofima AS i Tromsø. Labarbeid og skriving har blitt gjennomført ved IBT i Trondheim. Jeg ønsker å rette en takk til alle som har vært involvert i arbeidet med masteroppgaven min.

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Torill

Abstract

During harvest of snow crab (*Chionoecetes opilio*) by Norwegian vessels, the rest raw material is discarded into the ocean. This rest raw material is an abundant source of proteins, lipids and pigments, which can be purified and applied in production of food and feed. The aim of this thesis is to study how to increase the utilisation of the snow crab by recovering proteins, lipids and astaxanthin from the rest raw material in a sustainable manner.

A literature search was performed in order to identify the different methods for recovery of proteins, lipids and pigments from crustacean rest raw material, and determine which methods that were more environmentally friendly and efficient. Methods found in the literature search was acid and alkaline treatments and ensilation, enzymatic hydrolysis, heat treatment, oil extraction and supercritical fluid extraction. The traditional acid and alkaline treatments were identified as less environmentally friendly and less efficient in qualitative recovery.

In the experimental part of the study, two batches of snow crab rest raw material harvested in the Barents Sea in November 2017 was studied. Batch 1 and 2 were processed differently at slaughter and consisted of carapaces with intestines and empty carapaces, respectively. The chemical composition of both batches was determined. Both enzymatic hydrolysis with Alcalase® 2.4 L, Protamex®, Papain GSM80 and Corolase® 2TS as hydrolytic agents and heat treatment was performed to recover proteins and lipids. Enzymatic hydrolysis with Corolase® 2TS was combined with subsequent heat treatment of the hydrolytic sludge with rapeseed oil. This was done to first recover proteins in hydrolysate and then recover astaxanthin in rapeseed oil. This was compared to plain heat treatment with rapeseed oil and to supercritical extraction of astaxanthin.

The determined chemical composition of the two snow crab batches showed that the batch with carapaces including intestines was richer in lipids and astaxanthin. The protease Corolase® 2TS was the most efficient protease in recovery of soluble protein in hydrolysate. Enzymatic hydrolysis of batch 2 with Corolase gave 27,8±0,8% deproteinisation at 65°C, 1 hour, 30 g sample, water to sample ratio 1:2 and enzyme to substrate ratio (E/S) 0,1%. Heat treatment was not as efficient as the use of protease in recovery of protein, and recovery of protein in water fraction decreased at temperatures over 65°C. Neither enzymatic hydrolysis nor heat treatment could be used to extract lipids at this small scale. The combination

experiment with enzymatic hydrolysis and heat treatment with rapeseed oil recovered protein in water fraction, and astaxanthin in the rapeseed oil. The highest recovery of astaxanthin was 25,95±1,92% of total amount of astaxanthin, and was achieved when the treatment mix was stored overnight (-20°C) before separation of fractions. Without storing, plain heating of un-treated rest raw material with rapeseed oil gave the same recovery of pigment as when combining with a pre-treatment with Corolase. The supercritical fluid extraction was shown to have too strong solubility powers, extracting other compounds together with the astaxanthin.

The results show that the processing of the snow crabs at slaughter is determining for the nutritional composition of the rest raw material. The lipid content of the snow crab is low and the lipid is therefore difficult to extract from the material. Enzymatic proteases can be used in recovery of proteins from snow crab rest raw material. The proteases are not as efficient as the use of acid and alkaline treatments, and the main challenge is extensive demineralisation of the crab shells hindering access of the proteases. Proteases are found to be more efficient than heat treatment. Recovery of astaxanthin with rapeseed oil is most efficient when heating rest raw material together with rapeseed oil without pre-treatment and then storing the mixture overnight before separating the astaxanthin enriched oil from the snow crab sludge material. The use of protease compared to control samples has been shown to enhance extraction, but the heat load during enzymatic hydrolysis is assumed to degrade the astaxanthin. This leads to a loss of color in the astaxanthin, and the identification of absorbance of astaxanthin in spectrophotometric analysis is decreased. Supercritical fluid extraction of astaxanthin from snow crab should be optimised before comparison to the recovery with rapeseed oil. The rest raw material generated through snow crab harvest is a good source of protein and pigment. Milder processes than the traditional chemical treatments can be used to recover protein and astaxanthin, but the demineralisation of the snow crab shells material hinders access to the proteins and pigments and thus hinders efficient recovery.

Sammendrag

Den norske fangsten av snøkrabbe (*Chionoecetes opilio*) genererer store mengder restråstoff som kastes på havet. Dette restråstoffet er en god kilde for proteiner, lipider og astaxantin til bruk i produksjon av mat og fôr. Målet med denne oppgaven er å studere hvordan utnyttelsen av snøkrabben kan forbedres ved å ekstrahere ut proteiner, lipider og astaxantin fra restråstoffet på en bærekraftig måte.

Et litteratursøk ble gjennomført for å kartlegge hvilke ekstraksjonsmetoder som brukes på restråstoff fra skalldyr, og hvilke av disse som er mest miljøvennlige og effektive. Metodene som ble funnet var syre- og basebehandlinger og ensilering, enzymhydrolyse, varmebehandling, oljeekstraksjon og superkritisk væskeekstraksjon. De mer tradisjonelle metodene med bruk av sterke syrer og baser ble identifiserte som mindre miljøvennlige, og mindre effektive i ekstraksjon av forbindelser med høy kvalitet.

I den eksperimentelle delen av studiet ble to partier med restråstoff fra snøkrabbe studert. Restråstoffet kom fra fangst av snøkrabbe i Barentshavet i november 2017. Parti 1 var slaktet for hånd på land, og inneholdt skall, innvoller og hemolymfe. Parti 2 var slaktet på båt og inneholdt dermed kun tomme skall. Det kjemiske innholdet i begge partiene ble bestemt. Både enzymhydrolyse med Alcalase® 2.4 L, Protamex®, Papain GSM80 og Corolase® 2TS, og varmebehandling ble gjennomført for å ekstrahere proteiner og lipider. Enzymatisk hydrolyse med Corolase® 2TS ble kombinert med påfølgende varmebehandling av hydrolytisk slam sammen med rapsolje. Dette ble gjennomført for å ekstrahere proteiner ved enzymhydrolyse, og dernest astaxantin i rapsolje. Dette ble sammenlignet med enkel varmebehandling av ubehandlet restråstoffsammen med rapsoilje og superkritisk væskeekstraksjon for å avgjøre hva som er den beste metoden for ekstraksjon av astaxantin fra restråstoff av snøkrabbe.

Den kjemiske sammensetningen bestemt i parti 1 og 2 viste at partiet med innvoller og hemolymfe hadde et høyere innhold av lipider og astaxantin, og derfor kan regnes som mer næringsrik. Totalt protein ble bare bestemt for parti 2. Den kommersielle proteasen Corolase® 2TS var mest effektiv i ekstraksjon av løselige proteiner. Enzymatisk hydrolyse av parti 2 med Corolase gav 27,8±0,8% deproteinisering ved 65°C etter 1 time, med 30 g prøve, vann til prøve ratio 1:2, og enzym til prøve ratio (E/S) 0,1%. Varmebehandling var ikke like

effektivt som bruk av protease, og ekstraksjon av løselig protein avtok når temperaturen oversteg 65°C. Hverken enzymhydrolyse eller varmebehandling kunne brukes til å ekstrahere og isolere lipider. Kombinasjonseksperimentet med enzymhydrolyse og varmebehandling med rapsolje ekstraherte løselig protein i vannfase og astaxantin i rapsoljen. Høyeste ekstraksjon av pigment var 25,95±1,92% av totalt astaxantininnhold, og ble oppnådd når varmebehandlet slam og rapsolje fikk stå lagret over natten (-20°C) før oljen ble separert fra slammet. Uten lagring gav enkel varmebehandling med og uten forberedende enzymhydrolyse samme mengde ekstrahert astaxantin i rapsoljen. Superkritisk væskeekstraksjon ble vist å ha for sterk løselighet, og ekstraherte andre forbindelser sammen med astaxantin.

Resultatene viser at prosessering ved slakt av snøkrabbe påvirker næringsinnholdet i restråstoffet. Lipidinnholdet i snøkrabben var lavt i dette studiet, og lipid var derfor vanskelig å isolere på denne skalaen. Enzymatiske proteaser kan brukes til å ekstrahere protein fra restråstoff av snøkrabbe. Proteasene er ikke like effektive som bruk av syre- og basebehandlinger, og hovedutfordringen er mineralisering av krabbeskallet som hindrer proteasene adgang til proteinene. Proteaser er mer effektive enn varmebehandling i ekstraksjon av protein. Ekstraksjon av astaxantin med rapsolje er mest effektivt ved varmebehandling sammen med rapsolje og lagring av slam sammen med rapsoljen etter behandling, uten forberedende enzymhydrolyse. Bruk av enzymhydrolyse som forberedende behandling påvirket ekstraksjon av astaxantin positivt, sammenlignet med respektive kontrollprøver. Men varmeeksponeringen ved avslutning av hydrolysen på 90°C antas å degradere astaxantin. Dermed har enkel varmebehandling sammen med rapsolje blitt vist å være mer effektivt. Superkritisk væskeekstraksjon av astaxantin fra restråstoff av snøkrabbe bør optimeres før sammenligning med oljeekstraksjonen. Restråstoffet som genereres ved fangst av snøkrabbe er en god kilde til protein og astaxantin. Mildere prosesser enn tradisjonell syre- og basebehandling kan brukes til å ekstrahere ut protein og astaxantin, men demineralisering av snøkrabbeskallet hindrer tilgang til proteinene, og hindrer dermed også effektiv ekstraksjon.

Abbreviations

Abs	Absorbance
AL	Alcalase® 2.4 L
B1	Batch 1
B2	Batch 2
CO	Corolase® 2TS
dw	Dry weight
FPLC	Fast protein liquid chromatography
HPLC	High pressure liquid chromatography
NA	Not available
ND	No data
NO	Blank/control
OF	Oil fraction
PA	Papain GSM80
PR	Protamex®
RRM	Rest raw material
SCF	Supercritical fluid
SD	Standard deviation
SF	Sludge fraction
SFE	Supercritical fluid extraction
WF	Water fraction
ww	Wet weight
w/dw	Weight per dry weight
x g	Times gravity
w/ww	Weight per wet weight

CONTENTS

1. Introduction

1.1. CRUSTACEAN REST RAW MATERIAL

The use of marine resources for food and feed is increasing, with higher production values than ever. In 2016, world fish production (fisheries and aquaculture) was 171 million tonnes. This includes fish, crustaceans, molluscs and other aquatic animals, and excludes aquatic mammals, reptiles, seaweed and plants. It is estimated that the annual global fisheries and farming of crustaceans in 2016 was more than 14 million tonnes (Lage-Yutsy et al. 2011; FAO 2016; FAO 2018[b]). Crustaceans are a large group of arthropods, and include shrimps, crabs, prawns, lobsters, crayfish, barnacles and krill (Fredrick and Ravichandran 2012). In 2018 the total fisheries of crustaceans in Norway was 278 238 tonnes (SSB 2019). From the harvest of crustaceans, about 50-75% of the total weight ends up as rest raw material (RRM) (Kaur and Dhillon 2015; Hamed et al. 2016; Fredrick and Ravichandran 2012). The discarded biomass is cut-offs, by-catch and processing waste (Lage-Yutsy et al. 2011).

Within the RRM, distinction is made between *by-products* that cannot be sold as products for human consumption without further treatment, and *waste* that can be used neither for human consumption nor in animal feed at all. The waste has to be burned, composted or destroyed (Rustad et al. 2011). Another description of the material not directly used as food or feed is *add-on products* (Kristinsson, H., 2019, Bioprosp_19 lecture: "Consumers and Markets for Marine Ingredients and Products"). In this study, I have chosen to describe the studied raw material as *rest raw material*. RRM tends to have a more positive wording than by-products, and a more descriptive wording than add-on products.

The generation of RRM opens up for a possibility of adding value to the fishing and farming of crustacean species. Several species are thought to contain useful and valuable compounds. Crustacean RRM can be utilised for their proteins, oils, pigments, minerals, antioxidants, flavourings and other biopolymers. The use of crustacean proteins as an alternative to traditional animal protein supplement in food could be important when supplying the increasing population with healthy and nutritious food in the future. In addition, the extractable molecules can be useful in a wide range of industries, eg. in feed production for aquaculture and in cosmetics (Lage-Yutsy et al. 2011).

The seafood markets are experiencing increased competition within fisheries and aquaculture production (Gunasekaran et al. 2015). By expanding the horizon of what is possible to extract from the biomass taken up from the oceans, the industry is in the position of creating products that might add value to maintain economic viability and ensure a more sustainable development. The increased seafood production leads to increased generation of discarded RRMs, without appropriate discard options for unused material (Lage-Yutsy et al. 2011; Hamed et al. 2016). Today, large amounts of non-utilised RRM are combusted or used as landfill/deposition. Biodegradation of crustacean materials is slow, and increased utilisation could resolve environmental concerns as well as economical (Lage-Yutsy et al. 2011; Sachindra and Mahendrakar 2005; Shahidi and Synowiecki 1991; Gunasekaran et al. 2015); (Hamed et al. 2016; Arabia et al. 2013; FAO 2018[b]; Kristinsson and Rasco 2010). As different parts of the crab RRM has distinctive compound compositions, application of RRM depends on which parts of the organism that is available in the RRM (Shahidi and Synowiecki 1991; Soundarapandian et al. 2013).

The question in target of this study is *how can proteins, lipids and astaxanthin be extracted from snow crab (Chionoecetes opilio) rest raw material in a sustainable manner?* This is an attempt to aid the improvement of harvest and production of snow crab products, and contribute to a sustainable development in utilisation of marine resources.

1.1.1. The snow crab

The snow crab (Figure 1.1) inhabits the north-western Atlantic, Canada, Greenland and some parts of the Northern Pacific (Lage-Yutsy et al. 2011; HI 2019). The first commercial fisheries of snow crab were in the Gulf of St. Lawrence, 1966. In the Barents Sea the snow crab was first observed in 1996. Since then the fisheries of snow crab has increased, and in 2016 Norway exported 3953 tonnes frozen clusters of snow crab, equal to 331 million NOK (Lorentzen et al. 2018). By March 2018 three new fields of snow crab habitats were discovered in the Barents Sea and these are expected to be inhabited within ten years (FAO 2018[a]). The snow crab prefers low temperatures, lower than the king crab (*Paralithdodes camtschaticus*), and a north-eastern distribution is therefore expected (HI 2019). Knowledge about the nature of the snow crab in the Barents Sea is limited. The Norwegian fisheries of snow crab are located in the protected area around Svalbard. Earlier, Norwegian vessels had access to Russian areas, but Russia ended this agreement with Norway in January 2017. A decrease in number of vessels harvesting snow crab was therefore expected as from 2017

(Lorentzen et al. 2018). Indeed, the quota for 2017 was set to 4000 tons, but the fisheries only harvested 3061 tonnes (HI 2019). Quota for 2019 is set to 4000 tonnes (Lovdata 2019). Still, the snow crab stock of the Barents Sea is growing, and so the fisheries are expected to increase in the coming years (FAO 2018[a]).

Figure 1.1: Snow crab (Chionoecetes opilio), photo: Lidunn Mosaker Boge, Nofima AS.

In the Barents Sea, the snow crab lives at temperatures between -0,7 – 3,4°C, depending on the life stage (Alvsvåg et al. 2009). The average snow crab from a commercial catch is 11 cm in carapace width and 400 g to 1,3 kg. The females are mainly smaller in width than 9,5 cm, and the males reach their maximum shell width at 14 cm. It is the males that constitute the commercial part of the stock, as the sexes inhabit different depth locations. The males are valuable for 3-4 years after the last molt because the shell is naturally biodegraded over the years (Lorentzen et al. 2018; HI 2019; Conan and Comeau 1986; Lovrich and Sainte-Marie 1997; Lage-Yutsy et al. 2011). The edible meat in snow crab is 4 walking legs and 1 claw from each side of the body, called clusters (Lorentzen et al. 2018). The RRM of snow crab constitutes 30% of the total weight. The parts removed and not commercially exploited are the shell (cephalothorax or carapace), digestive systems (including hepatopancreas) and physiological liquid (hemolymph) (Beaulieu et al. 2009). However, the actual RRM that can be brought to land will vary.

One Norwegian producer brings live crabs on shore and performs slaughter there, but the remaining Norwegian harvest is slaughtered and cooked on the boat (99%) (Lorentzen et al. 2018). The RRM generated by slaughter on land can include intestines and hemolymph. Most Norwegian boats use a slaughter machine from Bader. In this machine, intestines and hemolymph gets disassembled from the shells during slaughter, and is washed away. This RRM thus only include empty carapaces (**Figure 1.2**), (in an email from Siikavoupio, S. Dr. philos (Sten.Siikavuopio@Nofima.no) 24.10.18). According to Norwegian law, Ocean resource law, chapter 4, §15, all harvested fish is to be taken to land (Lovdata 2008), and this includes crustaceans (Regjeringen 2007). However, dead or dying snow crab (rest raw material) do not have to be brought in (Lovdata 2005). Today there is no commercial value in the snow crab rest raw material (Lorentzen et al. 2018).

Vilasoa-Martínez et al. (2007) emphasises in their study of protein and amino acid content of snow crab RRM that other studies do not specify whether discard meat is removed from the shells before experiments are started (Vilasoa-Martínez et al. 2007). In this study, it is assumed that all studies using RRM processed by slaughter machines on boat have RRM containing mainly carapaces and very little digestive system, hemolymph or discard meat, unless other is stated.

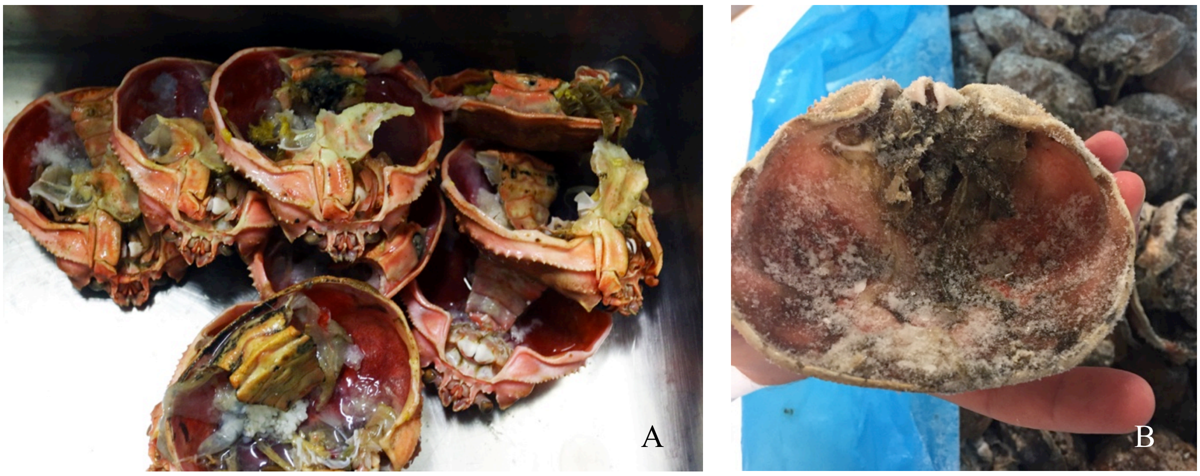

Figure 1.2: Snow crab rest raw material processed on boat, without digestive system, hemolymph or discard meat. Figure B shows the rest raw material used in batch 2. Photo (A): Lidunn Mosaker-Boge, Nofima AS (B): Torill Blix Bakkelund.

Some studies have described the chemical composition of snow crab RRM (**Table 1.1**). The composition of the RRM is used to determine the yield in fractions after processing of the RRM. The chemical composition of snow crab RRM vary with season, sex and processing at slaughter (eg. empty carapaces or including intestines) (Pires et al. 2017; Arabia et al. 2013; Hamed et al. 2016; Soundarapandian et al. 2013; Beaulieu et al. 2009).

Table 1.1: Chemical composition of snow crab RRM determined in four different studies.

Chemical feature	Amount	Reference
Dry matter (g/100 g w/ww)	22,2	(Beaulieu et al. 2009)
	28,0	(Lage-Yutsy et al. 2011)
Protein (g/100 g w/dw)	17,8	(Shahidi and Synowiecki 1991)
	42,9	(Beaulieu et al. 2009)
	34,2	(Lage-Yutsy et al. 2011)
	35,5	(Vilasoa-Martínez et al. 2007)
Lipid (g/100 g w/dw)	14,8	(Beaulieu et al. 2009)
	17,1	(Lage-Yutsy et al. 2011)
	0,1-1,4	(Shahidi and Synowiecki 1991)
Minerals (g/100 g w/dw)	25,7	(Beaulieu et al. 2009)
	28,5	(Lage-Yutsy et al. 2011)
Astaxanthin (µg/g w/dw)	71,7	(Lage-Yutsy et al. 2011)
	94,9	(Vilasoa-Martínez et al. 2008)
	99,1	(Shahidi and Synowiecki 1991)

1.1.2. Crustacean anatomy and physiology

The information about crustaceans is limited and only a handful studies have determined the chemical composition of snow crab RRM (Pires et al. 2017). The overview of snow crab anatomy and physiology in this study is based on some general knowledge about crustacean anatomy and physiology.

Crustaceans, like all other arthropods, are covered by an exoskeleton. The density, thickness and mechanical resistance are varying between species. The build-up and function is the same; providing support, resisting mechanical load and ensuring protection against the environment. But crustaceans stand out from other arthropods by having a high degree of mineralisation in the exoskeleton, providing mechanical rigidity. In crabs this mineralisation is mainly calcite or amorphous calcium carbonate ($CaCO_3^-$). The crustacean exoskeleton consists of two main layers, the epicuticle and the procuticle. The epicuticle is a thin layer covered with wax (lipids), providing the specimens with a waterproof coating (aquatic species). Beneath the thin epicuticle is the main structural part, the procuticle. This layer is divided into exocuticle (outer) and endocuticle (inner). The endocuticle is the main part of the total exoskeleton, imparting 90% of the volume. The exocuticle is stacked more densely than the endocuticle, and has layer spacing three times smaller (Chen et al. 2008; Diaz-Rojas et al. 2006). The main components of the shells are chitin (20-30%), ash (20-30%) and proteins (30-40%), thoroughly intertwined in the different layers. The proteins are what make the shell a living tissue, while chitin and ash are imparting strength to the exoskeletons structure. Chitin in crustacean shells are fibrous and associated with protein in chitin-protein complexes (Kaur and Dhillon 2015; Se-Kwon 2014; Hamed et al. 2016). The chitin-protein complexes are further covered with calcium carbonate ($CaCO_3^-$), carotenoids (mainly astaxanthin) and lipids (Shahidi and Synowiecki 1991; Kaur and Dhillon 2015).

Crustacean species occupy a variety of habitats, and the feeding and nutritional demands are adapted thereafter. Intake of proteins, lipids and ash depend on the species ability to synthesise and store compounds, in addition to the degree of calcification (Saborowski 2015). The omnivore snow crab diet consists of algae, other crustaceans and molluscs (Lage-Yutsy et al. 2011). Crustacean species digest carbohydrates like starch, cellulose and laminaran. The carbohydrates are used for glycogen storage, energy, chitin synthesis and synthesis of fatty acids and sterols (Saborowski 2015).

1.1.3. Proteins

According to Mukhin and Novikov (2001), invertebrates in the Barents Sea are rich in peptides and amino acids that can substitute todays nutritional supplements (Mukhin and Novikov 2001). The reported amount of protein in snow crab RRM varies from 17,8-42,9 g/100 g (w/dw) (Shahidi and Synowiecki 1991; Lage-Yutsy et al. 2011; Vilasoa-Martínez et al. 2007; Beaulieu et al. 2009). Beaulieu et al. (2009) analysed the content of amino acids in fractions from enzymatic hydrolysis of snow crab RRM, and demonstrated preparation of protein hydrolysates with low molecular weight peptides (≤30kDa), and essential amino acids. Essential amino acids are those that the human body cannot produce, and thus has to be obtained through the diet (Beaulieu et al. 2009).

1.1.4. Marine lipids

The main lipid content in crustaceans is phospholipids in cell membranes. The storage of lipids depends on species, and northern crustacean species (eg. snow crab) mostly use wax esters for long-term storage of lipids. Short-term storage of lipids mainly consists of triacylglycerol, and it is used for metabolic activity, thus replaced frequently. The midgut gland is the main storage compartment. (Loftsson et al. 2016; Saborowski 2015). Marine crustaceans have also been shown to contain high levels of fatty acids, and especially in the hepatopancreas. This tissue contains about 30% lipids, more than the lipid content in the liver of several terrestrial mammals (Chapelle 1977). Marine oils generally contain high amounts of polyunsaturated fatty acids (PUFA), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and are the main source of long chain omega-3 PUFAs (Loftsson et al. 2016; Saborowski 2015). Beaulieu et al. (2009) performed enzymatic hydrolysis of snow crab RRM, and obtained an oil fraction in addition to the liquid and sludge fraction. The oil fraction mainly contained monounsaturated fatty acids (MUFAs, 53,1% of total fatty acid methyl esters) and secondly PUFAs (22,2% of total fatty acid methyl esters). Amongst the PUFAs, EPA and DHA dominated with a ratio of omega-6 to omega-3 of 1:10. A low omega-6/omega-3 ratio indicates high nutritional value in the human diet, as it is suppressive to several diseases, eg. cardiovascular diseases, cancer and inflammatory/autoimmune diseases. This makes the oil extracted from snow crab RRM a desired product that could add value to the cluster production (Beaulieu et al. 2009; Simopoulos 2002, Lage-Yutsy et al. 2011).

1.1.5. Ash

Macrominerals in crustaceans are calcium, phosphorous, potassium, sodium and magnesium. Microminerals are cobber, iron, mangan, selenium and zinc. In the ash of the shells, $CaCO_3$ is the most important inorganic compound. Another less abundant mineral deposition is calciumphosphate ($CaPO_4^-$). In the epicuticle, minerals are deposited in channels of the bilaminar basal layer, normal to the surface. Most of the salts are depostited in the endocuticle, and hardened by calcium (Ca). In the molting, the animals loose calcium. This is regained from the water, over the gills. Ash storage is conserved in calcified concretions in the cardiac stomach. Pre-molt, ash is stored in mid-gut gland (Saborowski 2015). The amount of ash in the muscles of snow crab differs between the sexes. Males contain more ash than females, but less than berried females (egg-bearing crabs) (Woll 2006, Soundarapandian et al. 2013; Roer and Dillaman 1984).

1.1.6. Astaxanthin

Carotenoid pigments exist as 600 different pigments in nature. This group of lipophilic compounds is divided into two families, the hydrocarbons and the xanthophylls. The former are simply compounds build up by carbon and hydrogen atoms, and the latter are oxygenised derivatives containing OH-groups, oxi-groups or a combination of these. The structure of the carotenoids is based on lycopene, with 40 carbons or more, and two terminal ring structures. Along the carbon structure, the atoms are joined by conjugated double bonds, a polyene system. It is this system that gives the distinctive colour and chemical characteristics. Crustacean species are abundant in the carotenoids carotene and its oxidised form, astaxanthin (Shavandi et al. 2019; Higuera-Ciapara et al. 2006). Astaxanthin (3,3′-dihydroxy-β, β-carotene-4,4′-dione, M_w = 596,8 Da) is a ketocarotenoid, and it is considered as one of the most important and economically valuable pigments. It is used in agriculture and aquaculture feed as a supplement to improve coloration of flesh and eggs (Cremades et al. 2001). Orange to red colouring of the salmon flesh is considered as a quality trait of salmon products, but the salmon do not have the ability to produce astaxanthin themselves. It can be absorbed through the diet, and is thus added of the salmon feed. Increased aquaculture production demands increased feed production, and natural sources of astaxanthin are now being sought as synthetically prepared astaxanthin stands for the majority of the astaxanthin production, but is of high cost (Higuera-Ciapara et al. 2006; Lage-Yutsy et al. 2011; Pu et al. 2010; Chen and Meyers 1982).

In crustaceans, astaxanthin accumulates in and gives red to orange coloration of the shells, scavenges free radicals and protects cells against oxidation. In some species it can have an impact on nutrient utilisation and growth. It is bound non-covalently toeither proteins (carotenoproteins), proteins and lipids (carotenolipoproteins) or to chitin, with ester and imine bonds, in addition to existing in free form (Higuera-Ciapara et al. 2006; Caramujo, de Carvalho et al. 2012; Schiedt et al. 1993; Armenta and Guerrero-Legarreta 2009). As a carotenoprotein complex it is water soluble and more stable than the free form (Chakrabarti 2002; Schiedt et al. 1993). The astaxanthin content of crustacean by-products has been described, and the determined amount of astaxanthin in snow crab RRM is about 71,7 - 99,07 µg/g (w/dw). The carapace back contains the highest amounts of astaxanthin (Lage-Yutsy et al. 2011; Shahidi and Synowiecki 1991; Vilasoa-Martínez et al. 2008).

1.2. METHODS OF RECOVERY

When rest raw material is to be used in food and feed because of its content of a specific compound, it is advantageous for the compound to be purified before application. This way, the other compounds of the rest raw material are avoided. Purification is for example important when using crustacean rest raw material in feed for aquaculture to add astaxanthin to the feed. The high content of ash and chitin in the rest raw material would decrease the digestibility of the feed in the salmon (Higuera-Ciapara et al. 2006).

The methods of recovery used in the experimental part of this study were chosen based on information retrieved in the literature search. The complex and rigid organisation of protein, minerals, pigment and chitin in the crustacean shells demands efficient method for separation of the compounds. Traditional methods use strong alkaline and acid treatments and high temperatures (Kaur and Dhillon, 2015). These are efficient removal of the protein, ash, lipids and pigments, but these components will be of poorer quality. It is therefore sought to find alternative methods for extraction that are milder, more environmentally friendly and at the same time efficient (Valdez-Peña et al. 2010).

1.2.1. Enzymatic hydrolysis

Enzymatic hydrolysis (EH) disrupts tissues and cell membranes, allowing the extraction of various biomolecules (Beaulieu et al. 2009). The hydrolysis depends on the temperature, pH, enzyme activity, enzyme to substrate ratio (E/S) and duration of hydrolysis. This has to be described and customised for different combinations of enzymes, crustacean species and their tissues (Mukhin and Novikov 2001). Sources of enzymes are mammalian, plant or microbial material. Enzymatic hydrolysis results in a water-soluble fraction, a sludge fraction, and usually some lipids will be released as an oil fraction. The water fraction is also designated hydrolysate, and contains proteins and some minerals (Madeira et al. 2017; Arabia et al. 2013). The sediment fraction contains all components that are non-soluble and compounds that could not be released in the process, eg. minerals, some of the proteins, chitin, lipids and pigments (Šližytė et al. 2014; Lage-Yutsy et al. 2011).

The material to be hydrolysed is mixed with water. This reduces viscosity and eases homogenisation of the sample, in order to enhance the enzymatic reaction and increase the yield of protein from the material. Addition of water does however affect emulsion, and emulsified lipids can end up in the water fraction instead of in a separate oil fraction. This can be avoided by decreasing the addition of water (Šližytė et al. 2014).

The aim of an enzymatic hydrolysis when recovering protein and lipids is to increase the solubility of proteins in the water fraction, and release lipids in an oil fraction. The enzymes will cleave peptide bonds in the proteins of the material, and generate peptides of lower molecular weight, and free amino acids. The degree of hydrolysis is defined by Rutherford (2010) as a measure of proportion of cleaved peptide bonds, based on the total amount of peptide bonds in a protein hydrolysate (Rutherford 2010). The size of the peptides in a hydrolysate, hydrophobicity and amino acid composition affects taste and bitterness. Smaller peptides (<10 amino acid residues) are more rigid and cannot cover the overall hydrophobicity of peptide side chains. The hydrophobicity of side chains increase the bitter taste of hydrolysates, thus smaller peptides in the hydrolysate give more bitter taste (Aspevik et al. 2016). The use of snow crab hydrolysate could be an added value to the production of clusters, eg. as an alternative protein source in production of prepared food.

Degree of hydrolysis is increased as more peptides are cleaved with increasing temperature and enzyme concentration. At higher temperatures, the enzyme will be thermally denatured, depending on heat stability. At the end of the hydrolysis, the hydrolysed material should be exposed to temperatures around 75-100°C for 5-20 minutes to inactivate the enzyme. Adding acid or alkaline solutions can regulate the pH of the hydrolysis mixture in order to fulfil the optimal conditions of the protease used. Usually the pH of the mixture will decrease during the treatment because of the generation of free amino acids. In the case of crustacean material, pH can also increase by the release of carbon cations in demineralisation (Šližytė et al. 2014; Kaupang and Whitaker 2016 (unpublished); Shavandi et al. 2019; Kristinsson and Rasco 2010).

The enzymes used in extraction of compounds destined for food or feed production has to be food-grade. In the case of microbial enzymes the organism used has to be non-pathogenic (Beaulieu et al. 2009). Most enzymes used in food research are proteases, other enzymes are carbonases and lipases. The studies with commercial proteases are numerous, and these are widely used on an industrial scale (Kristinsson and Rasco 2010). The main challenge is to determine the protease suited for a given material as different proteases have different specificity. It is also necessary to determine optimal conditions for this specific combination of enzyme and substrate, improve taste defects and develop an economic production (Mukhin and Novikov 2001, Cahú et al. 2012; Kristinsson and Rasco 2010).

Beaulieu et al. (2009) used the commercial protease Protamex® (E/S 0,001%, 40°C, pH 8, 1 hour) on snow crab rest raw material. In liquid fraction after hydrolysis, centrifugation and decantation, they achieved 60,64% recovery of protein in the hydrolysate (Beaulieu et al. 2009). The authors did not calculate the recovery directly, but it was found based on mass balance of dry weight through the treatment process, and protein analysis of liquid fraction after decantation. Few other available studies have performed enzymatic hydrolysis on snow crab rest raw material. The discussion is therefore based on reported results from studies using other crustacean and some fish species as well.

Proteases: Endo- and exopeptidases

Proteases are active molecules that have the ability to cleave peptide bonds in proteins. These are found in both eukaryotic and prokaryotic organisms (Rao et al. 1998). At the cleaving of a peptide bond between two amino acid residues, a -COOH-group and a -NH-group is generated (Li et al. 2013). Proteases are classified as either endopeptidases or exopeptidases (Rao et al. 1998).

Exopeptidases are only active near either the amino- or carboxy terminal of a given peptide or protein, the N- or C-terminal, respectively. Aminopeptidases hydrolyse peptide bonds near the N-terminal, removing mono-, di- or tripeptides from the molecule. The aminopeptidases are especially attracted to and remove methionine ends from proteins and peptides. Carboxypeptidases hydrolyse and remove mono- or dipeptides. The carboxypeptidases are divided into subgroups describing the amino acid residue in respective active seats; Serine-, metallo-, cysteine carboxypeptidases (Rao et al. 1998, Kristinsson and Rasco 2010).

The endopeptidases are active in the inner segments of peptides and proteins, usually at specific residues. This is because free amino- and carboxygroups at N- and C-terminal has a repelling effect on the enzyme. The activity of endoproteases generates peptides of larger sizes. This enzyme group is further divided into the subgroups serine, aspartic, cysteine and metallo proteases. The subgroups are characterised by the amino acid residue in respective active seats, just as the subgroups of the exopeptidases (Rao et al. 1998, Kristinsson and Rasco 2010).

Both endo- and exopeptidases are also characterised and often named by the pH-area of their optimal activity, meaning acidic, neutral or alkaline. Lastly, the proteases are divided into families based on amino acid sequence and evolutionary relations (Rao et al. 1998). The association specificity and cleaving of the enzyme are naturally evolved, but the range of tolerable conditions for activity of commercial proteases can be engineered (Li et al. 2013). Broad specificity is an advantage as it gives higher yield of peptides from the material. It also allows gentle release of lipids, with recovery of polar lipids in liquid phases (Dumay et al. 2004, Dumay et al. 2006). More specific proteases may produce peptides of specific sizes and characteristics. Combining endo- and exopeptidases will give a more total degradation, as larger peptides are cleaved off, and amino acid residues are cleaved off both from the starting proteins and the peptides generated in the process (Kristinsson and Rasco 2010). The commercial proteases used in this study are described below. They were all selected because of frequent appearance in the literature studied, and Protamex® and Corolase® 2TS were especially chosen because of their ability of generating non-bitter, neutral hydrolysates.

AlcalaseX 2.4 L

Alcalase® 2.4 L (Alcalase, AL) is a non-specific serine-type endopeptidase from *Bacillus licheniformis* (Toldrá et al. 2018). Optimal conditions for activity are pH 6,7-9 and temperature 50-70°C (Charoenphun, Cheirsilp et al. 2013). AL is often used as a hydrolysis agent in different food production procedures, and specifically on calcium-chelating peptides (Žuža et al. 2017; Toldrá et al. 2018).

ProtamexX®

Protamex® (Protamex, PR) is an endolytic serine-type protease with broad specificity towards hydrophobic proteins, and originates from *Bacillus sp*. Optimal conditions are pH 5,5-7, and temperature 35-60°C. PR is a commonly used industrial enzyme used for calcium-chelating peptides, fish material, antiallergenic activity in cereals, and processing of soy bean. The protease is efficient in production of non-bitter hydrolysates (Sigma-Aldrich; Sung et al. 2014; Toldrá et al. 2018; Šližytė et al. 2016; Beaulieu et al. 2009).

PapainX GSM80

Papain GSM80 (Papain, PA) is a cysteine-type peptidase with origin from papaya (*Carcica papaya*) latex. Optimal conditions are pH 5-7 and temperature 65-80°C. PA cleaves peptide bonds of the basic amino acids like glycine and leucine, and hydrolyses esters and amides. Conventional application has been on collagen, for antiallergenic activity in cereals, diary processing and meat tenderisation (Sigma Aldrich A; Sung et al. 2014; Amri and Mamboya 2012; Toldrá et al. 2018).

CorolaseX® 2TS

Corolase® 2TS (Corolase, CO) is an endopeptidase with broad specificity and origin from *Bacillus thermoproteolyticus* and *Bacillus stearothermophylus.* The producer (Enzymes) does not specify optimal conditions, but neutral pH and tolerance for high temperatures are stated (AB Enzymes; Meinlschmidt et al. 2016). The conventional application areas for CO are cereals and soy beans (Toldrá et al. 2018). This protease is also proven as efficient in producing taste neutral hydrolysates (Arnesen et al. 2017).

1.2.2. Heat treatment

Extraction of proteins and lipids from natural sources can also be done by heat treatment (HT). This method involves a heat load (temperature and time) leading to destruction of phospholipid cell walls in the material, and release of fats in addition to degradation of proteins. This usually gives three fractions or phases; a solid fraction, a water fraction and an oil fraction (Carvajal et al. 2014; FAO 1986). The temperature necessary for release of oil depends on the species and material in question. Generally, fat cell walls break down at 50°C, however this may not be the case with all materials. Most proteins will be denatured at 75°C. Thus, a successful heating process depends on the species and the desired product (FAO 1986). The challenge of this process is to maintain a stable and even temperature through the material. Further, higher temperatures will increase the oxidation of lipids and thus affect the quality of the oil. Carvajal et al. (2014) used heating to produce fish oil from herring (*Clupea harengus L.*). The study compared heat treatment at temperature intervals of 50-60°C and 80-90°C (5 minutes) to enzymatic hydrolysis (Papain, Bromelain and Alcalase® 2.4 L, (15, 30, 45 and 60 minutes). Oil recovered by heat treatment was of higher quality than oil recovered by enzymatic hydrolysis, in means of oxidative status (Carvajal et al. 2014). In addition to the thermal degradation of proteins by heat treatment, the endogenous enzymes present in the material originally will degrade proteins as well. Šližytė et al. (2014) found that this activity declined with increased temperature over 60°C. This is supported by other studies looking at the activity of endogenous enzymes in cold-water fish (Kaupang and Whitaker 2016 (unpublished); Šližytė et al. 2005; Šližytė et al. 2014).

1.2.3. Pigment recovery using oil

Pigment extraction from natural sources requires by the use of organic solvents demands several extraction steps and it is time-consuming. It is also reckoned as a analytical method rather than industrial method (Sachindra et al. 2006). Development of milder, more efficient and cheap methods for pigment extraction have been sought (López et al. 2004). Many pigments are oil soluble, and several studies describe the extraction of pigments from crustacean RRM using vegetable or animal oil (Sachindra and Mahendrakar 2005; Shahidi and Synowiecki 1991). The main step is to heat the material to be treated together with oil, eg. in a water bath. The material to be treated can either be pre-treated with eg. proteases or acids, in order to demineralise and deproteinisate the material before addition of oil. After heating, centrifugation of the treatment mix generates a solid fraction, a liquid water fraction and an oil fraction. Filtration can be included before or after centrifugation, to enhance separation of fractions. In the method proposed by Pu et al. (2010), flax seed oil was added to homogenised raw material (ratio 1:1), before heating the mixture for 1 hour at 60ºC with stirring. The separation of oil was done by centrifugation. Shahidi and Synowiecki (1991) used cod liver oil to extract carotenoids from snow crab RRM, and studied the effects of temperature and oil to raw material ratio. Best results were achieved with oil to raw material ratio 1:2 and 60°C, which gave 74% recovery of total carotenoid content (Shahidi and Synowiecki 1991). Sachindra and Mahendrakar (2005) compared groundnut oil, gingelly oil (sesame), mustard oil, soy oil, coconut oil, rice bran oil and refined sunflower oil in recovery of carotenoids form shrimp (*Penaeus indicus*). They found that refined sunflower oil at an oil to raw material ratio 2:1, at 70°C for 150 minutes gave highest recovery of carotenoids with 26,3$\pm$2,31 μg/g raw material (total astaxanthin content in crude material is not specified) (Sachindra and Mahendrakar 2005).

The extraction method, temperature, treatment time, oil type, RRM particle size, oil to raw material ratio, total pigment content of raw material, species and lipid composition in organism, can affect the recovery of pigments in oil (Sachindra and Mahendrakar 2005). Regarding RRM particle size, the use of proteolytic enzymes can affect the outcome of the process (Pu et al. 2010). The pigment-enriched oil can be directly utilised, eg. for coloration of salmonid fish when added to fish feed (Shahidi and Synowiecki 1991). The oil used in this study, rapeseed oil, was chosen because of its low cost.

1.2.4. Supercritical fluid extraction (SFE)

Supercritical fluid extraction is the utilisation of a supercritical fluid (SCF) for extraction of a specific compound from a natural source. It has wide application, with the possibility of extracting caffeine from coffee, antioxidants from organic material, and purifying nanoparticles (Krichnavaruk et al. 2008). A supercritical fluid has a physiochemical property between a gas and a liquid, and it is able to diffuse through fine matrix better than conventional organic solvents (Félix-Valenzuela et al. 2001; Krichnavaruk et al. 2008). Different fluids have been tested (hexane, pentane, butane, nitrous oxide, sulphur hexafluoride and fluorinated hydrocarbons), but the most used is carbon dioxide (CO_2).

Supercritical CO_2 (SC-CO_2) has become a favourite as it is readily available and safe to use, is non-toxic, it can be used at low pressures and near-room temperature with low cost, and is easily up-scaled to industrial scale. The only inconvenience is the high investments cost. The use of this method requires knowledge about the solubility of the solute to be extracted, the structure of the raw material and specific location of the solute in the material (Reverchon and De Marco 2006). CO_2 is a non-polar solvent, and SC-CO_2 is thus not suitable for extraction of more polar solvents. The solubility of the solvent in SC-CO_2 is crucial for efficient operation and result. A co-solvent can therefore be added in smaller amounts to assist SC-CO_2 in the extraction. The co-solvent interacts with both the SC-CO_2 and the solute, and can increase the contacting surface area and enhance extraction. Astaxanthin is a liphophilic (non-polar) molecule. The molecule is relatively large (M_w = 596,8 Da), with a low volatility and low solubility in SC-CO_2. A co-solvent is thus required for SFE of astaxanthin from raw material, and ethanol (EtOH) is often used. This solvent requires elimination by evaporation after extraction. Another proposed solvent is vegetable oils, eg. soy, olive, hazel nut and canola oil. These will not require processing after extraction, and studies have shown extraction results comparable to the use of ethanol (Krichnavaruk et al. 2008). Co-solvents should be chosen carefully, as increased solubility (increased solvent powers) can also lead to poorer process selectivity for the target compound. The process starts by placing the extraction vessel with raw material, preferably freeze-dried and grinded, in the machine. SCF and the co-solvent flow through a depressurisation valve at the exit of the vessel to a separator. Because of the low pressure, extracted solutes are released from the gaseous medium and collected in the co-solvent (Reverchon and De Marco 2006).

Several studies have used SFE to recover pigment from crustacean rest raw material. Felix-Valenzuela et al. (2000) extracted pigment from demineralised crab (*Callinectes sapidus*) shell rest raw material. Solvent was CO_2 ethanol (90:10 M), flow 3,8-4,3 L/min, temperature 45-65°C, pressure 295-345 bar. They achieved the highest yield of 57% astaxanthin, at 45°C and 350 bar (Félix-Valenzuela et al. 2001). Charest et al. (2001) used SFE to extract astaxanthin from Louisiana crawfish (*Procambarus clarkii*), and at conditions CO_2 + 10% ethanol, flow 1,0-1,5 L/min, 50-70°C, 140-310 bar. They achieved an average of 88,51% recovery of astaxanthin. The material was cooked at ~80°C and freeze-dried before extraction. Addition of moisture did not affect recovery (Charest et al. 2001).

1.3. ANALYTICAL METHODS

1.3.1. Proteins

Total amino acid composition

Determination of total amino acid composition can be performed after Blackburn (1978) in order to estimate total protein content in the RRM. In determination of total amino acid composition, proteins and polypeptides are hydrolysed before separation and detection of single amino acids is performed with high-pressure liquid chromatography (HPLC). The hydrolytic agent is required to have broad specificity in order to increase cleaving of peptide bonds. In addition, hydrolysis is challenged by steric hindrance within the proteins bulky side chains, and macromolecular structure of the proteins. HCl is widely used as hydrolytic agent, at high temperature (105°C) and with 24 hours treatment time (Blackburn 1978). In separation and detection using HPLC, the stationary phase of the instrument consists of small particles, and high-pressure forces the liquid phase with molecules in target through the beds (Nelson and Cox 2013). Detected amino acids are plotted on a chromatogram and this is compared to chromatograms of known amino acids. Amino acids with polar side chains like serine and threonine are fragile to hydrolysis by the acid treatment, and are not expected to be detected (Blackburn 1978). This is accounted for in the calculation of total amino acid composition. But as this determination can be imprecise compared to the actual content, the use of total amino acid composition to describe total protein content is considered as an estimate.

Soluble protein content

The Lowry method (1951) is a widely used procedure to estimate the content of soluble protein in a solution, eg. a hydrolysate from enzymatic fractioning. It is one of many copper-ion based methods, with a resulting blue mixture whose increasing colour intensity correlates with an increasing amount of protein in solution. Two distinctive steps in this procedure leads to a coloration of the mixture. Firstly, the solution to be analysed is added an Alkaline copper reagent (2 % Na_2CO_3 in 0,1 M NaOH, 1 % $CuSO_4$, and 2 % potassium sodium tartrate, ratio 10:0,1:0,1, respectively). The cupric ions from $CuSO_4$ bind to the backbone of peptides by binding to the nitrogen groups. Cupric ions are stabilised by potassium sodium tartrate from the reagent. The reaction is complete after 5-10 minutes. Folin Ciocalteu reagent (phophomolybdic-phosphotungstic acid, diluted in water 1:3) is added, and reduced by the presence of protein bound cupric. This generates a blue colour within 3-30 minutes. The Folin reagent performs best at pH around 10, and this is achieved by the presence of NaOH and Na_2CO_3 in the alkaline reagent. The absorption of the mixture is read at 750 nm for high sensitivity to low concentrations of proteins. Standard protein samples with known concentrations, eg. bovine albumin serum, is also mixed with reagents and absorbance read. The absorbance is plotted against the known protein concentrations, and a relation between colour intensity and concentration is determined by linear regression ($R^2 \leq 1$). The relation of colour to amount of protein is not linear, as different proteins will bind differently to cupric and thus reduce the Folin reagent to a varying degree. However, it gives an estimate on the content of soluble protein, which is advantageous when comparing different hydrolytic products, or for analysis of a large number of similar protein samples (Lowry et al. 1951; Chang and Zhang 2017).

Analytical determination of protein is often done by wet chemistry methods (eg. Kjeldahl), instrumental methods based on combustion of nitrogen, near- and min-IR spectroscopic methods or colorimetric reaction of peptides with a reagent, eg. the biuret and bicinchoninic acid (BCA) methods), Bradford Dye-binding method and the anionic dye-binging method (Chang and Zhang 2017). Total amino acid hydrolysis was chosen because it uses 6 M HCl and is heated for 24 hours, thus it is a harsh method that can degrade the rigid crustacean material. The Lowry method was chosen for its ability to estimate soluble protein, and because it is frequently used in studies on rest raw material.

Free amino groups and degree of hydrolysis

The formol titration procedure (Taylor 1957) is used to determine the degree of hydrolysis in a solution or material. It is based on titration of protein solutions to an end point, and the amount of titrant needed to reach the end point can be used to calculate the amount of free amino groups. From this the degree of hydrolysis can be calculated based on total protein content in the sample. For this procedure, one can either choose to do a direct or an indirect titration. For a direct titration the solution to be analysed is added neutralised formaldehyde (CH_2O, ~39%) and titrated with NaOH (0,1 M) to pH ~9. Direct titration measures total carboxyl groups of amino acids and peptides. An indirect titration requires a pre-titration for neutralisation of the solution to be analysed before addition of neutralised formaldehyde and titration to pH ~9. Indirect titration measures the total amino groups of amino acids, peptides, primary and secondary amines and ammonia. With both methods, it is the alkalinity of NH_2 or NH- groups that is altered by exposure to formaldehyde, and the required amount of alkaline titrant to reach the end point in the solution is used to determine the approximate amounts of free amino groups. According to Taylor (1957) the advised method is direct titration as this gives more theoretical accuracy because all amino acid residues are titrated from their original pH of solution to the end point. Indirect titration, on the other hand, might need less alkaline titrant (Taylor 1957). However, unknown solutions are advised to be pre-titrated in order to select an appropriate starting salinity (Northrop 1926). Different amino acids might require different end points in order to achieve the shift to an alkaline state. This makes the formol titration a way of estimating the quantity of free amino groups and is thus suitable for comparable results (Taylor 1957; Northrop 1926). Other methods for determination of degree of hydrolysis include pH-stat, trinitrobenzenesulfonic acid (TNBS), o-phthaldialdehyde and trichloroacetic acid soluble nitrogen (SN-TCA). According to Rutherford (2010) there are advantages for all methods (Rutherford 2010), and so the formol titration was chosen based on the frequent appearance in literature describing rest raw material.

Molecular weight distribution

Molecular weight distribution of peptides in a solution can be determined using gel filtration by fast protein liquid chromatography (FPLC) (Madadlou et al. 2011). Molecules are separated based on size in the column. An inert, porous gel filtration medium of spherical particles is packed in a column. The column is soaked in a buffer that fills the pores and space between the particles of the medium. When a sample is added to the column, molecules will elute from the column according to molecular size. Larger peptides travel faster, while smaller peptides linger in the stationary phase. UV is used to detect components by their time of elution, which is proportional to elution volume (V_e) by flow (mL/min). In addition, the concentration is indicated by the absorbance values of the components. A chromatogram describes absorbance at given elution volumes (Figure 1.3).

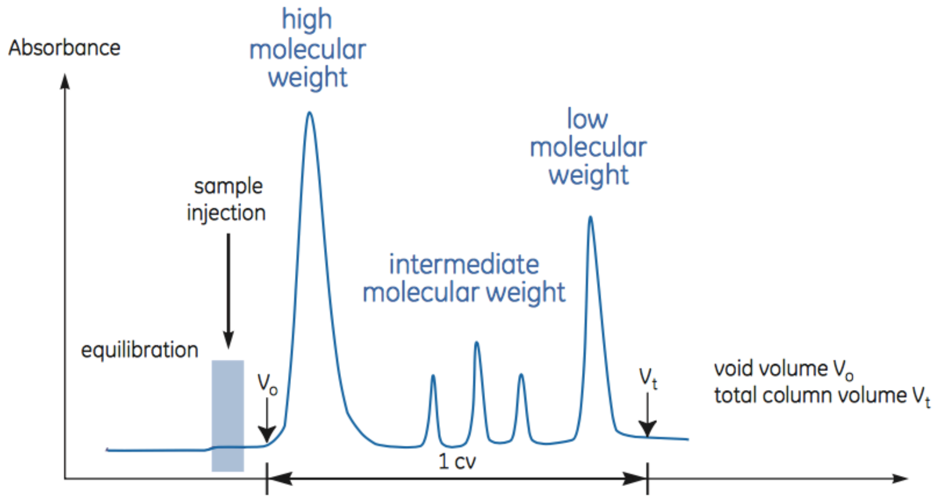

Figure 1.3: Illustration of a chromatogram generated by FPLC procedure. When the sample is injected the larger molecules are eluted and detected first. Smaller molecules linger. V_e is elution volume, V_t is the total volume of the column, V_o is void volume in the column (GE Healthcare).

A normalisation of the data is performed to determine sample behaviour independently of column dimensions. The partition coefficient K_{av} (chapter 3.3.8) is one such normalisation, using void volume (volume of the gel particles V_o), total column volume (V_t) and elution volume V_e. Standard samples of known molecular weight are used to determine a relation between K_{av} and molecular weight (GE Healthcare). Other methods of determination of molecular weight distribution exist, and these usually include size exclusion chromatography, separating molecules of different sizes and determining them thereafter. The choice of use of FPLC was based on availability of equipment in the lab.

1.3.2. Lipids and pigments

Total lipid composition

The determination of total lipid content can be performed by the Bligh and Dyer method (1959). Lipids are insoluble in water, and the extraction of lipids requires organic solvents (Nelson and Cox 2013). Further, marine tissues contain mostly unsaturated fatty acids, which requires gentle lipid extraction to minimise oxidative decomposition. The method developed by Bligh and Dyer involves homogenisation of sample with water, chloroform and methanol. This creates a monophasic solution as the methanol and chloroform mixes with the water in the sample. The chloroform does not permit hydrophobic clustering of the lipids, as would happen in water liquids. Additional dilution with water and chloroform changes the phase equilibrium of the mixture. The system evolves to a biphasic system with a heavy chloroform layer containing the lipids, and a light water-methanol layer containing non-lipid components from the sample. Centrifugation separates unextractable compounds from the chloroform phase. When removing the chloroform layer from the system, lipids are isolated (Bligh and Dyer 1959).

Another common method for determination of total lipid composition is Folch method (1957). The Bligh and Dyer method uses less solvent than the Folch method, and does not include the number of filtration steps (Iverson et al. 2001). Considering these advantages, the Bligh and Dyer method was chosen for determination of total lipid composition in this study.

Quantitative determination of astaxanthin

Astaxanthin can be quantitatively analysed using spectrophotometry (Metusalach et al. 1997). Spectrophotometry describes the reflectance of a material as a function of wavelength (NIST 2018). Carotenoids have a distinct absorption spectrum, which is a function of the chromophore structure. Extracted lipids expected to contain astaxanthin is diluted in an appropriate solvent, and the absorbance read at absorbance maximum for astaxanthin, 470 nm. For control samples, pure solvent or lipid without pigment is used. Absorbance is affect both by the natural structure of the polyene, by structural features in the astaxanthin molecule, and by the solvent. Following this, the absorbance A is used in an equation (VII) described in chapter 3.3.7 to calculate the concentration of astaxanthin in the lipid sample (Davies 1976; Tolasa et al. 2005; Metusalach et al. 1997).

Other methods like NMR and HPLC could be efficient in the determination of total astaxanthin content (Vilasoa-Martínez et al. 2008; Holtin et al. 2009; Lu et al. 2010). The use of spectrophotometry was however chosen based on availability of equipment in the lab and because the method is time efficient (Tichy et al. 2011).

2. Aim of the study

The main aim of this project is to study how proteins, lipids and astaxanthin can be extracted from snow crab rest raw material in a sustainable manner. Sustainable is here defined as environmentally friendly and efficient. Four aims listed below have been set in order to achieve the main aim.

- Perform a literature search in order to explore and compare methods for recovery of protein, lipid and pigment from crustacean rest raw material.
- Determine the chemical composition of two different batches of snow crab rest raw material, and discuss the effect of different slaughter processes of snow crabs.
- Use commercial proteases and heat treatment in the recovery of proteins and lipids from snow crab rest raw material, and compare the efficiency of the methods.
- Use oil extraction with and without pre-treatment with protease, as well as supercritical fluid extraction in the recovery of astaxanthin from snow crab rest raw material and compare the methods.

A project plan can be viewed in appendix A. This appendix also includes overview of all the work conducted in the lab, with batch 1 (B1) and 2 (B2), respectively. The determination of chemical composition of batch 1 and 2 is expected to reveal that presence of intestines and hemolymph in the rest raw material affect the nutritional value of the rest raw material.

The enzymatic hydrolysis is expected to give a higher recovery of protein compared to heat treatment, because adding proteases enhances degradation of proteins. Recovery of lipid is expected to be difficult because the experiments are run in a small scale, and the lipid content of snow crab is low compared to protein and ash. The use of enzymatic hydrolysis as pre-treatment for extraction of astaxathin in rapeseed oil is expected to aid the recovery as can enhance the degradation of the material. Finally the use of CO_2-extraction of astaxanthin is expected to be more efficient than extraction with oil as it has shown promising results in several studies. The knowledge generated in this study can be used in the development of a more sustainable fishery of snow crab, and add value to the production.

3. Materials and methods

3.1. LITERATURE STUDY

To explore the subjects introduced the literature collection databases Google Scholar, Research Gate, Springer and Science Direct was used in search of primary and secondary literature. The online database search terms used were crustacean*review, crustacean*by-products, hydrolysis*crab, chitin*crustacean, enzymatic*hydrolysis*shrimp, protein*recovery*crustaceans, hydrolysis*proteins*crab, deproteinisation, demineralisation, crustacean*exoskeleton*minerals, deproteinisation*seafood. Some article written before year 2000 were included, as they had been used in recent publications already included in this review. The search included the following areas of research; food science, biotechnology, microbiology and (aquatic) biochemistry.

3.2. REST RAW MATERIAL BATCHES AND PRE-TREATMENT

The rest raw material was obtained via Nofima AS in two batches, purchased from fishing boat Northeastern (H-27-AV), captured 9-16.11.17 (N 75°49,2 E 37°39,2), at ca 220-240 metres depth. The first batch (1 kg) was received in Trondheim 29.8.18 as minced crude material of carapace, digestive systems (including hepatopancreas) and physiological liquid (hemolymph). The second batch (9 kg) was received in Trondheim 24.10.18 as whole empty carapaces. These crabs had been slaughtered on the boat and the RRM contained solely cephalothorax. The material was coarsely ground from frozen state (Sinmag Mixer SM200) at the Department of Biotechnology and Food Science, Kalvskinnet, by help of engineer Trond Viggo Pettersen. After the first mincing, the material was kept in zip-lock bags at -40°C. The material was then thawed in water baths (~40°C, 20 minutes) and finely minced (Hobarth AE200, 10mm mesh hole size) at SINTEF Ocean, by help of engineer Marte Schei. During the thawing some water from the water bath got mixed into the material through wholes in the bags containing the material. Water was discarded out of the bags by decantation. The finely minced material was portioned into batches of about 500 g, and stored in lidded plastic containers at -40ºC. Before each experiment, batches were thawed in a water bath of about 40°C for 20 minutes. Occasionally the thawed RRM was returned to -20°C and thawed again before the next experiment, but this was never repeated more than once.

Within all experiments and analysis, equal samples dealt with are described as *parallels*. Repetitions of experiments are *replicas*. Not all experiments are repeated, and the results generated from these experiments are not considered more than indications of trends, as it is not statistically feasible to determine rigid trends without proving the results as reproducible.

3.3. QUANTITATIVE AND QUALITATIVE ANALYSIS OF CHEMICAL COMPOSITION

The chemical composition of batch 1 (B1) and 2 (B2) was determined. The features determined were dry weight and ash content, total protein, soluble protein, free amino groups and degree of hydrolysis, total lipid content and total astaxanthin content. All the analyses are performed on both batch 1 and 2, except for total amino acid composition, free amino groups and degree of hydrolysis that was not performed on batch 1, because this batch had been used up by the time the analysis was done. All analysis methods were also performed on fractions obtained from hydrolytic fractionation. If not stated otherwise, the method is performed similarly on RRM and on sludge and water fractions. For convenience, the mass of rest raw material, sludge and water fraction is assumed to have a density of 1g/mL.

3.3.1. Particle size

The particle size of the two batches 1 and 2 were determined by measurement (cm^2) of eight randomly selected particles from each batch. The comparison was based on average size ± SD.

3.3.2. Dry weight and ash

Dry weight (DW) and ash content was determined according to AOAC (1980). DW was determined, by heating a weighed amount of RRM in an oven at 105ºC for approximately 24 hours. The samples were cooled in a desiccator and the dried mass was measured. Dry weight in % w/ww was calculated using equation I.

Ash content was determined in six parallels by heating the dried material in an oven at 550°C for approximately 24 hours. The samples were cooled in a desiccator and the mass was measured. Ash content on % w/dw was calculated using equation II. Both analysis´ were performed with six parallels.

$$\frac{dry\ wweight\ raw\ material\ (g)}{wet\ weright\ raw\ material\ (g)} \times 100\% \qquad \text{I}$$

$$\frac{ash\ (g)}{raw\ material\ dry\ weight\ (g)} \times 100\% \qquad \text{II}$$

3.3.3. *Bligh & Dyer*

Total content of lipids was determined in 4 and 6 parallels for batch 1 and 2, respectively, according to a modified version of Bligh & Dyer (1959) (appendix B). Raw material (5-10 g) was added distilled water (H_2O, 16 mL), methanol (MeOH, 40 mL) and chloroform ($CHCl_3$, 20,0 mL) in a centrifuge cup. The mixture was homogenised for 2 minutes (IKA® T25 digital ULTRA THURRAX). The centrifuge cups were kept on ice at all times. After the first homogenisation, $CHCl_3$ (20,0 mL) was added before homogenisation for 40 seconds. Lastly, H_2O (20 mL) was added followed by homogenisation for 40 seconds. The mixture was centrifuged at 4080 *x g* (times gravity) for 10 minutes (RC5B Plus, Sorvall®). The bottom chloroform phase was pipetted out, and a sample (2 mL) from each parallel was evaporated on a heating block at 60 ºC for 1 hour under a stream of N_2-gas (Reacti-Vap™ Evaporating Unit Model 18780, Pierce). The dried lipid samples were cooled in a desiccator for 24 hours before measurement of mass and total lipid (%) was determined using equation III.

$$\text{Total lipid (\%)} = a \times b \times \frac{100}{c \times v} \qquad \text{III}$$

In the equation, *a* represents gram evaporated fat, *b* added chloroform, *c* mL evaporated chloroform and *v* is the weight of the final lipid sample.

3.3.4. *Total amino acid composition*

Determination of total amino acid composition was performed in order to estimate the total protein content of the RRM. The procedure was performed after Blackburn (1978). The RRM was freeze-dried overnight (Alpha 1-4 LD plus mod. 20182, Christ®). Dry samples (100 mg) were added HCl (6 M, 2mL), and the mixture was incubated at 105°C for 22 hours. Hydrolysed samples were cooled and transferred to 10 mL beakers using distilled water. The mixture was neutralised with NaOH to pH 7. The neutralised solution was filtered through Whatman glass microfibrefilter GF/F using suction and the volume adjusted to 10 mL. All parallels were diluted to 1:500, and filtered through 0,2 μm filter using a syringe. Finally, a sample (0,205 mL) was prepared for HPLC (Blackburn 1978). HPLC samples were stored at -20°C. Engineer Siri Stavrum at the Department of Biotechnology and Food Science ran the HPLC analysis. The analysis was performed with four parallels from the RRM.

3.3.5. *Lowry method*

The Lowry method was used to determine soluble protein content (Lowry et al. 1951). When estimating soluble protein in solid material, crude material and sludge material, extraction of soluble protein was first performed by dilution in water, centrifugation and filtration. Solid material samples (1 g) were dissolved in distilled water to total volume adjusted to 10 mL (1:10). The mixture was centrifuged (10 min, 1860 *x g*, Heraeus Multifuge X1R Centrifuge, Thermo Scientific). The liquid phase was filtered (Filterpaper Circle 589[1] Schwargband/ black ribbon 70 mm), and the volume was adjusted to 21 mL to get a stock solution with dilution 1:20. For the first analysis, three samples stock solution were diluted to 1:50, 1:100 and 1:1000 solutions. Results showed that dilution 1:100 was most appropriate for solid material, and so this dilution was used for the remaining samples. For analysis of water fractions the samples were diluted appropriately (1:250 - 1:400) and filtrated.

The Alkaline copper reagent was prepared with 1 % $CuSO_4$, 2 % potassium sodium tartrate and 2 % Na_2CO_3 in 0,1 M NaOH (1:1:100). The Folin reagent was prepared by diluting Folin Ciocalteu in water (1:3). All parallels were analysed in triplettes. Each sample (0,5 mL) was added the reagents and the absorbance was read at 750 nm (Genesis 10S UV-VIS Spectrophotometer, Thermo Scientific). The standard curve found by plotting the average absorbance against known concentrations of standard bovine serum albumin (BSA) samples was used to find the concentration of soluble protein in the snow crab RRM and hydrolytic fractions.

3.3.6. *Formol titration: Free amino groups and degree of hydrolysis*

The amount of free amino groups and degree of hydrolysis were determined by formol titration (Taylor 1957). Samples of raw material (~1,5 g) diluted in water (~50 mL) had a pH just above 7, and were therefore neutralised with formaldehyde to pH 7,0. Formaldehyde was titrated with 0,1 M sodium hydroxide (NaOH) to pH 8,5. The titrated formaldehyde (10 mL) was added to the neutralised raw material sample, and left for 5 minutes. The mixture was titrated with NaOH (0,1 M) to pH 8,5 (TitroLine® 7000, SI Analytics). The volume of NaOH used in the final titration was used in further calculations. The amount of free amino groups was found using equation IV, where *A* is mL NaOH used, *B* is concentration of NaOH (0,1 M), 14,007 is the molecular mass of nitrogen, C is mass of sample in grams, and 100 and 1000 are scaling factors to get the result in % free amino groups (w/ww). Degree of hydrolysis was calculated using equation V, where *D* is % free amino acids, *E* is % nitrogen found by dividing protein content with 6,25, and 100 is a scaling factor to get the result in % degree of hydrolysis (w/ww).

$$\% \text{ Free amino groups} = \frac{A \times B \times 14{,}007 \times 100}{C \times 1000} \quad \text{IV}$$

$$\% \text{ Degree of hydrolysis} = \frac{D \times 100}{E} \quad \text{V}$$

3.3.7. Spectrophotometric analysis of astaxanthin

Quantitative determination of astaxanthin content in lipids was performed after Tolasa et al. (2005). A sample of lipid (~0,2 g) extracted with Bligh and Dyer (1959) was dissolved in n-hexane (5 mL). The absorbance of the sample was read at absorbance 470-472 nm (Genesis 10S UV-VIS Spectrophotometer, Thermo Scientific). The analysis was performed in triplicates. Amount of astaxanthin in µg/g lipid was calculated using equation VI, where *A* is absorbance of the sample, *E* is a factor 2100 (the standard absorbance of 1% (w/v) astaxanthin solution at 472 nm in a 1 cm cyvette), *10000* is scaling factor to obtain the result in µg/g lipid dissolved in solute, and *C* is the concentration of lipid in n-hexane (Tolasa et al. 2005).

$$\text{Astaxanthin } (\mu g/g) = \frac{A}{E} \times 10000 \times \frac{1}{C} \quad \text{VI}$$

3.3.8. Molecular weight distributions

Molecular weight distributions of protein in hydrolysates were determined using fast protein liquid chromatography (FPLC). Sodium acetate buffer (0,05 M, pH 5) was filtered through a 0,2 µm filter using suction. The buffer was used both as mobile phase and for preparation of hydrolysates. Hydrolysate samples (1,2 g) were diluted in filtrated buffer (5 mL) and centrifuged (2907 *x g*, 10 min, 22°C, Heraeus Multifuge X1R Centrifuge, Thermo Scientific) before filtration through 0,2 µm syringe filter. Separation and detection of peptides were performed by a Superdex Peptide 10/300 GL column (Äkta FPLC UPC-900 + P-920, Amersham Biosciences). The column separates molecular weights between 100-7000 Da. The measured absorbance at 280 nm was plotted against retention volume, generating chromatograms with peaks indicating dominating molecular weights. Elution volumes were normalised using equation VII, where partition coefficient is determined with elution volume (V_e), void volume (V_o) and total column volume (V_t). A relation between K_{av} and molecular weight was determined using standard samples. K_{av} determined for vitamin B12 (M_w = 1,3 kDa), Aprotinin (M_w = 6,5 kDa) and cytochrome C (M_w = 12,4 kDa) was plotted against respective molecular weights, and a relation was determined by linear regression. Absorbance of all samples was determined once.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \qquad \text{VII}$$

3.4. ENZYMATIC HYDROLYSIS

The protocol for the enzymatic hydrolysis (EH) was based on knowledge obtained from lab work performed at Nofima AS 8.5.18 (appendix C) and after Beaulieu et al. (2009). Enzymatic hydrolysis was performed with four different proteases, Alcalase® 2.4 L (AL), Papain GSM80 (PA), Protamex® (PR) and Corolase® 2TS (CO). All proteases were used on batch 2, but only AL was used on batch 1. Details of number of replicas, parallels, mass of samples, temperature and hydrolysis time are given in **Table 3.1**. Figure 3.1 presents the flow scheme for the procedure. The pH in batch 1 and 2 was stable around 7, suitable for all proteases. The pH was therefore not regulated during enzymatic hydrolysis. This was also to use a time-efficient protocol without the need of adding acid or alkaline solutions. In that way, any up-scaling will be easier. Temperatures are based on optimal temperature ranges of each protease. In addition, the temperatures were kept as low as possible, considering the attempt to study how to conduct environmentally friendly processing of RRM. The mass of RRM sample needed was based on the resulting fraction mass and the planned further analysis. Also, as the access to snow crab RRM was quite limited, the use of RRM was held at a minimum and all experiments were performed at small scale. The enzyme to substrate ratio was 0,1%, and held equal for all treatments. All samples were added water with water to sample ratio 1:2. Fractions obtained from hydrolysis with CO showed higher values of soluble protein (chapter 4.3.4). This protease was therefore chosen for the combination experiments (chapter 3.6). Some of the replicas performed with this protease CO therefore deviated from the others in means of number of parallels. The two different temperatures 50 and 65°C for enzymatic hydrolysis with CO was chosen based on the increased amount of soluble protein generated with increased temperature in heat treatment experiments.

Table 3.1: Experimental details from enzymatic hydrolysis of snow crab RRM with proteases Alcalse® 2.4 L, Protamex®, Papain GSM80 and Corolase® 2TS.

Enzyme (batch)	Replicas	Parallels + control	Samples (g)	Temperature (°C)	Hydrolysis time (h)
AL (B1)	1	6 + 3	30	50	1,5
AL (B2)	4	5-6 + 3	30	50	1
PR (B2)	3	6 + 3	30	60	1
PA (B2)	4	6 + 3	30	65	1
CO(B2)	1-3	3-6	20-30	50	1
	4-6	2-3	20-30	65	1

The samples (~30 g) were mixed with water in 50 mL-tubes in water to sample ratio 1:2. All samples were immersed in the water bath for approximately 15-20 minutes in order to reach stable temperature. Lids were not tightened in order to release pressure at increased temperature. Enzyme was added, and during the following hydrolysis treatment (1 hour) all the sample tubes were shaken by hand about every 10 minutes in order to avoid accumulation of compact sediment on the bottom. After the end of hydrolysis, the tubes were transferred to a boiling water bath (90°C) for ten minutes in order to stop the enzyme activity. Mixtures were cooled in cold-water bath for 10 minutes. Subsequent centrifugation (9418 *x g*, 10 minutes, 22 °C, Heraeus Multifuge X1R Centrifuge, Thermo Scientific) generated a solid fraction (SF) and a water fraction (WF). In enzymatic hydrolysis of B1 with AL, an oil fraction was associated with WF (WF/OF).

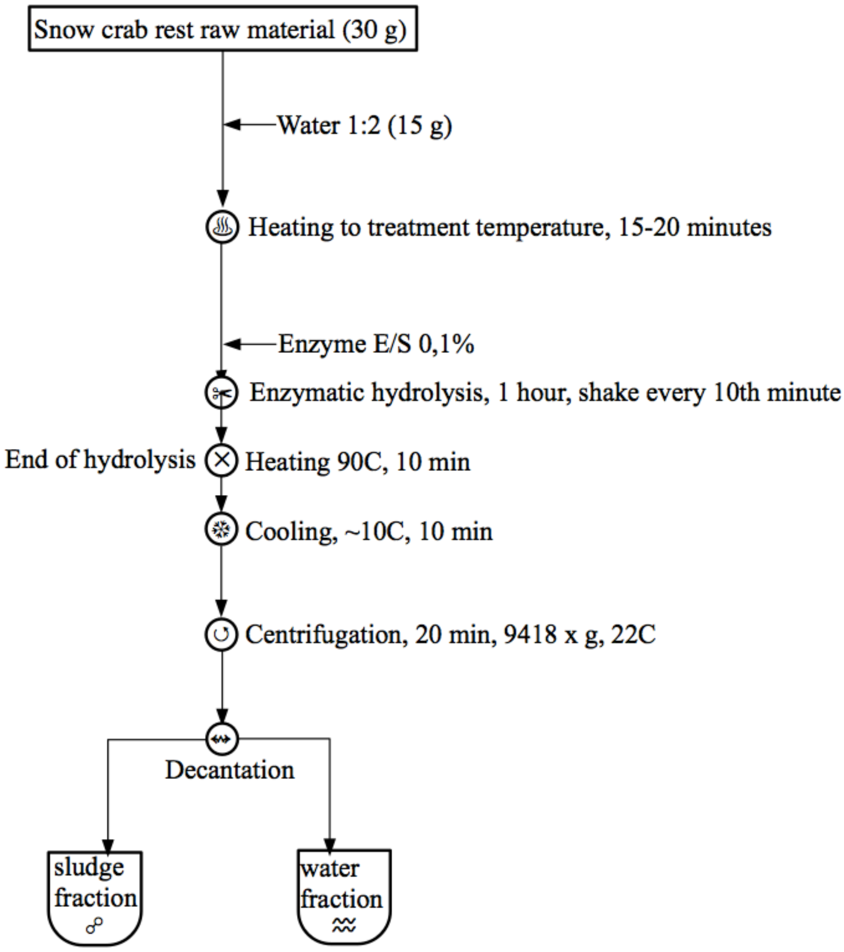

Figure 3.1: General flow sheet for enzymatic hydrolysis of snow crab rest raw material used in the experimental part of this thesis. For all experiments except the treatment with Corolase, centrifugation and decantation was repeated once with the water fraction. The C represents °C.

For hydrolysis experiments with AL (B1+B2), PA and PR the centrifugation and decantation was repeated for the WF. In these protocols, the second sludge fraction obtained was merged with the first sludge fraction. This merge was not done for AL (B1+B2). The second centrifugation did not appear to be necessary, as the finer sludge fraction was difficult to recombine with the first sludge fraction. The finer sludge fraction had a very small volume and low dry weight, and was difficult to remove from the experimental tube wall. This is further outlined and discussed in chapter 4.3.

All sludge and water fraction parallels obtained were weighed and subsequently merged with equivalent parallels within each replica. The merged fractions were stored at -20°C, or immediately analysed further. The mass of the fractions (%) was calculated based on original hydrolysis sample using equation VIII, where hydrolysis sample included water and enzyme added the RRM sample.

$$\text{Fraction (\%)} = \frac{fraction\ (g)}{original\ hydrolysis\ sample\ (g)} \times 100\% \qquad \text{VIII}$$

3.5. HEAT TREATMENT

Heat treatment (HT) was performed in 3 and 1 replica using a modified procedure of Carvajal et al. (2014). Samples of RRM were immersed in a water bath of 70°C (HT 70°C). Experimental details can be viewed in **Table 3.2**, and experimental procedure is presented in Figure 3.2. Replica 1-3 was performed using zip lock bags with RRM samples (35-50 g). These were not added water, but heat-treated plain. Replica 4 was performed as the enzymatic hydrolysis, using 50 mL tubes and samples (30) added water (water to sample ratio 1:2). The treatment was ended after 1 hour, and all samples were centrifuged (9418 *x g*, 20 minutes, 22°C, Heraeus Multifuge X1R Centrifuge, Thermo Scientific). Centrifuge conditions were performed as in enzymatic hydrolysis. Mass of SF and WF was measured, and parallels within every replica were merged with equivalent parallels. Dry weight samples were collected either before or after merging, and the fractions were stored at -20°C for supplementary analysis. Mass share of fractions were calculated according to equation VIII.

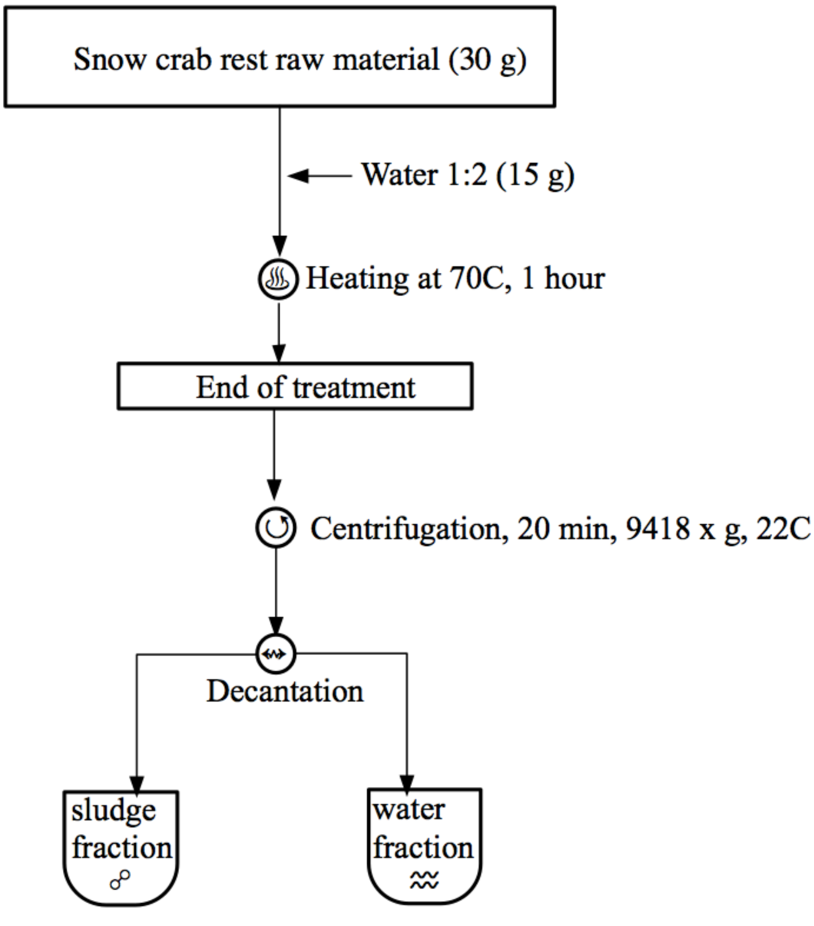

Figure 3.2: General flow sheet for heat treatment procedure. For replica 1-3, water was not added before the heating step. The C represents °C.

Table 3.2: Experimental details for heat treatment of snow crab RRM, replica 1-4.

Replica	Parallels	Samples (g)	Ratio water:sample	Temperature (°C)	Treatment time (h)
1-3	6	35-50	no water added	70	1
4	6	30	1:2	70	1

In addition to these independent heat treatment replicas at 70°C with and without added water, control samples from enzymatic hydrolysis experiments has been analysed as heat treatment samples. As control samples were RRM added water and exposed to different temperatures in water bath, the protocol is close to identical to heat treatment protocol. These samples are therefore considered heat-treated samples, and the chemical content of the fractions is compared.

3.6. COMBINATION EXPERIMENT AND PIGMENT RECOVERY

In an attempt to increase the yield of compounds from the RRM, enzymatic hydrolysis with Corolase® 2TS was combined with a subsequent heat treatment, and the addition of rapeseed oil for extraction of astaxanthin (Figure 3.3).

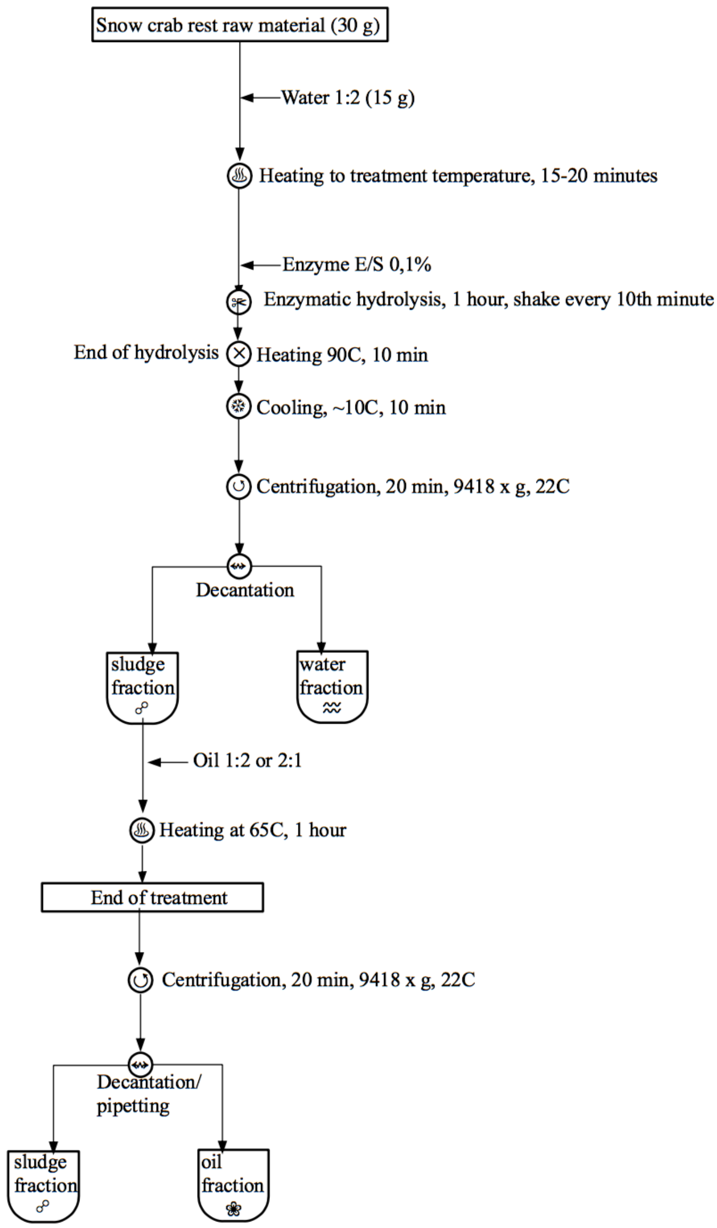

Figure 3.3: Flow sheet for combination experiment ($EH^{T}HT_{oil}$) with enzymatic hydrolysis followed by heating of sludge together with rapeseed oil. The C represents °C.

The combination experiment was also based on the experiments of Carvajal et al. (2014) with herring RRM (Carvajal et al. 2014), Sachindra and Mahendrakar (2005) use of vegetable oil for recovery of astaxanthin pigment from shrimp by-products (Sachindra and Mahendrakar 2005), and Hooshmand et al. (2017) work with sunflower oil and blue crab (*Portunus pelagicus*) and shrimp (*Penaeus semisulcatus*) (Hooshmand et al. 2017).

Combination experiments (CO^{T}+HT_{oil}) were performed in 1-2 replicas per experiment. Experimental details can be viewed in **Table 3.3**. Combinations are $CO^{50°C}HT^{65°C}_{oil\ 1:2}$, $CO^{65°C}HT^{65°C}_{oil\ 1:2}$, $CO^{65°C}HT^{65°C}_{oil\ 2:1}$ where superscripted values are treatment temperatures (°C) and *oil* is followed by the oil to sample ratio. Combination of enzymatic hydrolysis with CO at different temperatures (T) followed by heat treatment (65°C) and addition of rapeseed oil in different ratios was compared to heat treatment of RRM added rapeseed oil, $HT^{65°C}_{oil\ 1:2}$.

Table 3.3: Experimental details of the combination experiment. Column with experiment describes the combination of enzymatic hydrolysis treatment and heat treatment. Abbreviations are HT = heat treated (only heat treated once), CO = Corolase® 2TS, SF = sludge fraction, control = merged control samples from enzymatic hydrolysis.

Experiment	Rep.	Parallels + control	Samples (g)	Ratio oil : sample
$HT^{65°C}_{oil\ 1:2}$	2	6	20	1:2
$CO^{50°C}HT^{65°C}_{oil\ 1:2}$	2	3 + 3/5+2	20 - ≤10	1:2
$CO^{50°C}HT^{65°C}_{oil\ 1:2}$ (stored overnight -20°C)	1	3 + 3	20 - ≤10	1:2
$CO^{65°C}HT^{65°C}_{oil\ 1:2}$	2	4 + 4	10	1:2
$CO^{65°C}HT^{65°C}_{oil\ 2:1}$	2	4 +4 /3 + 2	15 - ≤10	2:1

For the combination experiments, the merged SF samples from enzymatic hydrolysis of RRM with protease CO at 50 and 65°C were used. Samples were measured into 50 mL tubes, and rapeseed oil was added. The heat treatment with oil was performed for 1 hour at 65°C, followed by centrifugation (9418 *x g*, 20 minutes, 22°C, Heraeus Multifuge X1R Centrifuge, Thermo Scientific). Results of soluble protein from heat treatment experiments (chapter 4.4.1) showed no significant benefit of higher temperatures than 65°C as the activity of endogenous proteases decreased. As this would also affect the release of astaxanthin, this was taken into consideration in the combination experiments. Fractions generated after heating with oil were separated by decantation and pipetting. This method of separation was performed for equally for all replicas except replica 2 of $CO^{50}HT^{65}_{oil\ 1:2}$. For this experiment, the mixture was centrifuged as above, and then stored at -20°C for about 12 hours. Thereafter, the mixture was centrifuged again, and separated by decantation and pipetting as above.

3.7. SUPERCRITICAL FLUID EXTRACTION

Extraction of pigment was also done by supercritical fluid extraction (SFT-110XW, Supercritical Fluid Technologies Inc.). The procedure was kindly performed by researcher Elena Shumilina at Dikiy Lab, Department of Biotechnology and Food Science, NTNU Trondheim (19. - 20.3.19). Material from batch 2 were thawed in a water bath (~40°C, 20 min) and placed into the processing vessel. The oven and restrictor block temperature were set to 60°C. Samples (15 g) were first soaked for 20 minutes at 4500 psi. Then samples were subjected to dynamic flow for 10 minutes with CO_2 and co-solvent ethanol (EtOH) at flow rates 10 and 0,5 ml/min, respectively. After soaking and dynamic flow, the procedure was repeated 3 times without ethanol. Resulting samples of pigment dissolved in ethanol was evaporated on a heating block at 30 ºC (3 hours) under a stream of N_2-gas until dry (Reacti-Vap™ Evaporating Unit Model 18780, Pierce). Final pigment samples were measured and the weight divided by the amount of original RRM sample used.

3.8. MASS BALANCE CALCULATIONS

The recovery of protein, lipid, ash and astaxanthin in processing of RRM is quantified by determining the mass balance of the compound. In this study, components in fractions generated by different treatments are described as *the change of* (equation IX), *the recovery of* (equation X) or *the concentration of* (equation XI), in comparison to the starting amount, or concentration of the component in the rest raw material. Equation X is equal to equation VIII *Recovery of fraction* (chapter 3.4), but these are separated to avoid confusion. Fraction represents the fraction obtained by treatment and separation of phases after treatment, either sludge fraction (SF), water fraction (WF) or oil fraction (OF). Whenever these equations are used, they are simply described in words as change, recovery and concentration, not with their numerations IX, X and XI.

$$\text{Change of component } (\Delta) = \text{component in RRM} - \text{component in fraction} \qquad \text{IX}$$

$$\text{Recovery of component } (\%) = \frac{mass\ of\ component\ in\ fraction}{mass\ of\ component\ in\ RRM} \times 100\% \qquad \text{X}$$

$$\text{Concentration of component} = \frac{Mass\ of\ component\ in\ fraction}{Mass\ of\ fraction} \qquad \text{XI}$$

For some of the comparisons, the mass balance principle is used to explain why the presence of one component affects the presence of another. Crustacean dry weigh material has four main components, ash, protein, lipids and chitin (Synowiecki and Al-Khateeb 2000). Thus, when comparing eg. batch 1 and 2, more ash in one of the batches than the other also imply that the protein content may be different. Or when comparing to hydrolytic fractions; if one treatment recovers more ash in the water fraction, then this is possibly at the expense of protein recovery in this fraction (Valdez-Peña et al. 2010).

3.9. STATISTICS AND STUDY DESIGN DETAILS

Data obtained in the experiments were analysed using Microsoft Excel. Standard deviation of average values was determined using the function (=STDAV). Students T-test was performed and differences were considered significant at p-values of 5%. Whenever significance is indicated with symbol, one star indicate p-value ≤ 0,05, two stars indicate p-value ≤ 0,02. If the p-value is lower than 0,001, the values is replaced by three stars.

4. Results and discussion

4.1. LITERATURE SEARCH - METHODS OF RECOVERY

A literature search was done in order to describe the methods performed to recover protein, lipid and pigment from crustacean rest raw material. This was done before the onset of the laboratory work, to evaluate which methods that are efficient and environmental friendly. The findings lead to the use of commercial proteases, heat treatment, oil extraction and supercritical CO_2 extraction. The results in this chapter describe the methods that were not used, in order to compare the methods throughout the discussion. These include the use of harsh acid and alkaline treatments and high temperatures, and are reckoned as more traditional. Later it will be discussed whether the chosen methods are equally or more efficient in the recovery of compounds than the more traditional methods. If so, it can be stated that it is possible to utilise snow crab rest raw material in a sustainable manner.

4.1.1. Acid and alkaline treatments

In the search for literature on the extraction of valuable components from crustacean RRM, chitin is frequently appearing. This is a biopolymer of high value, and crustacean material is the main source of natural chitin and its derivative chitosan (Shavandi et al. 2019; Healy et al. 2003). Efficient recovery of chitin demands total removal of minerals, proteins and pigments. Methods used are depending on being efficient in the removal in order to increase the recovery of chitin. Literature on chitin extraction has therefore been used to explore how proteins, lipids and pigments can be totally recovered from crustacean RRM. Due to the extensive complex organisation of chitin, proteins and minerals, chemical treatments for extraction are harsh and the chemical protocols involve strong acids and alkaline treatments and high temperatures (Kaur and Dhillon, 2015). Acid and alkaline treatments are time-efficient, and therefore the most used method. Three main steps are required to remove all chemical compounds from the inner chitin skeleton; Demineralisation by an acid treatment, deproteinisation by alkaline treatment, decoloration by organic solvents and chitin extraction (Hamed et al. 2016) (Figure 4.1). Various acids can be used in the demineralisation, but HCl is the preferred (Kaur and Dhillon, 2015). Figure 4.1 shows a flow chart for the chemical method of demineralisation, deproteinisation, decoloration and recovery of chitin, after Kaur and Dhillon (2015) and Hamed et al. (2016). Removal of lipids is also performed with organic solvents, eg. $CHCl_3$ and MeOH in Bligh and Dyer method or Folch method, but in the studies describing chitin purification, lipids are considered unproblematic compared to proteins and minerals, and thus usually not included.

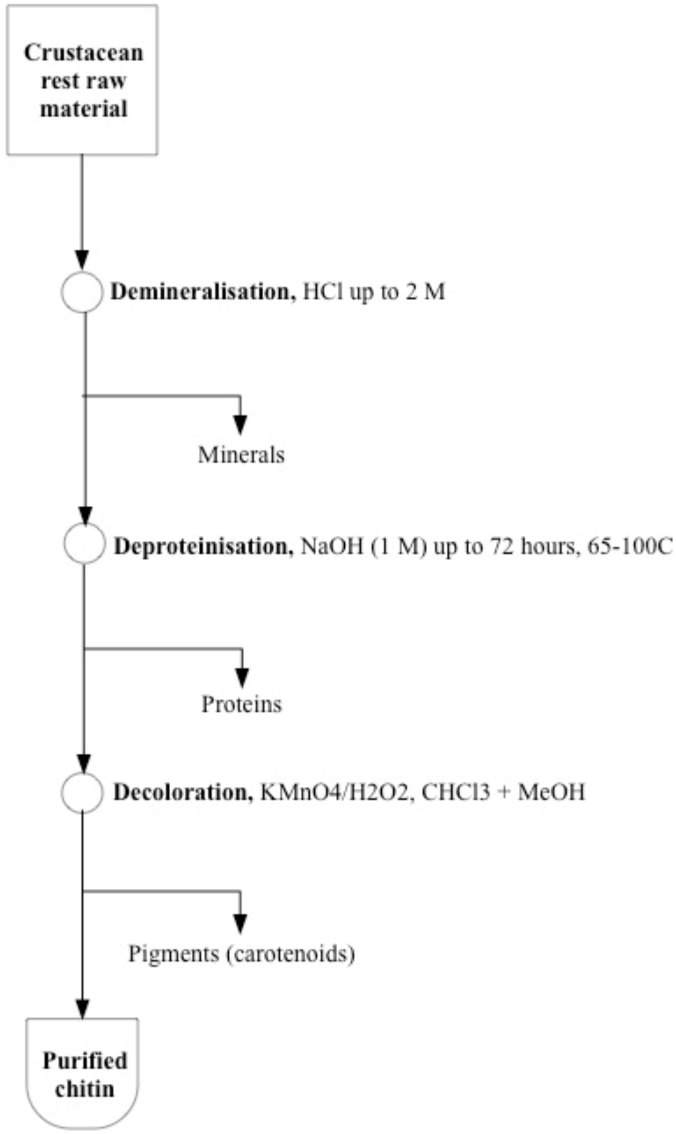

Figure 4.1: Traditional processing of crustacean rest raw material, by demineralisation, deproteinisation, decoloration and recovery of chitin by chemical methods. After Kaur and Dhillon (2015) and Hamed et al. (2016).

The use of HCl for demineralisation of *Parapenaeopsis stylifera* shrimp shell has been reported by Percot et al. (2003) to be achieved with 0,25 M HCl for 15 minutes (Percot et al. 2003). According to Kaur and Dhillon (2015), acid concentration can be up to 2 M for crustaceans in general (Kaur and Dhillon, 2015). This treatment almost completely removes calciumcarbonate ($CaCO_3^-$) and calciumphosphate ($CaPO_4^-$) (Kaur and Dhillon 2015; Hamed et al. 2016). Chemical deproteinisation is done by an alkali treatment, as NaOH degrades proteins. A protein liquid is generated during the process. The Percot study proposed a treatment of 1 M NaOH, 70°C, for 24 h, of the shrimp shells (Hamed et al. 2016; Percot et al. 2003). Again, Kaur and Dhillon (2015) states that the alkaline treatment can be up to 72 hours, and between 65-100°C. After demineralisation and deproteinisation the solution is added acetone in order to extract the remaining proteins and minerals. Pigments like carotenoids and melanin is removed by decoloration with 0,02% potassium permanganate at 60 °C, hydrogenperoxide, sodium hypochloride or organic solvent mixtures (Kaur and Dhillon 2015).

Ensilation, or acid silation, is utilisation of endogenous enzymes already present in the RRM for degradation of proteins. The material is added acid (ph 3,9-4,2) to create optimum conditions for the protolytic enzymes already present in the parts of the organism used. Grinding and mixing of the material ensures spread of the enzymes in the material. The acids will also react with the minerals in the material and perform demineralisation. The product of this process is a liquefaction of the RRM that is possible to use in feed as protein supply. Depending on the acid used, the product needs neutralisation before direct use in feed. Different acids are possible to use to control the pH, eg. hydrochloric acid (HCl), sulphuric acid (H_2SO_4) and formic acid (CH_2O_2). The strong HCl and H_2SO_4 have low costs but the product needs neutralisation before use in feed. Formic acid has a higher pH and so the product needs no/less neutralisation. RRM treated at 100°C for 15 minutes will not undergo autolysis degradation because the endogenous enzymes are deactivated at such high temperatures (Tatterson and Windsor 1974; Vieira et al. 2015). This gives reason to believe that crustacean RRM is depending on including hepatopancreas with digestive system in order to undergo degradation by endogenous enzymes. Empty carapaces without intestines are less likely to end up as a protein rich liquefaction because the endogenous enzymes are located in the midgut (Saborowski 2015).

Pigments have also been extracted from crustacean rest raw material using organic solvents. acetone, methanol, isopropyl alcohol and petroleum ether. Sachindra et al. (2006) reported optimalised extraction conditions at 50:50 mixture of isopropyl alcohol and hexane. This was in comparison to individually use of cetone, methanol, ethanol, isopropyl alcohol, ethyl acetate, ethyl methyl ketone, petroleum ether, hexane, and a mixture of acetone and hexane. The authors emphasise that the use of organic solvents is mainly for analytical procedures, and that extraction of pigments for commercial use is usually done with vegetable oils

(Sachindra et al. 2006). In the EU, the number of permitted acid and alkaline treatment solvents in food production is according to Directive 2009/32/EC, Annex I, limited. The levels of use is restricted depending on the type of food it is used for (EU 2009).

Even though the use of harsh chemicals for recovery of crustacean RRM products are time-efficient and removes close to all protein and minerals, the methods can have degradative effects on the resulting products. It increases the cost of production the process, the production is energy intensive, and causes environmental challenges. Disposal wastewater from the purification may need decontamination required by government legislation. And last but not least, products like minerals, proteins and pigments may be of poorer quality (Khor, 2014; Kaur and Dhillon 2015; Hamed et al. 2016; Shavandi et al. 2019).

4.1.2. Biological extraction - Lactic acid and/or hydrolytic bacteria

Despite the short reaction time of acid and alkaline treatments, the disadvantages with using strong acids and bases are that they are harmful to the environment and workers (Hamed et al. 2016). Fermentation, as a milder ensilation, has been studied, and studies using lactic acid as an extraction agent in demineralisation have given promising results. Lactic acid is an organic acid that can be produced by bacteria. This method has a lower cost, is not harmful to the environment in the same way as the chemical treatments, and resulting organic salts are available for further applications. Examples of uses are *Lactobacillus pentosus* (Arabia et al. 2013). The use of *Lactobacillus pentosus* gives simulataneous demineralisation and deproteinisation. The use of lactic acid bacteria requires addition of some energy source of carbohydrates, eg. cassava extract or lactose. The lactic acid is produced by the bacteria, and generates an environment with low pH. Further the lactic acid reacts with $CaCO_3^-$ and $CaPO_4^-$ as calcium lactate and phosphorus lactate, precipitates and is easily removed by washing (Arabia et al. 2013; Hamed et al. 2016).

Several studies have tried different raw material, bacteria cultures, carbohydrate additives and conditions. In studies with crayfish, scampi and prawn RRM, the fermentative lactic acid bacteria has been used as a pre-treatment in combination with chemical or enzymatical demineralisation and deproteinisation, respectively. By pre-treating the raw material, lower concentrations and amounts of acid and alkali was needed, 0,5 M HCl and 0,4 M NaOH, respectively (Arabia et al. 2013). An alternative to the use of single bacteria cultures is using mixtures consisting of several active bacterial cultures.

Also non lactic acid bacteria have been studied in the biological extraction of chitin. Jo et al. (2008) used a protease-producing *Serratia marcescens* FS-3 for fermentation of snow crab shells. The trial showed 84% deproteinisation after 7 days (Jo et al. 2008). This was supported by earlier studies using protease-producing bacterium *Pseudomonas aeruginosa* K-187 in fermentation of crab shell powder with 72% deproteinisation after 7 days (Oh et al. 2000). Yang et al. (2000) also showed recovery of proteins and ash using three different microorganisms, *Bacillus subtilus* Y-180, *Bacillus subtilus* CCRS 10029 and *Pseudomonas maltophilia* CCRS 10737. The shrimp, crab and lobster shells (species unspecified) were prepared by washing with tap water and subsequently powdered, and split into acid (HCl) treated and natural batches. Fermentation by all strains gave higher deproteinisation when the raw material was not treated with acid. *Bacillus subtilus* Y-180 achieved 88, 67 and 83% deproteinisation of shrimp, crab and lobster shell after 7 days of treatment, respectively (Yang et al. 2000).

According to a review by Arabia et al. (2013), the yield of proteins, chitin and ash from bacterial methods is close to the results from use of acid and alkaline treatments. They are not efficient enough to compete with the traditional methods of extraction because they are more time-consuming. The removal of proteins and minerals has been achieved at 40-99%, and to 23,3-99,7%, respectively, with various rest raw material species, bacterial species, carbon source and treatment duration. Before the biological method can be applied on an industrial level, optimalisation of conditions for demineralisation and deproteinisation of shell wastes must be done. In addition, the most efficient bacterium for the given crustacean species must be determined. Also, inexpensive carbon sources must be applied and available for large-scale fermentation processes (Arabia et al. 2013).

It is inevitable that an important advantage of using microbial extraction methods is the recovery of the other possibly valuable compounds like proteins, free amino acids, carotenoids and ash salts with higher quality, which may add value to the purification process (Kaur and Dhillon, 2015). This is also beneficial to the environmental impacts of the process. Viewing the process in a larger scale, the bacterial fermentation procedure will do less harm to the environment. However, the time required to extract compounds by bacterial fermentation is considerably longer and thus might be less desirable for producers.

The use of commercial proteases, heat treatment, oil extraction and supercritical fluid extraction for recovery of protein, lipids and pigments was also studied. As these methods are considered as mild, environmentally friendly and at the same time efficient, compared to the use of harsh acids and alkaline treatments, these methods were used in the laboratory work of this study (Cremades et al. 2001; Shavandi et al. 2019). The description of the method and comparison to the acid and alkaline treatments and the biological extraction is done in the introduction, chapter 1.2, and in the results and discussion of chapter 4.3 - 4.5.

Chitin is the main focus in recovery of compounds from crustacean waste. Recovery of chitin is depending on the removal of proteins and minerals, and studying recovery of chitin can therefore be used to study recovery of proteins and minerals. As crustacean material is the largest source of chitin, there are more studies describing chitin recovery than eg. protein and pigment lipid recovery. Chemical treatments are time-efficient, and remove proteins and minerals almost completely. The methods are however harsh to both the end products, the environment and are of high cost (Fernandes 2016; Kaur and Dhillon 2015). The results of this literature search will be further used in discussion of the use of proteases.

4.2. CHEMICAL COMPOSITION OF BATCH 1 AND 2

The first aim of the experimental work of this study was to determine the chemical composition of snow crab RRM, batch 1 and 2. The batches were containing carapace including intestines and hemolymph, and carapace only, respectively. This study has focused on the recovery of proteins, lipid and pigments, and the determination of chitin is therefore not performed.

The determined chemical content in snow crab RRM, batch 1 and 2 (**Table 4.1**), is to a varying degree comparable to the determined chemical content from other studies (**Table 1.1**, page 5). The chemical composition of snow crab rest raw material will vary with season, sex, and locality, in addition to the chosen methods of analysis (Beaulieu et al. 2009; Iverson et al. 2001). Information of season, sex and locality is not always available, but the chosen method of analysis is discussed.

Table 4.1: Chemical content and particle size of snow crab RRM, batch 1 and 2. Stars * indicate significant difference between the values of batch 1 and 2 (p≤0,05).

Feature		Batch 1	Batch 2
Particle size (cm^2)		1,2±0,7	0,5±0,6*
Dry weight % (g/100 g, w/ww)		18,4±0,05 (liquids) 28,8±3,8 (sediments)	32,7±1,8*
Protein content	Total amino acids % (g/100 g, w/dw)	NA	36,62±9,63
	Soluble proteins % (g/100 g, w/dw)	4,5±0,2	4,2±0,2
	Free amino groups % (g/100 g, w/dw)	2,2±0,2	1±0,1*
Lipids % (g/100 g, w/dw)		14,6±1,7	5,6±0,4**
Ash % (g/100 g, w/dw)		50,3±4,7	32,5±2,8***
Astaxanthin µg/g RRM (w/dw)		56,8±0,4	33,5±2,2**

4.2.1. Particle size

The average particle size from the two batches was determined. The different degree of mincing of the RRM B1 and B2, could affect the extraction experiments because increased mincing increases the surface of the material. The average size of particles in batch 1 was 1,2±0,7 cm2, and average size of particles in batch 2 was 0,5±0,6 cm2 (appendix D and **Table 4.1**). The average particle sizes of the batches are significantly different from each other. As the reaction tubes used in the experiments had a diameter of about 2 cm, the larger particles in batch 1 made the work more challenging. Larger particles had less ability to move in the reaction tube, and these mixtures were thus more difficult to mix with enzyme. This is further discussed in the following chapters. Where the protease is well mixed with the RRM, the results are expected to improve. Poorer mixing caused by larger particles is expected to give poorer recovery of protein.

4.2.2. Dryweight

The dry weight of snow crab RRM was determined. The RRM of batch 1 was roughly grounded, consisting of crushed solid material and liquid of dark colour. The liquid and solid parts were therefore analysed separately. The dry weight of the solid parts was further used to compare to batch 2, as the solid texture was more similar to batch 2 than the liquid texture. Raw data is presented in appendix D, results in **Table 4.1**. In the solid part of batch 1, dry weight was 28,8±3,8%. In the liquid part, dry weight was 18,4±0,05%. Batch 2 was more homogenous and more evenly grounded, described in chapter 3.2. The dry weight was 32,7±1,8% in batch 2. Batch 2 has a significantly higher dry weight than batch 1. Neither of the batches were mixed with water in the mincing.

Compared to the dry weight determined in the literature (22,2-28,0%), the values found in this study are about the same for batch 1 (28,8 %) and a little higher for batch 2 (32,7%). Considering that the rest raw material used in different studies is obtained from harvest at different seasons and localities, and processed differently, it is expected that the determined chemical composition is somewhat different. The processing at slaughter mainly determines the content of the RRM, as intestines are discarded when crabs are slaughtered by machine, but not when slaughtered by hand. RRM containing intestines and discard meat is expected to contain more water, as the presence of intestines will include water. Batch 1 included digestive system as it was processed in the lab, and batch 2 was empty carapaces obtained from slaughter on boat. The difference between the two batches 1 and 2 is therefore expected. Further, after molting the crab contains more water as it leaves it former shell and take in water in order to grow and gain a new shell (Hobbs and Lodge 2009). Therefore, depending on time since last molt, the water composition will vary.

Higher dry weight per batch will be more advantageous as it by definition will mean more compounds to extract from a smaller total volume. Meaning, a smaller wet volume of crab could contribute with more dry weight. The transport of RRM on shore includes an economic cost, as the boats in the Norwegian fisheries of snow crab are allowed to discard RRM into the sea. When using crustacean RRM for further utilisation, the RRM has to be available in high quality and regularly large amounts in order to contribute to commercial production (Venugopal 2016). This indicates that as much of the rest raw material as possible should be brought on shore. If RRM is to be further utilised, the boats have to charge for extra freezing and storing of the RRM. This would be at the expense of the cooked clusters, and is therefore estimated to cost 10 kr/kg RRM (in an email from Siikavoupio, S. Dr. philos (Sten.Siikavuopio@Nofima.no) 23.4.19). Considering the quota of 4000 tonnes, and the 1200 tonnes RRM generated (~30% of total harvest), bringing all RRM generated in the annual Norwegian fisheries on shore would cost 12 million NOK. High dryweight combined with high nutritional value per dry weight is therefore necessary to make further utilisation economically sustainable. This can be a challenge as the batches used in this study represent how low dry weight can contain high nutritional value, and high dry weight can contain less nutritional value (**Table 4.1**).

4.2.3. Ash content

The ash content of the RRM was determined. Raw data is presented in appendix D and final results in **Table 4.1**. The amount of ash in batch 1 and batch 2 was determined to 50,3±4,7 and 32,5±2,8% (w/dw) respectively. Batch 1 has significantly higher content of ash than batch 2. Considering the processing of the rest raw material, the ash content was expected to be different. The carapace of the crab is mineralised, and in addition there is a mineral storage located in the cardiac stomach (Soundarapandian et al. 2013). Ash content is therefore more likely to be present in the attached remaining intestines in batch 1, compared to the empty carapaces of batch 2. The processing at slaughter therefore affects the ash content of the RRM.As crabs molt (leave old carapace and grow new) to grow; the molting is affecting the chemical composition. When the crab has left its shell, it looses calcium. This is regained over the gills, and together with ash stored in the cardiac stomach before the molt, a new shell is restored (Hobbs and Lodge 2009). This means that depending on time since last molting, the ash content will vary. Crabs at different life stages (instars) molt at different times of the year.

Beaulieu et al (2009) used rest raw material including intestines and hemolymph, and is therefore expected to have similar chemical composition as batch 1. However, they determined the ash content to be 25,69%, which is not in agreement with the ash content in batch 1. Method of analysis is performed equally for all studies. Therefore, one can imply that the season could also affect the ash content. In Norway, it is not allowed to harvest the snow crab within the period 15th of June – 15th of September. This is in respect to that a larger part of the commercial available batch has recently molted, and the crabs are fragile (Regjeringen 2018). Based on the legal size of harvest (10 cm carapace width) the life stage of most harvested crabs can be assumed. But because the size and life stage is not necessarily a rigid relationship, and the terminal molt to maturity can vary between individuals, meaning that not all crabs of a given size will molt regularly (Lovdata 2005; Comeau et al. 1998). As the composition of mature crabs in one batch will vary, the effect of time since last molt on ash content is difficult to define. As both batch 1 and 2 are harvested in November this is not a matter of discussion. But it does indicate that commercially available batches harvested late autumn will contain more ash, and might not be affected by loss of ash in molting. More ash content in the rest raw material will lead to less available protein per dry weight, and so the content of ash is an important factor when discussing the increased utilisation of snow crab rest raw material.

4.2.4. Total amino acid composition

An estimate of the total protein content in batch 2 was performed by determining total amino acid composition with acid hydrolysis (HCl, 6M) and separation and detection of amino acids by HPLC. Results are presented in **Table 4.1**, and raw data can be viewed in appendix D. The analysis was not performed on batch 1 as this batch had been used up by the time of the analysis. The total amino acid composition of batch 2 was 36,6±9,6% (w/dw). In comparison to the determined protein content from the studies described in **Table 1.1** (17,8-42,9 %, w/dw), the protein content of batch is within expected range.

For determination of total protein content several methods were considered. Crustacean material is difficult in the sense of its molecular build up. As described in chapter 0, the crustacean shells consist of chitin polymers intertwined with proteins. These structures are enhanced by deposition of ash and are also associated with pigments. Protein will be locked into a matrix of chitin and ash. Detection and determination of proteins in the shells are thus depending on comprehensive hydrolysis to release as much protein as possible (Chen et al.

2008; Diaz-Rojas et al. 2006). Analytical determination of protein is often done by wet chemistry methods (eg. Kjeldahl), instrumental methods based on combustion of nitrogen, near- and min-IR spectroscopic methods or colorimetric reaction of peptides with a reagent (eg. Lowry). But these methods are all depending on soluble protein in the solution. This will not be appropriate when determining total protein content of the crustacean material.

All studies described in **Table 1.1** used Kjeldahl method for determination of protein content. The method for estimation of total protein in this study was chosen as it was expected to have a more efficient hydrolysis than the more standard method Kjeldahl. In addition, the access to equipment for Kjeldahl procedure was limited, thus HPLC was more convenient. The advantage of determination of total amino acid composition over Kjeldahl is the prolonged hydrolysis time and higher temperature. In addition, Kjeldahl detects nitrogen in the sample. Considering that crustacean material contain chitin, which contains nitrogen, a Kjeldahl analysis would require an additional chitin analysis. HPLC is therefore more time-efficient than Kjeldahl. Beaulieu reported the highest determined protein content (42,9%), which can be explained by the intestines present in their RRM.

The total amount of protein also gives an indication of the chitin content in batch 2. As all components other than chitin are estimated, the amount of chitin can be estimated to make up the remaining 20,9 % of the dry weight. This shows that the snow crab rest raw material of batch 2 is rich in protein, as this is the main component of the determined chemical composition. Looking at the dry weight of batch 1 and 2, one could assume that the protein content of batch 1 is lower than batch 2. The dry weight is composed of protein, lipid, ash and chitin. The dry weight is lower in batch 1, but both lipid and ash content is higher in batch 1. Unless the chitin content in batch is much lower than batch 2, the protein content should be lower.

4.2.5. Soluble protein

Determination of protein content in RRM was done with the Lowry method. Samples from batch 1 and 2 was properly diluted, centrifuged and filtrated. Raw data can be viewed in appendix D. The amount of soluble protein in RRM batch 1 and 2 was 4,9±0,2 and 4,2±0,2 % (w/dw), respectively (**Table 4.1**). The amount of soluble protein is not significantly different between the batches.

4.2.6. Free amino groups and degree of hydrolysis

Formol titration was performed after Taylor (1957) as an indirect titration (Taylor 1957). Raw data is presented in appendix D, and final results in **Table 4.1**. The concentration of free amino groups in batch 1 and 2 was 2,2±0,2 and 1±0,1% (w/dw), respectively. Batch 1 is significantly higher than batch 2.

Degree of hydrolysis in batch 2 (16,8±1,9%) is based on the total amount of protein in the RRM, described in chapter 4.2.4. As it was only batch 2 that was analysed for total protein content, the degree of hydrolysis was only determined for this batch. The values in **Table 4.1** are also therefore only given in free amino groups (g/100 g, w/dw), in order to be able to compare the two batches.

4.2.7. Total lipid

The total amount of lipid in the RRM was determined by a modified version of Bligh and Dyer (1959) (appendix B). The analysis was done with four and six parallels for batch 1 and 2, respectively. Raw data is presented in appendix D, and final results in **Table 4.1**. Batch 1 and 2 had a total lipid content of 14,6±1,7 and 5,6±0,4% (w/dw), respectively. Batch 1 had a significantly higher lipid content than batch 2.

The difference in lipid content was expected as batch 1 include intestines, and is therefore more likely to include some of the lipid stored in the crab. The comparison to the literature (0,1-17,1 %, w/dw) shows that lipid content of snow crab RRM vary. The season of harvest is not specified for all studies, thus it is difficult to define the effect of season on lipid content. Beaulieu et al. (14,82%, w/dw) used rest raw material including intestines, and is thus expected to have a higher lipid content than Lage-Yusty et al. (17,1%, w/dw), who used rest raw material without discard meat (Beaulieu et al. 2009; Lage-Yutsy et al. 2011). As intestines and discard meat is not necessarily the same, a clear difference in composition of between rest raw material in the literature is difficult to establish. But as Lage-Yusty emphasizes the importance of specifying the content of the RRM, and that the discard meat is removed, it is assumed that their rest raw material is similar to the RRM og batch 2 in this study.

The method of analysis could affect lipid content determined. Beaulieu et al. (2009) and Shahidi and Synowiecki (1991) (0,1-1,4 % w/dw) used versions of Bligh and Dyer (1959), Lage-Yusty et al. (2011) used Soxhlet system (solvent petroleumbenzine). Lage-Yusty reports that the findings of lipid in their studies are higher than all other studies they have compared to (Shahidi and Synowiecki 1991; Beaulieu et al. 2009; Lage-Yutsy et al. 2011). According to Clarke (1977), the determined lipid in Antarctic benthic prawn (*Chorismus antarcticus*) did not differ when using Bligh and Dyer compared to Soxhlet with chloroform as solvent (Clarke 1977). The reason for the large differences could therefore be the solvent petroleumbenzine or that Bligh and Dyer is less sensitive than Soxhlet. Other than this, based on the information about the origin of the RRM in the studies, it is difficult to determine any clear reasons for the difference in lipid content determined in different studies.

4.2.8. Pigment

The amount of astaxanthin in lipid from B1 and B2 was determined by spectrophotometric analysis. Results are based on average of 2 and 3 samples, respectively (**Table 4.1**). The raw data can be viewed in appendix D. The astaxanthin content in batch 1 and 2 was 56,8±0,4 and 33,5±2,2 µg/g RRM (w/dw), respectively. Batch 1 had a significantly higher content of astaxanthin than batch 2.

Astaxanthin accumulates in the back carapace of the crabs (Shahidi and Synowiecki 1991, Schiedt et al. 1993). The determined astaxanthin content was lower in batch 2 than both the reported values in the literature and than batch 1. Batch 1 also showed lower astaxanthin levels than the literature. This difference can be explained by astaxanthin destruction and by the choice of method. Astaxanthin are degraded by exposure to high temperatures, oxygen and light (Ambati et al. 2014, Takeungwongtrakul and Benjakul 2016; Niamnuy et al. 2008). Carotenoids can undergo *cis-trans* photoisomerisation at exposure to light, thermolability makes the carotenoids unstable when exposed to higher temperatures, and the exposure to oxygen can lead to oxidation. The degradation of astaxanthin leads to a loss of colour (Davies 1976; Bak et al. 1999; Chen and Meyers 1982), and the degraded astaxanthin can therefore be less detectable with spectrophotometric methods. Both batches were stored with exposure to light and oxygen. There is also a possibility that the RRM in batch 2 used to extract lipids and further analyse astaxanthin content had been thawed and freezed twice. This would increase the exposure to degrading factors, and decrease the detectability of the astaxanthin. The lipids from batch 1 had been stored at -20°C for about seven months before evaporation of chloroform (50°C) and spectrophotometric analysis. The lipid used for astaxanthin determination in batch 2 was stored in chloroform phase at -20°C for two days before evaporation of chloroform (50°C) and spectrophotometric analysis. Considering the difference in storing time, the storage at -20°C in chloroform is therefore not assumed to affect degradation of the astaxanthin. It should also be mentioned that the available lipid from batch 1 was scarce, and the analysis was therefore only performed in duplicate. Increasing the number of parallels for analysis could have given more stable results.

The difference between the RRM of this study and the literature is more prominent than the difference between batch 1 and 2. Studies described in **Table 1.1** shows that the determined astaxanthin content in snow crab rest raw material varies from 71,7-99,07 ug/g (w/dw) (Shahidi and Synowiecki 1991; Vilasoa-Martínez et al. 2008; Beaulieu et al. 2009; Lage-Yutsy et al. 2011). Vilasoa-Martinéz et al. (2008) and Lage-Yusty et al. (2011) extracted the carotenoids of the RRM with acetone, evaporated the solvent and dissolved the carotenoids in methanol-hexane-dichloromethane before detection of the specific carotenoids with UV-Vis and fluorescence detectors (HPLC-UV-FL) (Vilasoa-Martínez et al. 2008; Lage-Yutsy et al. 2011). Shahidi and Synowiecki determined the total carotenoid content of the snow crab RRM by extracting carotenoids with cod liver oil at 60°C (30 min) and vacuum drying, before the carotenoid content in the RRM was determined with spectrophotometric methods. Subsequently the carotenoid fractions were separated, extracted in chloroform three times and absorbance was read and compared to standards (Shahidi and Synowiecki 1991).

Considering the variation in method of determination of astaxanthin, the method used in this study can be assumed to be less sensitive. Also, the studies presented determined the astaxanthin and its derivatives, monoester and diester. This is included in the values of **Table 1.1**. If the spectrophotometric method of this study detects the derivatives as well, this would also affect the total amount of astaxanthin determined. According to Tichy et al. (2011), the disadvantage of spectrophotometric determination of pigments is overlapping spectrum of detection (Tichy et al. 2011). Thus, derivatives of astaxanthin can be assumed as completely or partly included in the determined astaxanthin content.

4.3. ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis was performed on batch 1 using Alcalase® 2.4 L (AL), and on batch 2 using Alcalase® 2.4 L, Protamex® (PR), Papain GSM80 (PA) and Corolase® 2TS (CO). All hydrolysis experiments were run in constant manner as described in chapter 3.4, **Table 3.1**. The conditions and method were held as constant as possible in order to obtain comparable results – heating in water bath for 20 minutes before adding enzyme, 50 mL reaction tubes and ending the hydrolysis by exposure to 90°C for 10 minutes. Hydrolysis temperature was customised for each enzyme, as proteases are temperature sensitive. Also, some changes were made with temperature for protease CO to embark on some optimisation, as this protease is not widely described for use on snow crab rest raw material. The hydrolysis with AL on batch 1 was run for 90 minutes, but the remaining enzymatic hydrolysis experiments were performed with 1 h hydrolysis time. Treatment was performed on 3-30 parallels and 1-6 replicas.

4.3.1. *Weight of fractions*

Enzymatic hydrolysis treatment generated sludge fractions and water fractions after centrifugation and separation by decantation. The raw data can be viewed in appendix E. The weight of the fractions is compared for all proteases in order to evaluate the efficiency of centrifugation and decantation as method of separation. **Figure 4.2** presents the weight of the fractions based on the weight of the total hydrolysis mix. Control samples are not shown as the figure is to illustrate the difference between the proteases. Within each replica, all water fraction parallels were merged and all sludge fraction parallels were merged. This was not done for Alcalase® 2.4 L (B1+2). The distribution of dry weight in the fractions is discussed in the next chapter.

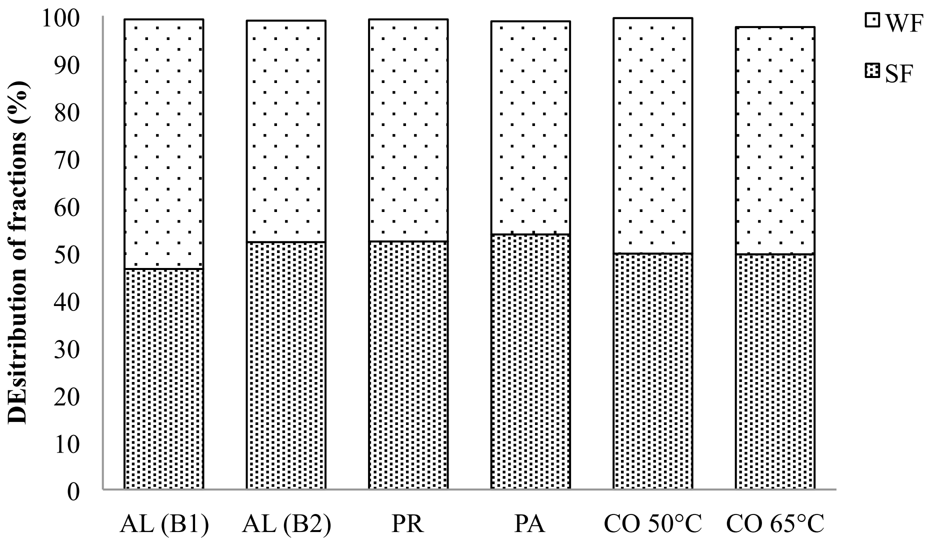

Figure 4.2: Distribution of sludge fraction (SF, shaded bars) and water fraction (WF) constituents of hydrolysis mix (%) after centrifugation and separation by decantation. Corolase are treated at two different temperatures (50° and 65°C), indicated in the horizontal axis description. Abbreviations are AL = Alcalase® 2.4 L, B1 = batch 1, B2 = batch 2, PR = Protamex®, PA = Papain GSM80 and CO = Corolase® 2TS.

4.3.2. *Dry weight of hydrolytic fractions*

The distribution of dry weight in the hydrolytic fractions was used to evaluate whether the separation by centrifugation and decantation was efficient (appendix F). Values of dry weight and ash show little difference between enzymatic treatments. The sludge fraction obtained by a second centrifugation of the hydrolysis mixture was finer and wetter than the main sludge. This is reflected in the low dry weight.

Table 4.2: Dry weight (%, w/ww) in fractions obtained in enzymatic hydrolysis using Alcalase® 2.4 L, Protamex®, Papain GSM80 and Corolase® 2TS. Alcalase is used on both batch 1 and, as marked. Treatments with different temperatures are marked with given temperature in superscript. Results are divided into sludge fractions and water fractions, and based on wet weight of the sample. Abbreviations are AL = Alcalase® 2.4 L, B1 = batch 1, SF2 = sludge fraction 2, B2 = batch 2, PR = Protamex®, PA = Papain GSM80, CO = Corolase® 2TS, NA = not analysed.

Enzyme	**SF**		**WF**	
(batch)	**Average dw ± SD dw/ww (%)**	**Control ± SD**	**Average dw ± SD w/ww (%)**	**Control ±SD**
AL (B1)	31,14±4,31	32,09±1,43	NA	NA
(SF2)	23,80±3,67	31,76±2,07	NA	NA
AL (B2)	29,95±2,24	NA	6,71±0,67	5,71±0,01
PR	30,30±1,81	32,22±0,91	6,93±0,08	6,55±0,55
PA	26,77±2,35	29,02±3,62	6,94±0,14	6,52±0,15
CO 50°C	33,26±6,15	33,60±1,76	7,67±0,07	5,88±0,09
CO 65°C	32,55±3,02	29,84±1,02	7,88±0,17	6,73±0,22

As most samples were 30 gram RRM mixed with water with water to sample ratio 1:2, the distribution of resulting hydrolytic fractions from the different protease treatments was expected to be equal. The method of isolation of fractions in the hydrolysis mix was centrifugation and separation by decantation. **Figure 4.2** represent the distribution of fractions, and shows that separation of fractions varies between the treatments. The largest difference is for AL (B2) and CO65. For fractions obtained by Alcalase on batch 2, the sludge fraction of enzyme treated samples is larger than the control sample sludge. The fractions treated with enzyme were generally more difficult to separate as the material contained particles more varying in size. The larger the sludge fractions are, the more water they contain. The smaller sludge fractions, and the control fractions, have less water. The treatments were run in 50-mL tubes with a diameter of about 2 cm. The particles of the RRM in batch 1 and 2 had a size of 1,2±0,7 and 0,5±0,6 cm^2, respectively. The centrifugation did not always compact the sludge completely, leaving pockets of liquid within the sludge. This led water getting trapped within the sludge. The decantation could therefore be claimed not to be an advantageous method of separation.

The fractions could probably have been separated by harder centrifugation to avoid the pockets of liquid in the sludge and/or filtration using suction. This would have given more efficient separation. Filtration could, however, risk some loss of material in the equipment, as observed by Beaulieu et al. (2009). That would not have been desired if up-scaling the procedure. In addition, filtration causes additional work with cleaning the filters to remove deposits. The vast number of samples to be processed was considered when choosing the more time-efficient method of decantation over filtration.

Beaulieu et al. (2009) centrifuged the hydrolysis mix twice after treatment (Beaulieu et al. 2009). The first centrifugation with the total hydrolysis mix, and the second with the water fraction decanted from the sludge. This was done in order to clarify the water fraction. Most of the protease treated samples in this study were centrifuged twice. The second sludge was only weighed individually for AL (B1) and there it had a low fraction of dry weight (23,8%), and the weight was only 4% of that of the first sludge fraction. Merging of the first and second sludge fraction was difficult. Considering the low fraction of dry weight and mass of the second sludge, the second centrifugation did not appear to be necessary in means of yield. It was also time-consuming. The second centrifugation was therefore removed from the protocol for the Corolase hydrolysis experiments. In addition, the amount of soluble protein in the second sludge fraction (chapter 4.3.4) was low, and so the presence of such a fraction in the water fraction would not present any advantage.

However, later in this study the second centrifugation showed to affect the recovery of astaxanthin. The sludge from Corolase treatment of RRM was further used in a combination experiment with heat treatment for extraction of astaxanthin. Results discussed later showed that some lipid was lost in the water fraction at hydrolysis with Corolase. Since the second sludge did not contain much soluble protein, it could be assumed to contain some lipid. Astaxanthin is soluble in lipid, and the loss of lipid also affected the yield of astaxanthin in the combination experiments. Clearing the water fraction with a second centrifugation would have given purer protein extracts, and increase the yield of astaxanthin in the sludge fraction.

4.3.3. *Recovery of ash in hydrolytic fractions*

Ash content was analysed in order to evaluate the demineralisation and recovery of ash in the hydrolysates. The results are presented in appendix F, and a summary is presented in **Table 4.3**. The recovery of ash is based on the relation between gram ash in the fractions and gram ash in the RRM used.

Table 4.3: Recovery of ash in hydrolytic sludge and water fractions. The values (%) are based on the total amount of ash in original RRM sample used for enzymatic hydrolysis and SD is the respective standard deviation. Abbreviations are AL = Alcalase® 2.4 L, B1 = batch 1, B2 = batch 2, Av. = average, SF = sludge fraction, WF = water fraction, PR = Protamex®, PA = Papain GSM80, CO = Corolase® 2TS.

Enzyme	Av. Recovery SF ± SD (%)	Control ± SD (%)	Av. Recovery WF ± SD (%)	Control ± SD (%)
AL (B1)	44,6 ± 10,7	44,9 ± 6,4	-	-
AL (B2)	67,8 ± 7,1	64,5 ± 12,0	9,7 ± 1,1	10,9 ± 0,1
PR	76,7 ± 8,3	78,1 ± 4,3	10,4 ± 0,3	10,4 ± 0,8
PA	62,2 ± 8,2	68,8 ± 15,3	9,3 ± 0,3	9,5 ± 0,2
CO 50°C	78,9 ± 4,3	73,6 ± 7,4	10,5 ± 0,1	10,2 ± 0,2
CO 65°C	96,3 ± 24,4	79,1 ± 10,3	10,1 ± 0,3	9,2 ± 0,7

Increased amount of ash in fractions would affect the amount of protein in the fraction. Increased demineralisation into water fractions could however also indicate increased degradation of the RRM and a more efficient protease. Studying the table, the loss of material is evident as none of the values for recovery of ash is 100% when adding recovery in sludge fraction to water fraction. The loss of material during treatment is described in appendix E. Corolase at 50 and 65°C has lower loss of material than the other proteases. This is in agreement with the table showing higher recovery values for CO 50°C and CO 65°C (when looking at the combined recovery in sludge and water fraction). **Figure 4.2** (page 54) shows that the loss of material during treatment is minor compared to the total hydrolysis treatment, as recovery of fractions is close to 100% in total.

Most of the further results for the composition of fractions obtained by enzymatic hydrolysis are based on wet weight. This is both done for convenience, but also in order to the reader to get a clearer idea of the content of the fractions and the change from RRM to fraction. All data given in w/ww for the chemical content of the RRM batch 1 and 2 can be viewed in appendix D. These values are also repeated when required in the appendices describing chemical content in hydrolytic fractions.

4.3.4. Recovery of proteins in hydrolytic fractions

Amount of soluble protein in fractions obtained by enzymatic hydrolysis was estimated with the Lowry method in order to evaluate the effect of the proteases (appendix G). The most relevant way of comparing the activity of the proteases in this study would be to look at the degree of deproteinisation, thus the recovery of protein from the RRM. This requires knowledge about the total amount of protein. Since total amount of protein was only estimated for the RRM of batch 2, the comparison of the activity of the proteases is in stead discussed by looking at the increased amount of solubilised protein. It is assumed that most of the protein and peptides in the water fraction is soluble (Arabia et al. 2013). The protein made soluble in the water fraction during enzymatic hydrolysis is compared to the starting amount of soluble protein in the RRM, and the increase is described as increase per amount of soluble protein in the original RRM.

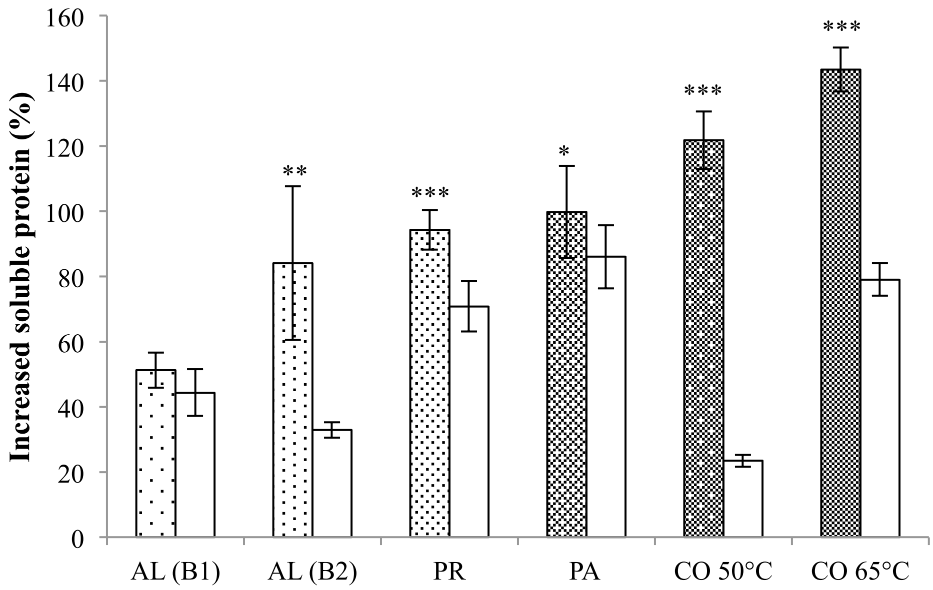

Figure 4.3: Increase in soluble protein (%) from RRM in water fractions obtained by enzymatic hydrolysis using Alcalase® 2.4 L (AL), Protamex® (PR), Papain GSM80 (PA) and Corolase® 2TS (CO) on snow crab rest raw material, batch 1 and 2 (B1 and B2). AL was used on both batch 1 and 2. CO is shown for fractions obtained at temperature 50° and 65°C. White bars are control samples, and the error bars shows standard deviation. Star indicates significant difference between protease treated and control fractions, where p≤0,05*, p≤0,01** and p≤0,001***.

Both sludge and water fraction were analysed in order to evaluate the separation step in the experiments. The results presented in **Figure 4.3** describe the increase in soluble protein by enzymatic hydrolysis with proteases Alcalase (AL), Protamex (PR), Papain (PA) and Corolase (CO). The activity of the proteases solubilises the proteins by degrading them into smaller peptides and amino acids, increasing the amount of soluble protein (Šližytė et al. 2014). Results are based on average values from 1-4 replicas. Hydrolysis with AL on batch 2 was repeated in 4 replicas, only results for replica 4 are shown, as the first 3 water fraction replicas had concentration of soluble protein half that of replica 4. This can be viewed in appendix G. The absorbance determined in the Lowry analysis shows overall lower absorbance for water fractions of replica 1-3 compared to 4. This could be because replica 1-3 and 4 are analysed with two different standard curves. In addition, some of the samples from replica 1-3 had lower absorbance values, which can be caused if reagents are not mixed quickly enough into the sample.

The results in **Figure 4.3** are presented by order of performance. It is therefore reasonable to ask whether the performance of enzymatic hydrolysis improved over time. This is however disproved as the fourth replica of PA treatment was performed months after the three first replicas. The increase of soluble protein of water fractions of replica 1-3 and replica 4, 87,5-140,7 % versus 96,5-97,9 %, shows that values from the same treatment is stable over time. This indicates that the order of performance by time did not affect the results.

Table 4.4: Results from Students T-test performed on average values of soluble protein (mg/g RRM) in water fractions generated by treatment of snow crab RRM with given enzyme.

Group 1	**Group 2**	**p-value**
AL (B1)	AL (B2)	0,034
AL (B2)	PR	0,389
PR	PA	0,267
CO 50°C	PA	0,001
CO 50°C	CO 65°C	0,003

Table 4.4 shows p-values from Students T-test for comparison of the protease treatments. The treatments that are closest to each other in increase of soluble protein are compared. Results show significant difference when comparing AL (B1) to AL (B2), CO 50°C compared to PA, and CO 50°C compared to CO 65°C. Values presented in **Figure 4.3** and **Table 4.4** show that Corolase is the most efficient protease on snow crab rest raw material, generating

significantly higher increase in amount of soluble protein than all other protease treatments. The increase in soluble protein with protease Corolase at 50° and 65°C, was 121,8±0,8 and 143,5±6,7 %, respectively. Increasing the temperature to 65°C has a significant effect on the yield of soluble protein in the water fraction. The treatment with CO at 65°C is thereby used in further comparison of enzymatic hydrolysis and heat treatment. The different treatment temperatures cause the difference in increase of soluble protein between the control samples. This is further discussed in chapter 4.4.1.

The recovery of soluble protein based on total protein content using Corolase was evaluated and compared to other studies. The deproteinisation represents how much of the total protein content that has been made soluble and extracted into the water fraction (recovery). The results show that 27,8±0,8% deproteinisation was achieved using Corolase at 65°C on the snow crab rest raw material of batch 2. Considering the hard texture of the raw material, it is expected that crustacean material is resistant to proteolysis, and that some protein will remain entrapped in the chitin-protein structure in the sludge (Dumay et al. 2004; Cremades et al. 2001).

Kaupang and Whitaker (2016) performed enzymatic hydrolysis of snow crab RRM and achieved 43 and 47% deproteinisation using Alcalase® 2.4 L and Protamex®, respectively (E/S 0,1%, 50°C, 1 hour, water:RRM 1:1) (Kaupang and Whitaker 2016 (unpublished)). Beaulieu et al. (2009) achieved 60,64% recovery of protein in snow crab hydrolysate using Protamex® (E/S 0,001%, 40°C, pH 8, 1 hour, water:RRM 1:1) (Beaulieu et al. 2009). Chakrabarti (2002) has studied the recovery of pigments and protein from brown shrimp (*Metapenaeus monoceros*) using the commercial enzymes Papain, Pepsin and Trypsin (pH 6,2, 4,6 and 7,6 respectively, 28°C, >3 h, E/S 0,0003% based on protein content, RRM:buffer 3:7). Trypsin had the highest recovery of protein with total 92,1%, while papain and pepsin gave 88,4 and 91,2%, respectively (Chakrabarti 2002). Valdez-Peña et al. (2010) also achieved deproteinisation with commercial proteases Alcalase® 2.4 L FG, Flavorzyme® 500 MG, Lysozyme (Inovapure 300), Papain and Trypsin VI on shrimp (*Litopenaues vannamei)* head discard (E/S 0,5 %, 37°C, pH 8, vacuum, 6 hours, powder sample:buffer 1:20), and Alcalase was the most efficient protease for deproteinisation (Valdez-Peña et al. 2010). The difference between the deproteinisation achieved in this study compared to the deproteinisation reported in the literature could be a result of the amount of water added. In this study the amount of water was restricted to water to sample ratio 1:2 in order to avoid emulsions in the water fraction. However, the more water added to the hydrolysis mixture before treatment, the higher the recovery of protein in the hydrolysate (Šližytė et al. 2005). The studies described above all had water or buffer to sample ratio above 1:1. Increasing the amount of water added to the sample in this study could increase the recovery of soluble protein in the hydrolysate. Also, most of the studies mentioned adjusted the pH of the hydrolysis mix. Optimisation of conditions for the protease will also aid in the recovery by enhancing the protease activity.

The use of proteases for deproteinisation of crustacean rest raw material is not as efficient as the use of harsh acid and alkaline treatments (Cremades et al. 2001). The difference between the use of acid and alkaline solutions compared to the proteases is the demineralisation step before deproteinisation, by removal of calciumcarbonate ($CaCO_3^-$) and calsiumphosphate ($CaPO_3^-$) (Hamed et al. 2016). Figure 4.1 and the findings of the literature search show how demineralisation by acid treatment is an important step before attempting the degradation of proteins in crustacean shells. By adding acids (eg. HCl, HNO_3, H_2SO_4), the crustacean material can be almost completely demineralised (Kaur and Dhillon 2015). The removal of minerals gives the proteases easier access to the proteins for degradation (Hamed et al. 2016; Kaur and Dhillon 2015; Jo et al. 2008; Synowiecki and Al-Khateeb 2000).

This demineralisation can be performed by pre-treatment with acids. But such a pre-treatment can however affect the structure and quality of the protein products (Fernandes 2016). Another alternative is fermentation of the material. By adding acid producing bacteria, the fermentation of the bacteria will react with the minerals and release them from the rigid material (Rao et al. 1998). A fermentative demineralisation is less harsh to the material, and unlike the use of strong acids, fermentation will not affect the target compounds (Hamed et al. 2016). Optimising the use of proteases would be beneficial as the use of enzymes is more environmentally friendly than the use of acid and alkaline treatments. These methods generate effluent waste-water containing chemicals, and it is energy intensive. The disadvantage of fermentation is that it is more time-consuming than the chemical treatments (Kaur and Dhillon 2015). If the obstacle of demineralisation before deproteinisation with proteases is solved, commercial proteases for recovery of proteins from crustacean RRM could compete with the efficient acid and alkaline treatments.

The material used by Beaulieau et al. (2009) had been stored at 0-4°C before processing (duration not specified) (Beaulieu et al. 2009). The material used by Kaupang and Whitaker (2016) had been thawed at 4°C overnight (Kaupang and Whitaker, 2016 (unpublished)). Storing before treatment can aid the degradation process as endogenous enzymes and bacterial enzymes in the RRM starts to degrade the material (Lorentzen et al. 2018). Søvik and Rustad (2004) studied the activity of the endogenous enzymes chymotrypsin and trypsin from intestines of different cod species at increasing temperature. This study found that these endogenous enzymes at pH 7 were active at 40°C, with declining activity at 50°C. At 70°C the activity had declined to about 10%. At 5°C the activity was close or equal to none (Søvik and Rustad 2004). Further published work shows that in cod rest raw material without intestines, proteolytic enzymes are not as efficient as in intestines, but that at pH 5 and 7 the activity still increases with temperature up to 50°C (Søvik and Rustad 2005). Heat stability for endogenous enzymes over 25°C has also been found for Atlantic salmon (*Salmo salar*) (Hultmann and Rustad 2004). Little research is done on the endogenous enzymes in crabs. Since both the cod and the salmon is adapted to colder water, the findings of the studies above can be applied to the snow crab as well. It is therefore not likely that much endogenous enzymes are active at 4°C storing of snow crab RRM.

In this study, the RRM was always thawed from -40°C, at about 40°C for 20 minutes. If this was considered as a heat treatment, there is a risk that the heat might have denatured the structural proteins in the material. This is caused by hydrophobic interactions between the proteins and lipids, or by aggregation of larger proteins/peptides. The access of the enzyme to the proteins will be reduced, and the yield of protein in the hydrolysate will decrease. The amount of insoluble sludge with phospholipids and lipids will increase (Šližytė et al. 2005). The endogenous enzymes will however not be affected by this thawing. Some degradation could be expected, but considering the short duration of thawing this is not very likely. The second batch of snow crab RRM did not include intestines, and is thus not expected to contain much endogenous enzymes, which are usually located in the midgut region (Saborowski 2015). The effect of endogenous enzymes on batch 2 is therefore expected to be minor. During thawing and storing the snow crab RRM should anyway be vacuum packed, in order to preserve the structure of the astaxanthin.

The total protein content of the Beaulieu et al. (2009) RRM (42,87%, w/dw) was higher than the protein of batch 2 (36,6 %, w/dw). Even though the original protein content is normalised when calculating the deproteinisation, a higher starting amount of protein could indicate that more protein is available in the RRM compared to lower amount of protein. The RRM used in the study of Beaulieu et al. (2009) contained cephalothorax shells, digestive systems including hepatopancreas and hemolymph (Beaulieu et al. 2009). This is different from the RRM of batch 2 in this study, which only consisted of cephalothorax shells. The protein present in the RRM of Beaulieu et al. (2009) will be in the shell, intertwined in chitin and minerals and pigment, but some will also be in the intestines. Protein in looser tissue will be more available than the protein of crab carapace. The difference in protein content between different studies also supports that the presence of intestines has a positive effect on the nutritional value of the RRM (chapter 4.2.4).

The recovery of protein when using Alcalase is significantly higher for batch 2 than batch 1. This is assumed to be an indication of that the increase in homogenisation for batch 2, and smaller particles affect the result. With smaller particles, the protease has easier access to the proteins because of increased surface area of the material. Alcalase for batch 1 is however only shown for one replica, and cannot be taken as more than an indication.

The change in soluble protein content from RRM samples to resulting sludge fractions was negative (appendix G). This was expected, as most of the available soluble protein should be recovered in the water fraction. The sludge did however contain some soluble protein, because remaining water in the sludge fractions will contain some remaining soluble protein. The finer sludge fraction obtained by a second centrifugation of water fraction of AL (B1) was also analysed and had about 96% decrease (negative change) in soluble protein. It contained 0,61 mg soluble protein per g RRM used. When comparing to the amount of soluble protein in the water fraction of 24,4 mg/g RRM used, it is evident that the second sludge will not contribute to the amount of soluble protein in the water fraction. This supports that the finer sludge should be removed from the water fraction. The finer sludge also represents a loss of dry weight from the main sludge fraction (minor but existing), possibly affecting any further utilisation of the sludge.

The use of commercial enzymes is not as efficient as the use of acids and bases in the removal of proteins. Demineralisation, deproteinisation and decoloration are necessary to fully remove all components from the chitin in the crustacean rest raw material. The use of commercial enzymes is limited by remaining ash in the substrate, as it limits the access of the protease. Some residual protein will remain in the chitin structure as a cause of this. That has also been demonstrated in this study.

The advantage of proteases over chemical recovery of proteins is that commercial enzymes avoid the high temperatures and harsh chemicals. According to Beaulieu et al (2009), both extraction of proteins and lipids from crustacean rest raw material benefit from use of commercial enzymes. Proteins recovered this way are found to have improved solubility, heat stability and water binding ability, in addition to increased nutritional quality compared to acid and alkaline treatments (Beaulieu et al. 2009).

4.3.5. Free amino groups in hydrolytic fractions

Free amino groups (mg/mL) in water fractions obtained by enzymatic hydrolysis were determined by indirect titration in formol titration procedure (chapter 3.3.6). Raw data is presented in appendix H and calculations in appendix D. Final results (mg/mL) are presented in **Table 4.5**. Star behind concentration indicate significant difference between enzyme treated samples and respective control samples. Average values are based on results from 2-10 parallels (2 samples analysed per merged replica).

Table 4.5: Free amino groups in water fractions obtained by enzymatic hydrolysis. Average value is based on 2-10 parallels. Star indicates statistical significant difference between enzyme treated samples and respective control samples.

Enzyme	Average mg/mL ± SD	Control ± SD
AL	4,53 ± 0,39	3,32±0,18
PR	3,23 ± 0,47	2,82±0,12
PA	4,20±0,98 *	2,98±0,38**
CO 50°C	3,43±0,43	3,04±0,23
CO 65°C	4,05±0,56 *	2,80±0,17*

The amount of free amino groups in the water fractions shows that the method of determination has not worked optimally. The standard deviations are high compared to the averages. The low amount of soluble protein in the hydrolysates is suspected to affect the result, making titration more challenging, as less free amino groups are available. But the results indicate the content of soluble protein in the hydrolysates. The use of proteases has increased the concentration of free amino groups for all treatments compared to the control. This is in agreement with the difference in soluble protein between treated samples and control samples. Only Papain and Corolase (65°C) showed significant difference between treatment and control. Increased amount of free amino groups by the use of proteases is also found by (Šližytė et al. 2005).

4.3.6. Molecular weight distributions in Corolase® 2TS hydrolysates

The molecular weight distribution in water fractions from enzymatic hydrolysis with Corolase® 2TS (CO) was determined. The hydrolysates from treatment with CO were studied as this hydrolysate showed the highest increase of soluble protein from the original RRM. Results from heat treatment at 50° and 65°C showed that the 15°C increase in temperature increased the yield of soluble protein in the water fraction. The hydrolysates from treatments with Corolase at 50° and 65°C were therefore studied to determine whether increase in temperature would change the molecular weight distribution as well.

Molecular weight distribution was determined by FPLC gel filtration. The column separates molecular weights between 100-7000 Da. Figure 4.4 and Figure 4.5 presents the chromatograms for water fraction from treatment with Corolase at 50 and 65°C, respectively. Raw data is presented in appendix J.

Most of the protein in the samples is contained in the two peaks. The last peak (second highest) was excluded as it indicates sizes smaller than 100 Da, which is outside the separation range of the column. The results show that the molecular weight distribution of hydrolysates from CO50°C was 2760 and 990 Da, and the molecular weight distribution of hydrolysates from CO65°C was 2760 and 950 Da. This indicates that a 15°C increase in temperature has not affected the sizes of the peptides made soluble in the hydrolysates.

When comparing the Corolase treated hydrolysates and the control samples, the activity of the protease does not seem to have affected the sizes of the peptides recovered from the RRM. The control samples for both hydrolysates are equal to the enzyme treated samples. This is in agreement with the analysis of free amino groups, which showed little difference between treated samples and control samples in amount of free amino groups. The absorbance detected is lower for control samples. This is in agreement with the significantly higher amount of soluble protein in protease treated hydrolysates than in controls. It can therefore be stated that the treatment with heat generates soluble peptides of a certain size, and the protease speeds this reaction. This is supported by (Šližytė et al. 2005). It is outside the scope of this study to discuss the further application of the hydrolysate based on the molecular weight distribution.

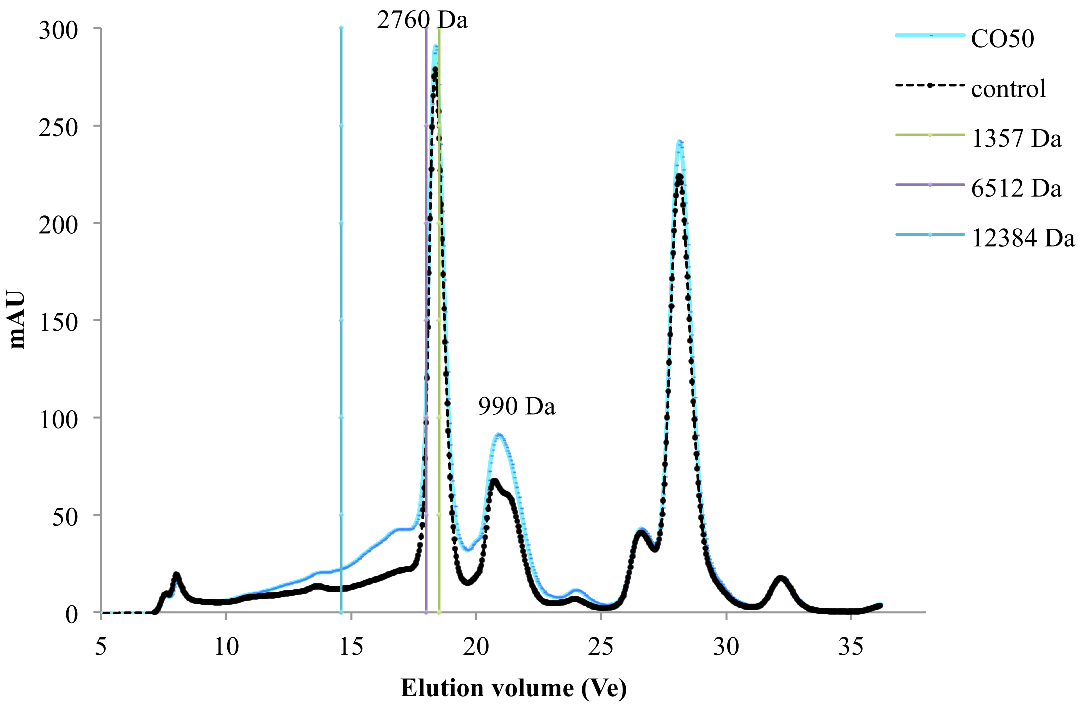

Figure 4.4: Chromatogram describing absorbance of samples and respective elution volumes based on retention time (min) and flow 0,5 mL/min, for water fraction from enzymatic hydrolysis using Corolase® 2TS as hydrolytic agent, at 50°C (blue line). Dotted line is control sample water fraction. Main peptide size groups for enzyme treated samples are indicated with molecular size (Da). The three lines represents the elution volumes of standard samples Cytochrome C (12384 Da), Aprotinin (6512 Da) and Vitamin B12 (1357 Da).

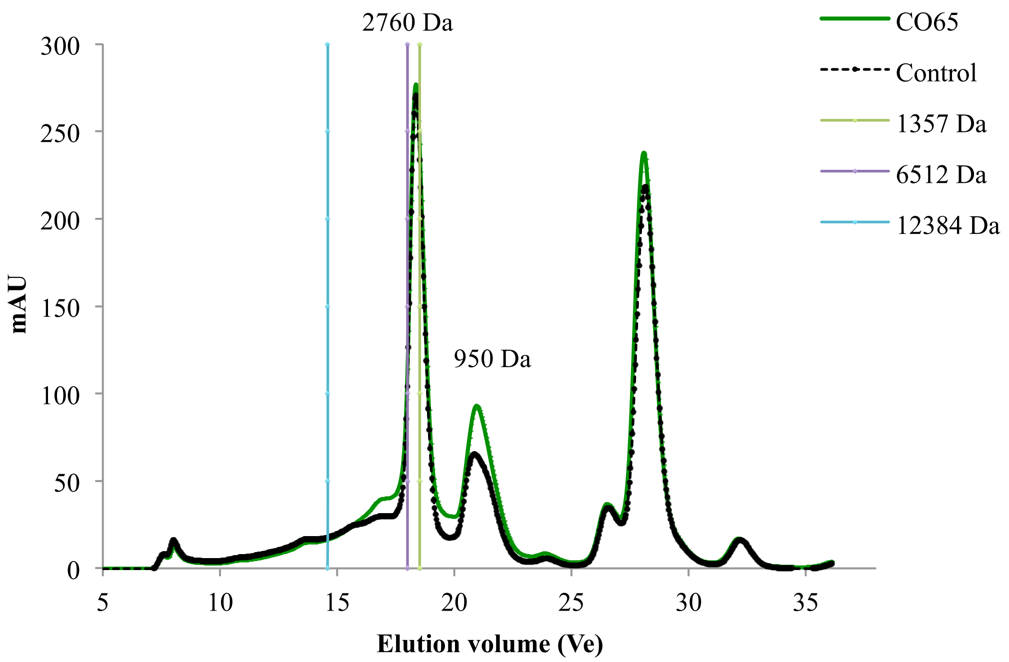

Figure 4.5: Chromatogram describing absorbance of samples and respective elution volumes based on retention time (min) and flow 0,5 mL/min, for water fraction from enzymatic hydrolysis using Corolase® 2TS as hydrolytic agent, at 65°C (green line). Dotted line is control sample water fraction. Main peptide size groups for enzyme treated samples are indicated with molecular size (Da). The three lines represents the elution volumes of standard samples Cytochrome C (12384 Da), Aprotinin (6512 Da) and Vitamin B12 (1357 Da).

4.3.7. Recovery of lipids in hydrolytic fractions

Lipid fractions were not possible to obtain and isolate in the centrifugation and separation of hydrolytic fractions. Since the experiments were all performed at a small scale (30 g RRM per sample), the available lipid that could theoretically be separated as an individual fraction was 1,26 and 0,55 g for batch 1 and 2, respectively (lipid content 4,21 and 1,83%, w/ww). The separation of such small fractions, with the chosen methods of separation is very difficult. The lipid recovered in the sludge and water fractions was therefore the further focus. When performing the fourth replica of Alcalase on batch 2, a thin, orange mass was scattered on the tube wall (Figure 4.6). Considering the pigmentation it is reasonable to assume that this was lipid. Being attached to the tube wall and of a very small mass, it was difficult to isolate. This was observed for several of the proteases.

Figure 4.6: Hydrolytic fractions obtained after treatment with ALcalase® 2.4 L on snow crab RRM of batch 2. The larger browm mass to the left is the sludge fraction, the liquid to the right is the water fraction and on the tube wall is a small orange oil fraction.

The total lipid content of fractions obtained by enzymatic hydrolysis was determined by Bligh and Dyer method (1959). Raw data and calculations can be viewed in appendix I and D, respectively. Results are presented in **Table 4.6** and Figure 4.7. Results for AL (B1) are considered excluded as they exceeded the total lipid content of RRM in batch 1 (4,21±0,5%). Other than this, the results are similar for all proteases. Fractions analysed for AL (B2) are from replica 1-3. As described in chapter 4.3.4, replica 1-3 had lower values of soluble protein in the water fractions than replica 4 and in WF from the other proteases. Regarding total lipid content, the WF of AL (B2) does not seem to differ much from the other proteases.

Table 4.6: Total lipid content (%) in fractions obtained by enzymatic hydrolysis using Alcalase® 2.4 L (AL), Protamex® (PR), Papain GSM80 (PA) and Corolase® 2TS (CO) on snow crab rest raw material, batch 1 and 2 (B1 and B2). AL is shown for both batch 1 and 2. CO is shown for fractions obtained at temperature 50° and 65°C (CO^{50} and CO^{65}). Abbreviations are SF = sludge fraction, Av. = average, WF = water fraction.

Enzyme	**SF**		**WF**	
	Av. total lipid (%) ± SD	**Control total lipid (%) ± SD**	**Av. total lipid (%) ± SD**	**Control total lipid (%) ± SD**
AL (B1)	11,22 ± 1,79	10,35 ± 0,85	1,79 ± 0,51	1,54 ± 0,29
AL (B2)	2,03 ± 0,34	2,35 ± 0,20	0,10 ± 0,05	0,09 ± 0,04
PR	2,03 ± 0,26	2,23 ± 0,23	0,12 ± 0,07	0,09 ± 0,06
PA	1,92 ± 0,17	2,09 ± 0,13	0,07 ± 0,04	0,05 ± 0,06
CO 50°C	2,23 ± 0,17	2,14 ± 0,29	NA	NA
CO 65°C	1,74 ± 0,22	1,70 ± 0,17	NA	NA

During the enzymatic treatment, the total lipid present in the RRM before hydrolysis has thus been recovered in the sludge and the water fraction. Figure 4.7 represent the distribution of total lipid between SF and WF, based on values of lipid composition from **Table 4.6** and fraction constituents from tables in appendix E. As the lipid content of Corolase WF was not measured, only lipid content of SF from this protease treatment is included. The figure shows that the recovery of lipid is not affected by the protease, as the recovery of lipid in the SF is not changing based on protease. There is however a greater loss of lipid or lipid in WF of Corolase at 65°C. Since the WF was not analysed, this is difficult to define. In the water fractions, small orange particles was observed floating on the surface. This could be accumulation of lipid, but the small size made it difficult to isolate and define this.

The recovery of lipid is not 100% when looking at the SF and WF combined. This either indicates some loss of material in the equipment or that the Bligh and Dyer method is not able to detect all lipids in a sample, as proposed by Iverson et al. (Iverson et al. 2001). Alternatively, the loss of lipid from the RRM to the fractions could be explained by the accumulation of the solid lipids observed in most of the protease treatments after centrifugation. The fraction was very small of size, and thus difficult to both isolate but also evenly mix into the sludge fraction. Therefore it is assumed that this fraction was not detected by the Bligh and Dyer analysis performed on the sludge fraction.

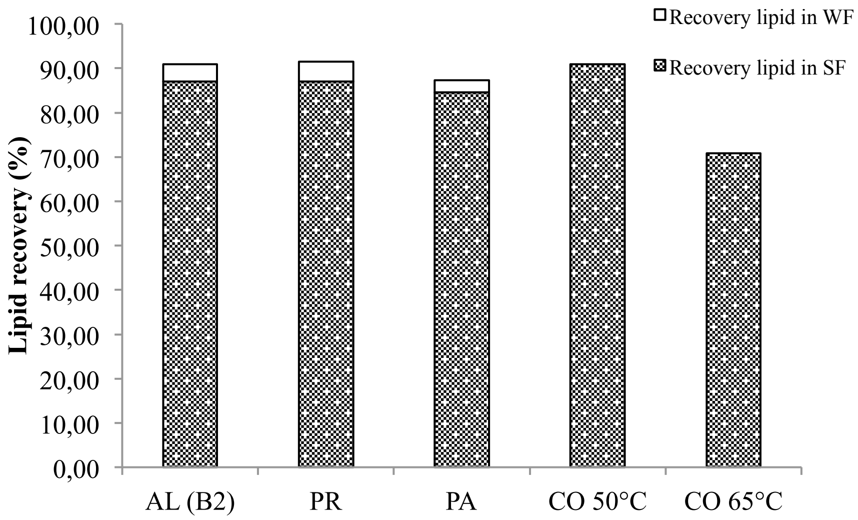

Figure 4.7: Recovery of lipid in sludge and water fractions after enzymatic treatment. The values (%) are based on the total amount of lipid in original RRM sample used for enzymatic hydrolysis. Top bars (blank) are recovery in water fractions, and bottom bars (shaded) are recovery in sludge fractions.

The lipid determined in the water fractions indicates some emulsion of the hydrolysate. The presence of lipid is not desired in the hydrolysate, which is supposed to mainly contain proteins. This can be affected by reducing the addition of water to the hydrolysis mix (Šližytė et al. 2014). When deciding on how much water to use in the enzyme hydrolysis, the high amount of water in the RRM was considered. Most studies use a water to sample ratio 1:1 and 2:1, and so the ratio 1:2 was considered as a restriction. A smaller amount of water added would probably decrease the amount of protein made soluble, as it would affect the performance of the enzyme, reducing liquid volume.

If the enzyme treatment had been performed at a larger scale, accumulation of a distinct oil fraction could have been possible. This was observed in the pilot enzyme treatments performed at Nofima AS in May 2018. Working at the small scale, studying oil fractions or lipid extraction was challenging. The difference in lipid available in the RRM of batch 1 and

2 is also evident in the results. More lipid seem to have been transferred to the WF of AL (B1) (**Table 4.6**). This was also visible after centrifugation and decantation, as a WF/OF fraction. Optimisation of the recovery of lipid could be performed if upscaling the experiments. Also, using RRM containing intestines could aid recovery of lipid. Commercial utilisation of snow crab lipids was proposed by Lage-Yusty et al. (2011), stating that the snow crab rest raw material could be a good source of marine lipids (Lage-Yutsy et al. 2011). This will require that it is the RRM containing intestines that is available. The choice of protease does not seem to affect the recovery, but it is difficult to draw any firm conclusions, considering the small scale of this study.

4.4. HEAT TREATMENT OF SNOW CRAB RRM BATCH 2

Heat treatment (HT, 70°C, 1 h) was performed on the RRM (B2) in order to compare this method of recovery of protein and lipids to the use of enzymatic hydrolysis. For the study of heat treatment, the control samples from enzymatic hydrolysis are used as additional replicas. The control samples (temperature 50, 60 and 65°C) are only mixed with water, exposed to heat for 1 hour and 20 minutes, heated at 90°C for 10 minutes, cooled, centrifuged and separated. The heat treatment experiment was run at 70°C, for 1 hour, was not exposed to 90°C, not cooled, but centrifuged and separated. Replica 1-3 was not added water before heat treatment, but the samples of replica 4 were diluted with a water to sample ratio 1:2. The differences in heat treatment between the HT samples and the enzymatic hydrolysis (50-65°C) control samples are therefore discussed. However the comparison was considered important because it is an efficient way of expanding the heat treatment experiment. In the appendices, the values for heat treatment in means of enzymatic hydrolysis control samples are presented together with the results form enzymatic hydrolysis (appendix E and G). The results from heat treatment at 70°C are presented in appendix K. This chapter describes the HT 70°C experiment, and all heat treatments will be compared.

The distribution of fractions can be viewed in Figure 4.8, as replica 1-3 and 4. Values are based on results presented in appendix K. Fractions obtained were sludge fraction and a water fraction. The increased amount of water in replica 4 is due to the addition of water (water to sample ratio 1:2) in this replica.

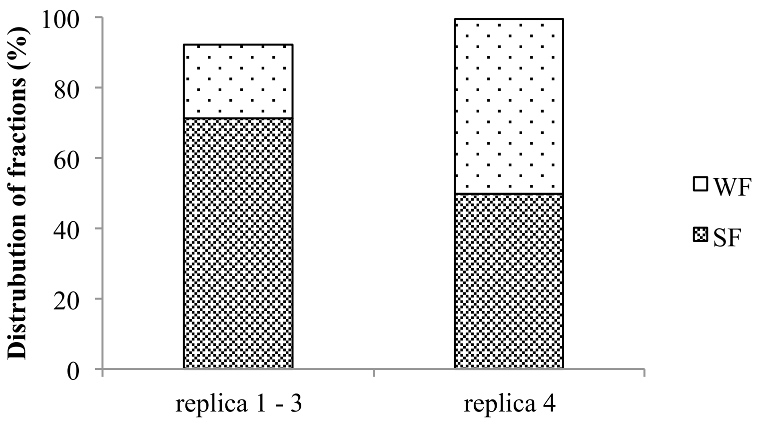

Figure 4.8: Distribution of sludge fraction (SF, shaded bars) and water fraction (WF) obtained by heat treatment (70°C, 1 h), after centrifugation and separation by decantation Replica 4 was added water (water:CM, 1:2) before treatment.

No oil fraction was visible after centrifugation of samples in HT 70°C. Some orange coloured matter was scattered on the tube surface after centrifugation of replica 4, assumed to be solid lipid (**Figure 4.9**), just as after enzymatic hydrolysis. Heat treatment gave more scattering of orange material than the enzymatic hydrolysis, but it was still too little to be isolated. Considering the small total amount of lipid in the rest raw material (1,83%, w/ww), isolation of an oil fraction at this small scale was determined not to be possible.

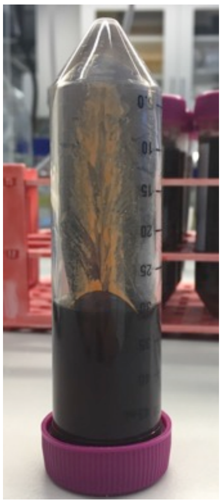

Figure 4.9: Heat-treated RRM after centrifugation. Orange material scattered on the tube surface is assumed to be a solid lipid fraction.

4.4.1. Increase of soluble protein by heat treatment

The concentration of soluble protein in water fractions from heat treatment experiments (temperature 50, 60, 65 and 70°C) was analysed by the Lowry method (appendix G and K). The % increase of soluble protein from RRM to water fractions by heat treatment and enzymatic hydrolysis was compared in order to evaluate if the heat treatment is comparable to the use of proteases. **Figure 4.10** presents the increase in soluble protein (%) from RRM to the water fractions obtained, including the amount of soluble protein recovered by Corolase treatment at 65°C. This gives an estimate on how much protein that it is possible to solubilise with each heat treatment. **Table 4.7** presents p-values for a comparison of different heat treatments and of the use of Corolase® 2TS. All WF from heat-treated RRM had significantly higher amount of soluble protein, than the RRM itself before treatment.

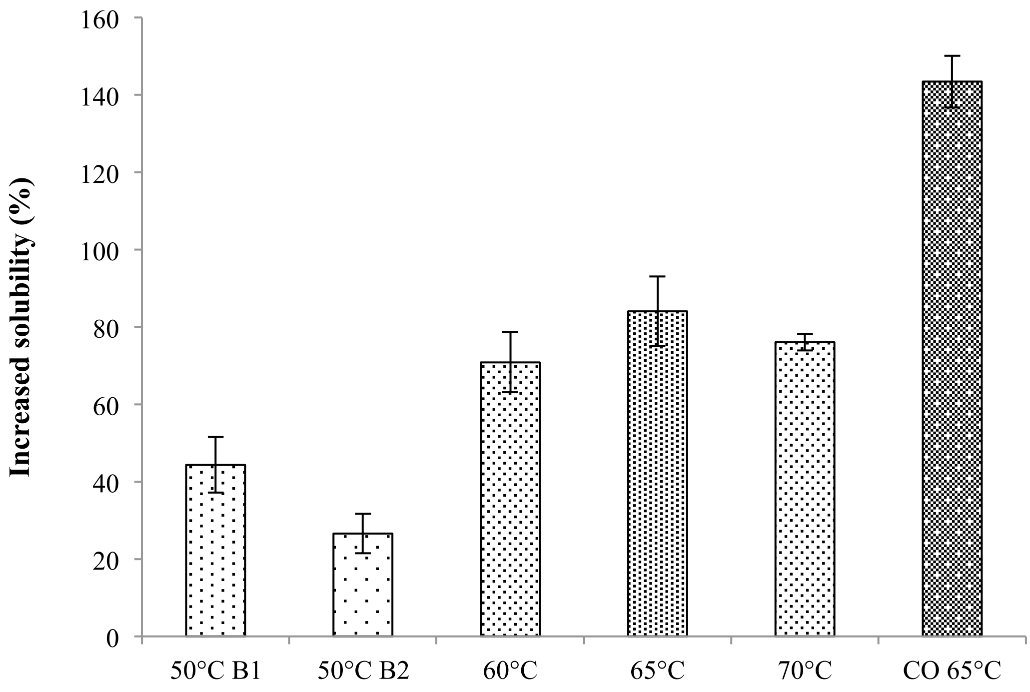

Figure 4.10: Increase in soluble protein (%) from RRM in water fractions obtained by heat treatment at given temperatures, and enzymatic hydrolysis using Corolase® 2TS (CO, 65°C) on snow crab RRM, batch 1 and 2 (B1 and B2). Treatment at 50°C is shown for both batch 1 and 2. The error bars shows standard deviation.

Table 4.7: Students T-test results for comparison of soluble protein content in water fractions from different heat treatments, and to the soluble protein content available in RRM before treatment. Amount of soluble protein in water fraction from enzymatic hydrolysis using Corolase® 2TS was also compared to the heat treatments. Only heat treatment at 50°C is performed on both batch 1 and 2, as indicated. Significant differences p≤0,001 are represented by three stars.

Group 1	Group 2	p-value
HT50°C (B1)	HT50°C (B2)	0,034
HT50°C (B2)	HT60°C	***
HT60°C	HT65°C	0,001
HT65°C	HT70°C	0,021
HT65°C	CO65°C	***

Figure 4.10 shows that increased temperature increases the amount of soluble protein in the water fraction, up to treatment at 70°C. The increase of soluble protein by heat treatment at 70°C is significantly lower than at heat treatment at 65°C. The heat treatment time was shorter, and did not include exposure to 90°C for 10 minutes, as was the case for all control samples. This could explain the lower increase in soluble protein at 70°C compared to 65°C. Increasing the treatment time to 1 hour and 20 minutes could increase the extraction of soluble protein in the heat treatment at 70°C.

However, as discussed earlier, the enzymes in the snow crab rest raw material are depending on temperature. Endogenous enzymes in cod species intestines at pH 7 have declining activity at 50°C, and at 70°C the activity is down to 10% (Søvik and Rustad 2004). Stoknes and Rustad (1995) reported the same for Atlantic salmon (*Salmo salar*). Proteolytic enzymes degrading the muscle of the salmon are most active at 65°C, and have declined to neglectable activity at 70°C (Stoknes and Rustad 1995). These studies shows that the enzymes present in the snow crab can be assumed to be unstable at the higher 50-70°C, and activity is declining at 70°C. The main degradation therefore happens at the heating up to hydrolysis temperature. This is supported by (Šližytė et al. 2014). In addition, at 70°C the protein of the rest raw material is expected to denature and aggregate, decreasing the detected soluble protein (Šližytė et al. 2005).

Even though batch 2 is not expected to contain much enzyme because of its lack of intestines, the heat dependence of endogenous enzymes is reflected in the results. If heat treatment is to be further investigated, the temperature should not be increased above 65°C. Since time also could affect the extraction, it could be interesting to perform the heat treatment at 1,5 hours or 2 hours, and compare to the use of protease.

Recovery of proteins by heat treatment is significantly lower than with protease, both at 65°C. Heat treatment is thereby not comparable to the efficiency of the use of commercial proteases. The protocol is similar to ensilation protocol with activation of endogenous enzymes by controlling temperature. However, the ensilation method controls the pH as well, and this is what makes simple heat treatment more sustainable than ensilation. The ensilation also usually demands neutralisation of the final protein liquid product. Values of deproteinisation of crab shells with chemical ensilation were difficult to retrieve. Several studies use the word ensilation when describing fermentation of RRM for demineralisation and deproteinisation. Even though lactic acid is less harsh than higher concentrations of HCl, the fermentation process has been determined as less harmful than ensilation. Further, since heat treatment in this study could not recovery as much protein as the use of Corolase, and Corolase could not recover as much protein as the use of fermentation reported in the literature, heat treatment is determined to be less efficient than ensilation and fermentation. Thus, even though heat treatment is more environmentally friendly (compared to chemical ensilation using HCl or H_2SO_4), it is not as efficient and thus not a sustainable choice of method.

The study of Søvik and Rustad (2005) reports that the activity of proteolytic enzymes in cod rest raw material is higher at pH 3, regardless of temperature, and highest activity is at 35°C. When pH is increased to 5 and 7, the activity increases if the temperature is also increased (Søvik and Rustad 2005). Since the type of enzymes in the snow crab rest raw material was not identified in this study, the activity of the proteases can only be assumed. Any activity of lipases is not discussed. However, the increasing activity of endogenous enzymes up to 65°C during heat treatment of the RRM in this study reflects the high pH (~pH 7) of the material. The low amount of solubilised protein also reflects the high pH, as the endogenous enzymes will not be as active at this pH. Finally, the results show that at 70°C the endogenous enzymes are deactivated. If the pH had been decreased, with fermentation or acid ensilation, the activity could have been increased as well. Then the temperatures could have been lowered. In this case, fermentation is advised over acid ensilation, as it is more environmental friendly.

As the results of increase in soluble protein are based on 2-14 parallels from 1-4 replicas, and averages of 2 values are not as valid as averages of 14 values. The heat treatment at 70°C was performed in 4 replicas, but water was only added before treatment in the last replica (4). This affected the increase of soluble protein extracted, and the replicas not added water were therefore not included in the results presented in the figure. The results should therefore be considered as indications of trends.

4.4.2. Free amino groups in heat treatment water fractions

Free amino groups (mg/mL) in water fractions obtained by heat treatment at various temperatures were determined by indirect titration in a formol titration procedure (chapter 3.3.6). Raw data is presented in appendix H and K (HT 70°C), and calculations in appendix D. Final results (mg/mL) are presented in Figure 4.11 together with the results from formol titration of water fractions obtained when using Corolase at 65°C. As CO 65°C gave highest amount of soluble protein compared to other proteases and heat treatments, this treatment was chosen for comparison. Average values are based on results from 2-10 parallels (2 samples analysed per replica/control).

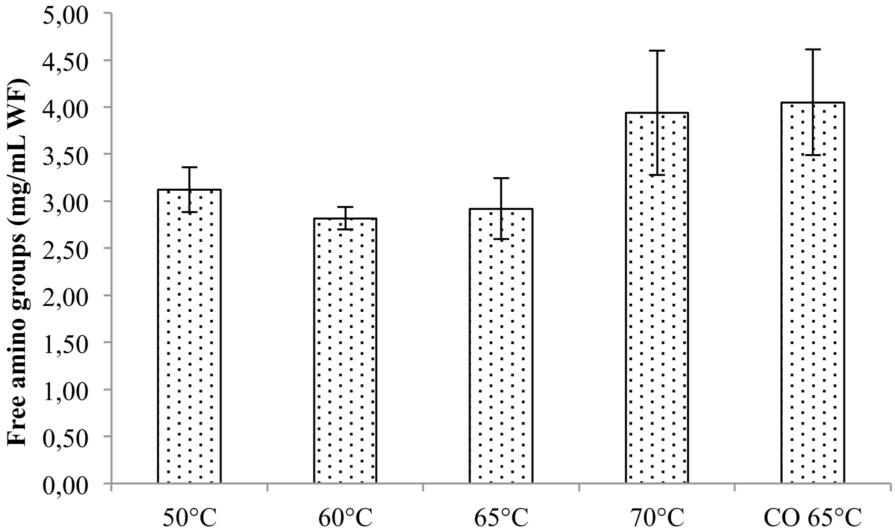

Figure 4.11: Free amino groups in water fractions obtained by heat treatment at various temperatures, and enzymatic hydrolysis using Corolase® 2TS (CO, 65°C). Values are averages based on 2-10 parallels from 1-4 replicas. Error bars represented standard deviation.

The results shows that increase in temperature increase the amount of free amino groups. This correlates with the increase of soluble protein with increasing temperature. There is however more free amino groups in the water fractions generated from heat treatment at 70°C than at lower temperatures. This could indicate that higher temperatures caused more degradation of soluble proteins, generating more free amino groups. As described for the analysis of free amino groups in hydrolytic hydrolysates, the small amount of soluble protein available in the water fractions could have made formol titration less accurate.

4.5. PIGMENT RECOVERY

The recovery of astaxanthin pigment from snow crab rest raw material was attempted with three methods; A combination of enzymatic hydrolysis at different temperatures (T) followed by heat treatment (65°C) with addition of rapeseed oil in different ratios ($\mathrm{CO}^{T^\circ C}$+ $\mathrm{HT}_{\mathrm{oil}}^{65^\circ C}$), heat treatment of RRM and addition of rapeseed oil ($\mathrm{HT}_{\mathrm{oil}\ 1:2}^{65^\circ C}$), and supercritical fluid extraction (SFE) of astaxanthin from crude material.

Since Corolase® 2TS was shown as most efficient in the degradation of the snow crab RRM (chapter 4.3.4), this protease was chosen to be used in the combination experiments for recovery of astaxanthin. One of the combination experiments included 12 hours storing at -20°C after treatment and centrifugation, before separation of fractions. The next two chapters describe results from astaxanthin recovery with $\mathrm{CO}^{T^\circ C}$+$\mathrm{HT}_{\mathrm{oil}}^{65^\circ C}$ and $\mathrm{HT}_{\mathrm{oil}\ 1:2}^{65^\circ C}$.

4.5.1. Yield of fractions

Sludge fractions from enzymatic hydrolysis mixed with rapeseed oil was heated at 65°C for 1 hour ($CO^{T°C}HT_{oil}^{65°C}$), described and illustrated in chapter 3.6. This was also performed with crude material ($HT_{oil\ 1:2}^{65°C}$). SF was separated from WF and OF by centrifugation and subsequent decantation of the liquid fractions. In the removed liquid with water fraction and oil fraction, heavy water-soluble droplets descended to the bottom of the tube and the lighter oil could be separated from WF by pipetting. The sludge fraction and oil fraction was measured; water fraction was discarded because of small volume. The yield of the fractions, the amount they made out of original sludge and oil, were determined, presented in **Table 4.8**, raw data in appendix L.

Table 4.8: Yield of fraction in heat treatment of crude material and enzyme sludge fractions added rapeseed oil. Sludge fraction and oil fraction constituents are based on original crude/sludge fraction and rapeseed oil, respectively. Heat treatment did not have any control samples, and OF from $CO^{65°C}HT_{oil\ 2:1}^{65°C}$ was not measured, thus no data.

Treatment	**SF**		**OF**	
	Average yield (%)	**Control**	**Average yield (%)**	**Control**
$\mathbf{HT_{oil\ 1:2}^{65°C}}$	74,84±13,36	-	81,68±1,89	-
$\mathbf{CO^{50°C}HT_{oil\ 1:2}^{65°C}}$	95,89±4,05	99,59±5,61	69,78±6,79	72,23±9,38
$\mathbf{CO^{50°C}HT_{oil\ 1:2}^{65°C}}$ (stored -20°C, 12 h)	105,72±7,90	113,40±0,46	78,24±7,70	69,20±2,54
$\mathbf{CO^{65°C}HT_{oil\ 1:2}^{65°C}}$	98,18±2,96	97,84±2,99	71,83±6,00	76,69±3,27
$\mathbf{CO^{65°C}HT_{oil\ 2:1}^{65°C}}$	103,39±9,03	100,91±17,36	ND	ND

The results demonstrate the efficiency of the method, but also the loss of oil in the sludge and/or water fraction. Cohesion of oil to water surface and the difference in density caused some difficulties with separating oil fraction from water fraction. Using a syringe was also attempted, but this also led to loss of oil fraction. For further experiments, using a separation funnel and filtration by muslin cloth, as in the literature, is advised. Assuming total separation is possible, calculations of astaxanthin content in the next chapter are made with the total amount of rapeseed oil added. This is assumed to be reasonable, as the remaining rapeseed oil associated to the solid sludge will have the same concentration of astaxanthin at separation.

4.5.2. Astaxanthin recovery with rapeseed oil

Astaxanthin content of rapeseed oil from heat treatment ($HT^{65°C}_{oil\ 1:2}$) and combination experiment ($CO^{T°C}$+$HT^{65°C}_{oil}$) was measured using spectrophotometry (chapter 3.3.7). The results of recovery are presented in appendix L. Results for recovery of astaxanthin from original RRM sample, and enzyme treated SF sample is presented in Figure 4.12. Assumed loss of astaxanthin with lipids or in water-soluble carotenoprotein complexes, in water fraction during enzymatic hydrolysis required a comparison of recovery from original sample and recovery from sludge sample. When studying the figure only bars with equal patterns can be compared, except when looking at the effect of protease treatment compared to control samples.

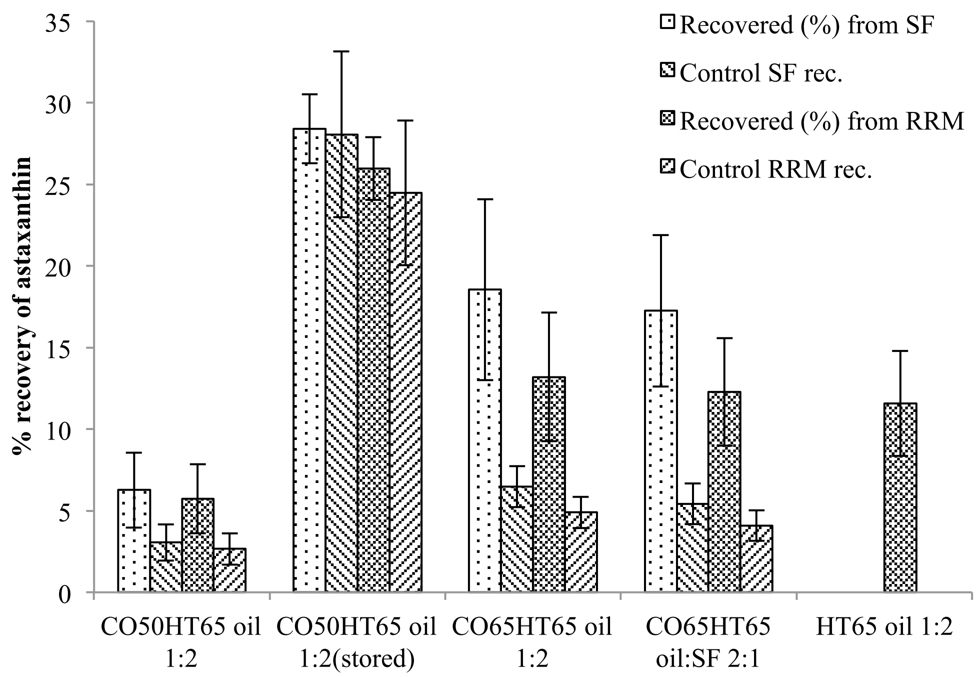

Figure 4.12: Astaxanthin recovered (%) in rapeseed oil by heat treatment (HT65 oil 1:2), and by combining enzymatic hydrolysis using Corolase® 2TS (CO) at 50°C and 65°C with different combinations of heat treatment temperature and oil to sludge ratio. Dotted bars are % astaxanthin recovered from sludge fractions (SF rec.), and shaded bars are % astaxanthin recovered from the original RRM sample (RRM rec.). Error bars represent standard deviation. Numbers in the horizontal axis descriptions presents the temperature at which the Corolase (CO) treatment was held, eg. CO50HT65 oil 1:2 present results from enzymatic hydrolysis at 50°C and subsequent heating of obtained sludge at 65°C with oil to sludge ratio 1:2. One Corolase treatment was stored overnight at -20°C after heating with oil, before separation of fractions.

For all combination experiments, **Figure 4.2** shows that there is a difference in the recovery of the astaxanthin from the original RRM and from the sludge fractions. The amount of astaxanthin in the snow crab lipid is assumed to be constant. By determining the total lipid content of the sludge fractions, the amount of astaxanthin in the sludge fractions is calculated (appendix L). Because there is also an overall material loss during enzymatic hydrolysis in addition to the possibility of loosing astaxanthin as carotenoproteins in the hydrolysate, the amount of astaxanthin lost is not further commented other than together with lipid loss. As discussed in chapter 4.3.7, some lipid was lost in the enzymatic hydrolysis experiment, both in the equipment and the water fraction. As there are less lipids in the sludge fraction than in the original RRM, there is also less astaxanthin. The recovery of astaxanthin is therefore different depending on to which starting sample one is comparing the result. When looking at the recovery of astaxanthin from the RRM to the rapeseed oil after combination treatment of enzymatic hydrolysis and heat treatment (shaded bars), the recovery is lower than the recovery is from the sludge fractions (dotted bars). This is a trend independent of the conditions for enzymatic hydrolysis, and amount of oil added to the heat treatment. There is a significant difference between recovery form RRM and recovery from SF for all treatment except $\mathrm{CO}^{50°C}\mathrm{HT}^{65°C}_{\mathrm{oil}\ 1:2}$ stored and not stored overnight (**Table 4.9**). The enzymatic hydrolysis with Corolase was not performed with the second centrifugation. This means that some sludge remained in the water fraction, and this is assumed to be minerals and lipids.

In order to avoid the loss of astaxanthin in the combination experiments, one could either run the extraction of astaxanthin to oil together with enzymatic hydrolysis, or optimise the enzymatic hydrolysis to reduce emulsions and sludge in the water fractions. As hydrolysis with oil could lead to rapeseed oil disturbing the hydrolysate, optimisation of the method should be performed, eg. with two centrifugations. In addition, by performing the combination method in a continuous production line, as proposed for enzymatic hydrolysis and subsequent heat treatment by Šližytė et al. (2014), the loss of material could be avoided (Šližytė et al. 2014).

Table 4.9: Results from Students T-test for comparison of different treatments in astaxanthin recovery. Abbreviations are CO = Corolase, HT = heat treatment, SF = sludge fraction, RRM = rest raw material, rec = recovery from. P-values lower than 0,001 are indicted with three stars.

Comparison of	Group 1	Group 2	p-value
The astaxanthin recovery from RRM vs recovery from SF	$CO^{50°C}HT^{65°C}_{oil\ 1:2}$ rec SF	$CO^{50°C}HT^{65°C}_{oil\ 1:2}$ rec RRM	0,402
	$CO^{50°C}HT^{65°C}_{oil\ 1:2}$(stored) rec SF	$CO^{50°C}HT^{65°C}_{oil\ 1:2}$ (stored) rec RRM	0,021
	$CO^{65°C}HT^{65°C}_{oil\ 1:2}$ rec SF	$CO^{65°C}HT^{65°C}_{oil\ 1:2}$ rec RRM	***
	$CO^{65°C}HT^{65°C}_{oil\ 2:1}$rec SF	$CO^{65°C}HT^{65°C}_{oil\ 2:1}$rec RRM	***
Effect of 12 hours storing (-20°C)	$CO^{50°C}HT^{65°C}_{oil\ 1:2}$ (stored) rec SF	$CO^{65°C}HT^{65°C}_{oil\ 1:2}$ rec SF	***
Effect of protease (difference from control samples)	$CO^{50°C}HT^{65°C}_{oil\ 1:2}$ rec SF	control	***
	$CO^{50°C}HT^{65°C}_{oil\ 1:2}$(stored) rec SF	control	0,853
	$CO^{65°C}HT^{65°C}_{oil\ 1:2}$ rec SF	control	***
	$CO^{65°C}HT^{65°C}_{oil\ 2:1}$rec SF	control	***
	$CO^{50°C}HT^{65°C}_{oil\ 1:2}$ rec RRM	$HT^{65°C}_{oil\ 1:2}$	***
	$CO^{65°C}HT^{65°C}_{oil\ 1:2}$ rec RRM	$HT^{65°C}_{oil\ 1:2}$	0,100
Effect of increased temperature	$CO^{50°C}HT^{65°C}_{oil\ 1:2}$ rec SF	$CO^{65°C}HT^{65°C}_{oil\ 1:2}$ rec SF	***
Effect of increasing oil:sample ratio	$CO^{65°C}HT^{65°C}_{oil\ 1:2}$ rec SF	$CO^{65°C}HT^{65°C}_{oil\ 2:1}$ rec SF	0,401

The use of Corolase has been shown to improve the extraction of astaxanthin. All control samples for the recovery of astaxanthin from the sludge fraction are significantly lower than the respective protease treated samples. The activity of the protease will release more astaxanthin, as this is bound to the chitin-protein complexes in the crab shells.

However, heat treatment ($HT^{65°C}_{oil\ 1:2}$) gives the same recovery of astaxanthin as when combining with Corolase® 2TS. The recovery from the RRM is significantly higher for the heat-treated samples than the combination experiment $CO^{50°C}HT^{65°C}_{oil\ 1:2}$. This can be viewed when comparing the results from $CO^{50°C}HT^{65°C}_{oil\ 1:2}$, $CO^{65°C}HT^{65°C}_{oil\ 1:2}$, $CO^{65°C}HT^{65°C}_{oil\ 2:1}$ to $HT^{65°C}_{oil\ 1:2}$. The addition of a protease treatment should recover more astaxanthin since the degradation by proteases will release more pigment from the material. But there is no significant difference between $CO^{65°C}HT^{65°C}_{oil\ 1:2}$ recovery from RRM and $HT^{65°C}_{oil\ 1:2}$. As the astaxanthin is fragile to heat (Pu et al. 2010), the heat load at enzymatic treatment leads to the same recovery by enzymatic treatment as with only heat treatment.

The results also show that the recovery by only heat-treating RRM with rapeseed oil is higher than the control samples. Since less astaxanthin is detected in control samples when heat treatment is combined with a preparatory enzymatic hydrolysis compared to only heat treatment, the heat exposure at enzymatic hydrolysis could impose a negative impact on the astaxanthin. The heat treatment and the highest temperature for enzymatic treatment are both at 65°C. It can therefore be assumed that it is the exposure to 90°C for 10 minutes to end hydrolysis that degrades the astaxanthin. In addition, the heat load (heat multiplied by duration) by combination experiment is larger as the material goes through two heat exposures - enzymatic hydrolysis followed by heat treatment with rapeseed oil.

Compared to the 74% recovery of astaxanthin reported by Shahidi and Synowiecki (1991) using cod liver oil (60°C, 30 minutes, no pre-treatment) (Shahidi and Synowiecki 1991), the amount of astaxanthin recovered in this study was low. The cod liver oil is marine, and will be more similar to the lipids of the snow crab than vegetable oils. This has been disregarded in this comparison. The highest recovery was 25,95±1,92%, with $\mathrm{CO}^{50^{\circ}C}\mathrm{HT}_{\mathrm{oil}\ 1:2}^{65^{\circ}C}$, stored -20°C overnight. This could be affected by degradation of astaxanthin by both oxygen and heat. The RRM was not stored under vacuum, and so oxygen exposure during storing and thawing could have caused degradation. Also, heat exposure during both thawing and enzymatic hydrolysis is assumed to have caused oxidation. The heat effect of the enzymatic hydrolysis is evident when comparing the astaxanthin recovered by simple heat treatment ($\mathrm{HT}_{\mathrm{oil}\ 1:2}^{65^{\circ}C}$) and enzymatic hydrolysis at 50°C ($\mathrm{CO}^{50^{\circ}C}\mathrm{HT}_{\mathrm{oil}\ 1:2}^{65^{\circ}C}$).

The effect of increased temperature correlates to the increased amount of soluble protein extracted when increasing temperature (chapter 4.3.4). Astaxanthin recovered from the sludge fraction in the treatments $\mathrm{CO}^{50^{\circ}C}\mathrm{HT}_{\mathrm{oil}\ 1:2}^{65^{\circ}C}$ and $\mathrm{CO}^{65^{\circ}C}\mathrm{HT}_{\mathrm{oil}\ 1:2}^{65^{\circ}C}$ was significantly different. By increasing the temperature at the enzymatic hydrolysis by 15 °C, the recovery of astaxanthin is increased from 6,3 to 18,5% recovery.

Increasing the amount of oil added to the sludge samples did not appear to affect the recovery of astaxanthin. **Figure 4.2** shows how the recovery both from the RRM and the SF is equal when oil to samples ratio is 1:2 and 2:1. This indicates that the oil is not a limiting factor, and rapeseed oil can be saturated with astaxantin with the given extraction conditions. This is not in agreement to the findings of Hooshmand et al. (2017). They optimised their protocol to oil to sample ratio 5:1, 78°C, 95 minutes (Hooshmand et al. 2017).

The main factor that affected the recovery of astaxanthin in rapeseed oil was storing the samples after centrifugation, before separation. The astaxanthin recovered from the sludge fraction with $\mathrm{CO}^{50^{\circ}C}\mathrm{HT}^{65^{\circ}C}_{\mathrm{oil}\ 1:2}$ and 12 hours storing at -20°C was significantly higher than the recovery of astaxanthin from sludge fraction with $\mathrm{CO}^{65^{\circ}C}\mathrm{HT}^{65^{\circ}C}_{\mathrm{oil}\ 1:2}$. These were compared as the recovery of astaxanthin with $\mathrm{CO}^{65^{\circ}C}\mathrm{HT}^{65^{\circ}C}_{\mathrm{oil}\ 1:2}$ without storing was significantly higher than the $\mathrm{CO}^{50^{\circ}C}\mathrm{HT}^{65^{\circ}C}_{\mathrm{oil}\ 1:2}$, but significantly lower than the recovery with storing. For the samples stored overnight, the protease does not affect the result significantly. The loss of astaxanthin in the enzymatic hydrolysis is still an issue independently of storing, as the recovery is significantly different from both RRM and SF.

Optimisation of the recovery of astaxanthin in vegetable oil should include vacuum storing and slow thawing of RRM before enzymatic hydrolysis. In order to avoid the degradation of astaxanthin during the exposure to 90°C for ending of hydrolysis, the sludge to be heated with oil can be separated from the water fraction before ending hydrolysis. The water fraction containing the recovered protein is then exposed to 90°C. Since storing showed increased recovery of astaxanthin, the sludge should be immediately mixed with oil and further heated for a prolonged time, and at lower temperature than used in this thesis. Also, more stirring/shaking of reaction tubes during the heating with oil could increase the reaction surface for transfer of astaxanthin to the oil.

Other oils should be exploited. Refined vegetable oils are sensitive to heat, and reduced quality could have affected the astaxanthin recovery. However, Sachindra (2005) showed that refined sunflower oil was the best candidate for recovery of astaxanthin, compared to other non-refined oils (Sachindra and Mahendrakar 2005).

4.5.3. Supercritical fluid extraction

The results from CO_2 extraction of pigment from snow crab rest raw material can be viewed in appendix M. The average extracted solute was 969,9 μg/g raw material (w/ww). Considering the total amount of astaxanthin in the raw material being 11,78±0,88 μg/g (w/ww), the results from SFE are considered high. It is proposed that this can be due to residual ethanol in the samples, smaller particles that was glimpsed in the resulting samples, or extraction of familiar compounds to astaxanthin because of too strong solubility power of the solvents. Some critics can be given to the method performed the determination of the total astaxanthin content in batch 2. But looking at total amount of astaxanthin determined by other studies, 51,6 - 119,6 ug/g (w/ww) (Lage-Yutsy et al. 2011; Shahidi and Synowiecki 1991; Vilasoa-Martínez et al. 2008), the amount extracted with SFE is still high. The extracted material was not further analysed. In the literature, recovery of 88,51% astaxanthin from Lousiana crawfish has been reported (Charest et al. 2001). This is also higher than the astaxanthin recovered in the rapeseed oil by heating and storing (discussed above), and so the further optimisation of CO_2-extraction should be attempted.

4.6. FURTHER WORK

For further work, the rest raw material not containing intestines should be in focus. Even though it is the presence of intestines and hemolymph that gives highest nutritional value in means of lipid and astaxanthin, this has not been shown for proteins, this should, however, also be further studied. It is the rest raw material without intestines that is the actual available rest raw material, due to that most slaughter is preceded on the boats. Alternatively, the slaughter machines should be optimised to keep intestines. The astaxanthin and its derivaties in the rest raw material should be characterised, and the differences between presence of intestines and empty carapaces should be studied. Up-scaling the experiments can make isolation of oil fractions at enzymatic hydrolysis and heat treatment possible. For the analysis of total protein content, the results of a Kjeldahl analysis and the HPLC for total amino acid determination can be compared. The most convenient method for analysis of total protein in crustacean material should then be discussed.

For optimisation of enzymatic hydrolysis, several factors can be changed. The RRM can be dried and grinded to a fine powder in order to increase the surface area and the access to the proteins and astaxanthin. This will, however, increase the cost of the process. Increasing the amount of water added to the sample will most likely increase the recovery of soluble protein in the hydrolysate. Increasing the water to sample ratio to 1:1 or even 2:1 does not necessarily affect emulsion too much. Most of the studies mentioned adjusted the pH of the hydrolysis mix, and this adjustment can be done in order to affect the activity of the protease. But in order to keep the treatment of the RRM simple and environmentally friendly, the addition of any acids or alkaline treatments should be kept at a minimum. This will also increase the cost of the process. The use of fermentation as a preparatory step can aid the demineralisation of the material, and this should be further studied. Fermentation is a good, environmentally friendly alternative to acid treatment (Arabia et al. 2013). If the obstacle of demineralisation before deproteinisation with proteases is solved, the use of commercial proteases for recovery of proteins from crustacean RRM can compete with the efficient acid and alkaline treatments. The molecular weight distribution of other protease hydrolysates should also be studied to compare the characteristics of Corolase hydrolysate to other proteases. Corolase is known to generate hydrolysates with non-bitter taste (Arnesen et al. 2017), and comparison to other proteases on snow crab material should be discussed to optimise combination of material and different proteases. The endogenous enzyme activity in the snow crab RRM should be studied in order to optimise alternative pre-treatment with storing, or to optimise heat treatment at different temperatures with water to recover proteins.

Recovery of astaxanthin in oil should be further studied with different vegetable oils. Animal oils like cod oil can also be explored, but the marine resources that are already scarce should be avoided. The pre-treatment with enzymatic hydrolysis should be further explored with removing the sludge from the water fraction before ending the hydrolysis only in the water fraction. The sludge can be heated with oil immediately. Optimisation of the combination of heat treatment duration, temperature and oil to sample ratio should be further studied. In addition, the exposure of the RRM to oxygen, heat and light is advised kept at a minimum in order to avoid degradation of the astaxanthin. Vacuum packing the minced or grinded material, and further storing it at low temperatures and in darkness can solve this. The recovery of chitin from snow crab should also be further studied, as this is a valuable biopolymer. This will increase the utilisation of the snow crab.

For optimisation the use of supercritical fluid extraction should be performed on dried and grinded samples. The solvents seem to have too strong solubility power, and decreasing the amount of ethanol used as co-solvent could solve this. The elimination of ethanol from the final product should be performed in burned glass tubes, and the duration of elimination of ethanol should be prolonged. Pre-treatment with proteases could improve the results. But as above, this should be done without exposure of the sludge to 90°C. Other co-solvents like vegetable oils should be explored, as this will give a final product of pigment enriched oil that is ready to use without need for further processing (Krichnavaruk et al. 2008).

5. Conclusion

There are different treatments available to degrade crustacean rest raw material and recover protein, lipid and pigment. The literature search found that the use of alkaline and acid treatments are most used, and that it is the chitin that is mostly focused on in the utilisation of crustacean rest raw material. Other methods described were ensilation and fermentation, enzymatic hydrolysis, heat treatment, as well as the use of organic solvents, oil extraction and supercritical fluid extraction for recovery of pigment. The methods determined to be milder and more sustainable to use was enzymatic hydrolysis, mild heat treatment, oil extraction of pigments and supercritical CO_2 extraction.

The rest raw material generated during snow crab harvest is a good source of protein and pigment. The processing of the snow crabs at slaughter is determining for the chemical composition of the rest raw material. This was expected since they do not manage to keep the intestines attached to the carapace during slaughter on the boats. This leads to loss of lipid and pigment. In this study it is not determined whether the loss of intestines leads to loss of protein.

Enzymatic proteases can be used in recovery of proteins from snow crab rest raw material. The proteases are not as efficient as the use of acid and alkaline treatments, and the main challenge is extensive demineralisation of the crab shells hindering access of the proteases. Proteases are found to be more efficient than heat treatment. A solution could be to integrate the use of fermentation to demineralise the material before adding proteases for deproteinisation. The lipid content of the snow crab is low and the lipid is therefore difficult to extract from the material. Recovery of astaxanthin with rapeseed oil is most efficient when heating rest raw material together with rapeseed oil without pre-treatment and then storing the mixture overnight before separating the astaxanthin enriched oil from the snow crab material. The use of protease compared to control samples has been shown to enhance extraction significantly, but the heat load during enzymatic hydrolysis is assumed to degrade the astaxanthin. Supercritical fluid extraction of astaxanthin showed too strong solubility powers of the ethanol and supercritical CO_2. The protocol should be optimised before comparison to the recovery with rapeseed oil. The rest raw material generated through snow crab harvest is a good source of protein and pigment. Milder processes than the traditional acid and alkaline treatments can be used to recover protein and astaxanthin, but the demineralisation of the snow crab shells material hinders access to the proteins and pigments and thus hinders efficient recovery.

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Figure resources

Figure 1.1: Photo by Lidunn Mosaker Boge, Nofima AS From <https://nofima.no/nyhet/2017/06/samler-all-kunnskap-om-krabbe/>

Figure 1.2: Photo A: Lidunn Mosaker-Boge, Nofima AS, From <https://nofima.no/nyhet/2016/02/snokrabbe-kan-bli-baerekraft-vinner/>; Photo B: Private.

Figure 1.3: Illustration from GE Healthcare Gel filtration Principles and Methods 18-1022-18. From <https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/General_Information/1/ge-gel-filtration.pdf>

Figure 4.6: Photo: Private.

Figure 4.9: Photo: Private.

Appendices

A. Project plan

A project plan (Table A.1) was made in the preface of this project. It has been revisited several times, and has been used in both lab work as well as in the discussion of the results. An overview of the lab work conducted on batch 1 and 2 (figure A.1 and figure A.2) is also based on the project plan.

Table A.1: Project plan describing main aim, and aims, issues and solutions for conduction of the study.

Main aim	Aims	Issue	Solution
How can proteins, lipids and astaxanthin be extracted from snow crab rest raw material in a sustainable manner?	Perform a literature search in order to explore and compare methods for recovery of protein, lipid and pigment from crustacean rest raw material.	*What methods of extraction are used to increase the utilisation of crustacean rest raw material?*	Describe methods, and the quantity and quality of the products.
		What methods are most sustainable to use?	Discuss the advantages and disadvantages of traditional and modern methods of extraction of different compounds from crustacean rest raw material
	Determine the chemical composition of two different batches of snow crab rest raw material, and discuss the effect of different slaughter processes of snow crabs.	*Which compounds are possible to extract from snow crab rest raw material?*	Describe selected chemical components, proteins, lipids and astaxanthin.
		What methods are used to analyse chemical content of proteins, lipids and astaxanthin?	Explain analytical methods: Lowry, Bligh and Dyer, FPLC, HPLC, formol titration, spectrophotometric astaxanthin analysis.
		Which parts of the snow crab rest raw material is most nutritious?	Compare determined chemical content in batch 1 and 2.
		What degree of homogenisation of rest raw material is necessary?	Compare the particle size of homogenised batch 1 and 2, based on recovery of protein.
	Use commercial proteases and heat treatment in the recovery of proteins and lipids from snow crab rest raw material, and compare the efficiency of the methods.	*What are commercial proteases, why are they useful?*	Describe enzymatic hydrolysis, conditions, fractions obtained and the characteristics of the fractions.
			Describe proteases (exogenous and endogenous.
			Describe chosen proteases used in lab work: Alcalase® 2.4 L, Protamex®, Papain GSM80 and Corolase® 2TS.
		Which protease(s) are most efficient in recovery of proteins and lipids?	Use commercial proteases in recovery of protein, lipid and astaxanthin form snow crab rest raw material.
		What fractions contain most protein and lipid after enzymatic hydrolysis?	Analyse protein, lipid and ash content of fractions obtained by enzymatic hydrolysis with chosed proteases.
		How can heat treatment be used for extraction of proteins, lipids and astaxanthin?	Described traditional heat treatment.
			Perform heat treatment of rest raw material
			Analyse protein, lipid, astaxanthin and ash content of fractions obtained by heat treatment
	Use oil extraction with and without pre-treatment with protease, as well as supercritical fluid extraction in the recovery of astaxanthin from snow crab rest raw material and compare the methods.	*How can oil (vegetabile/animal oil) be used in extraction of astaxanthin from snow crab rest raw material?*	Describe the use of oil in extraction of astaxanthin
			Use rapeseed oil to extract astaxanthin in 1) heat treatment, 2) heat treatment with preparatory enzymatic hydrolysis
			Compare astaxanthin extracted by heat treatment + oil to combination experiment + oil
		What is the most efficient method in extraction of astaxanthin?	Describe supercritical fluid extraction for recovery of astaxanthin
			Use CO_2-extraction to recover astaxanthin from snow crab rest raw material
			Compare the use of rapeseed oil to CO_2-extraction in the recovery of astaxanthin.

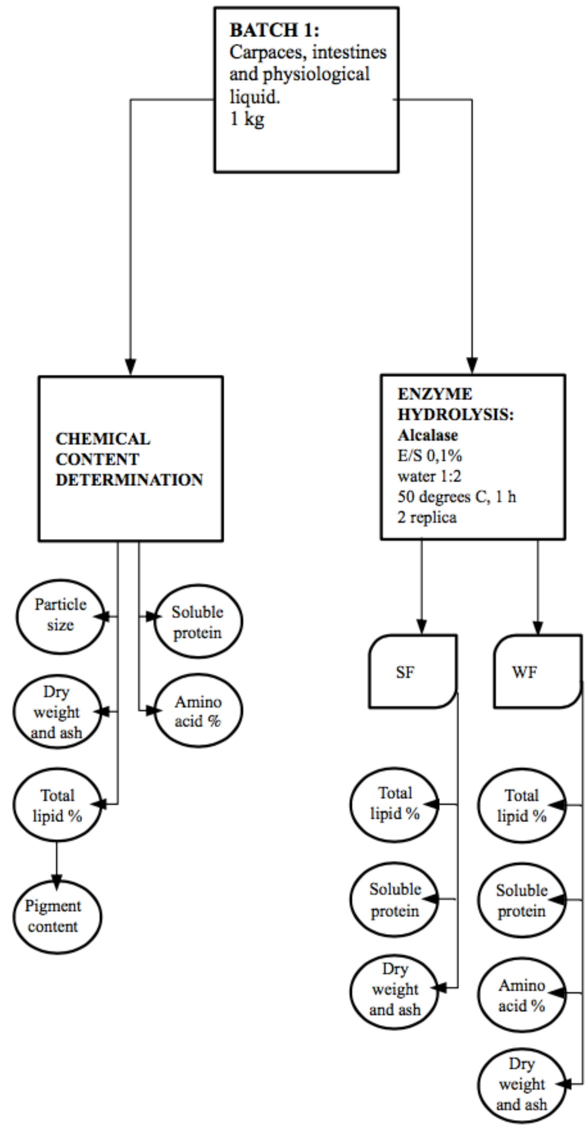

Figure A.1: Overview of lab work conducted on snow crab rest raw material, batch 1. Abbreviations are SF = sludge fraction, WF = water fraction, C = °C.

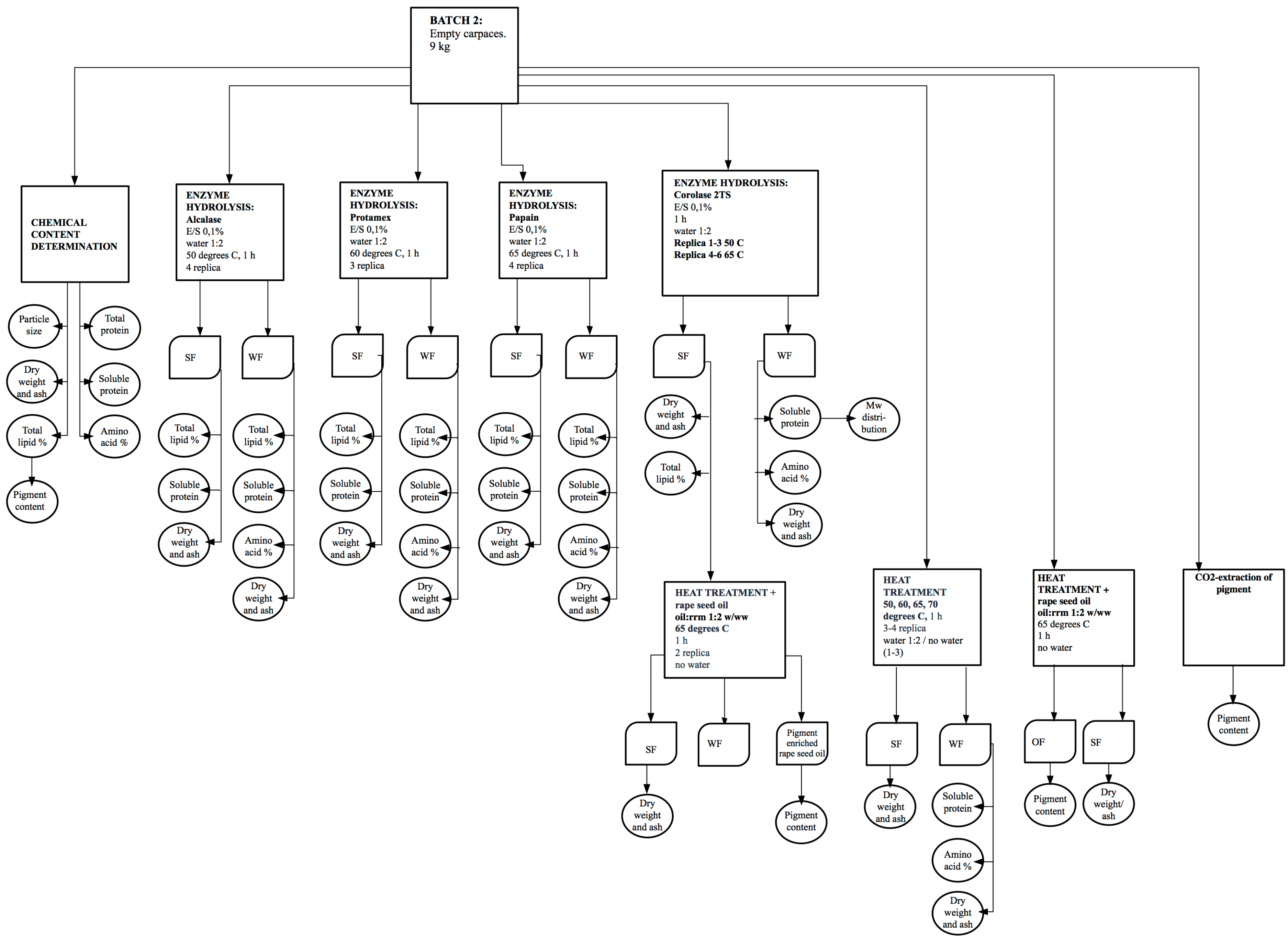

Figure A.2: Overview of for lab work conducted with batch 2 of snow crab rest raw material. Abbreviations are SF = sludge fraction, WF = water fraction, C = °C.

B. Modified method Bligh and Dyer (1959)

SINTEF Fiskeri og havbruk

Utarbeidet av Johanna Halvorsen, SINTEF

FORMÅL/OMFANG:
Ekstrahering av lipid. Beregning av totalt lipidinnhold. Modifisert utgave av: Bligh, E.G & Dyer, W.J (1959). A rapid method of total lipid extraction and purification. Canadian J. Biochem. 37:911-917.

GJENNOMFØRING:

5-10 g prøve (noter nøyaktig vekt, 2 desimaler)
Tilsett
16 ml H_2O
40 ml MeOH
og 20,0 ml $CHCl_3$

Homogeniser 2 min

(ved all homogenisering holdes sentrifugekopper på is i avtrekk – dette for å minimalisere avdamping og øke nøyaktigheten)

Tilsett 20,0 ml $CHCl_3$ og homogeniser 40 sek
Tilsett 20 ml H_2O , Homogeniser 40 sek.
Sentrifuger ved 5000 rpm i 15 min.

Pipetter ut det underste laget (kloroformfasen) .
For å bestemme totalt lipidinnhold, ta straks en nøyaktig mengde av kloroformfasen (f.eks. 2,0 ml) over i et veid reagensrør. Kloroformen dampes av på varmeblokk (60°C) og med tilførsel av N_2-gass.

Etter avkjøling i eksikator (minst 1 time), veies røret med fettet og % lipid beregnes av innveid prøve.

Resten av kloroformfasen kan dampes inn for andre undersøkelser – dette gjøres på rotavapor i avtrekk. Oppsamlet kondensat avhendes som løsemidler med halogener.

Metoden kan skaleres opp og ned.

BEREGNING AV % T0TALT LIPIDINNHOLD:
a*b*100/(c*v)
a = g inndampa fett, b = tilsatt ml kloroform, c = ml inndampa kloroform og v = innveid g prøve.

REGISTRERINGER
Resultater legges i de enkelte prosjektmappene

AVFALL
Klorformavfall helles i beholder merket løsemidler med halogener, som finnes i under avtrekket på rom 413 Bruk blå hansker ved denne prosedyren.

C. Hydrolysis experiment, Nofima AS 8.5.18

Materials and method

Enzymatic hydrolysis was performed in a 7100 system (Distek Inc., North Brunswick, NJ). 215.84 g crude crab material was weighted and 216.78 g water was added for a 1:1 mixture. The hydrolysis was set to 60 minutes at 50°C, with 10 minutes at 90°C to end the reactions. The mixture reached 50°C after about 26 minutes. Then the 26 minutes was set as time 0 (t0) for the 60 minutes hydrolysis. A sample of about 1 mL for SEC-HPLC was removed from the mixture. Enzyme/substrate concentration was set to 0,1% of wet weight. This indicated a volume of 176 µL for the first enzyme Celluclast (1,22 g/mL). Enzyme was added at t0. Then the hydrolysis was run for 60 minutes and samples of 1 mL were collected at t5, t15, t30, t45 and t60. At t30 Protamex was added after sampling. At t60 the temperature was raised to 90°C, and the mixture reached this temperature after about 20 minutes. Then the mixture was kept at 90°C for 10 minutes to end the reactions. In experiment 2 the mixture was held at 90°C for about 20 minutes. The pH was measured at t0 before adding of enzyme, at t30 (experiment 2), t45 (experiment 1) and t60.

After the hydrolysis the remaining solid fraction was sifted out and weighted. The liquid fraction was weighted and centrifuged at 7000 *x g* for 20 min (Jouan KR4i centrifuge, VWR). The hydrolysis experiment was repeated, but with 251 g crude crab material and 1% enzyme. The results for both experiments are presented in the results section designated experiment 1 and 2, respectively.

Results

The results from enzymatic hydrolysis, experiment 1 and 2, are presented in **Table C.1**. Considering these experiments to be mainly for the purpose of method training, the fractions were not further analysed. Centrifugation of liquid fraction gave a liquid mixture with orange lipid fractions floating on the surface of the water fraction. Lipid fraction was not separated from the water fraction.

Table C.1: Components and results of enzymatic hydrolysis. Values are described for experiment 1 and 2. Abbreviations are LF = liquid fraction, SF = sludge fraction.

Mixture	Experiment 1	Experiment 2
Crude crab material (g)	215,84	251,01
Water added (g)	216,78	251,51
Celluclast (g)	0,215	2,51
Protamex (g)	0,215	2,51
Total hydrolysis mixture (g)	433,05	507,54
LF (g)	386	402,2
LF % (g/100 g total hydrolysis mix.)	89,13	79,24
SF (g)	37,97	51,59
SF % (g/100 g total hydrolysis mix.)	8,76	10,16

Remarks: HPLC samples were lost due to technical mistakes.

D. Chemical composition batch 1 and 2

Analysis of chemical composition and structure of batch 1 and 2 was performed. Raw material data is presented in this appendix. Methods are described in chapter 2.3.

1. Particle size

Particle size was determined by measuring cm^2 of a random selection of particles from each batch. Results are presented in **Table D.1**.

Table D.1: Raw material data from determination of particle size of snow crab rest raw material, batch 1 and 2.

		Side 1	Side 2	cm^2	Average cm^2	SD	p-value
Batch 1	a	1,4	1,5	2,1	1,24	0,6911	0,039
	b	1,2	0,7	0,84			
	c	1	1	1			
	d	1,8	0,5	0,9			
	e	1,5	0,8	1,2			
	f	0,7	0,4	0,28			
	g	1,2	1	1,2			
	h	1,6	1,5	2,4			
Batch 2	a	1	0,5	0,5	0,5125	0,5752	
	b	0,3	0,5	0,15			
	c	0,5	0,5	0,25			
	d	0,3	0,3	0,09			
	e	0,5	0,8	0,4			
	f	0,4	0,6	0,24			
	g	1	0,6	0,6			
	h	1,7	1,1	1,87			

2. Dry weight and ash

Dry weight and ash determination was performed by heating for 24 hours at 105°C and 550°C, respectively. Results for batch 1 and 2 are presented in **Table D.2**. Average % content of both dry weight and ash shows statistically significant difference ($p<0,05$) between batch 1 and 2. For batch 2 the p-value was $\leq 0,001$, indicated with ***. The results was converted to total ash content % (w/dw) based on average dry weight content of 28,79±3,83 and 32,71±1,77% in batch 1 and 2 respectively. The ash content on basis of w/ww is also included as these value are used in comparison of proteolytic fractions to original RRM.

Table D.2: Raw material data for dry weight and ash content of snow crab rest raw material, batch 1 and 2.

Batch	Nr	Sample (g, ww)	Dry weight (g)	Dw % (w/ww)	Average % w/ww	SD	p-value	Ash (g)	Ash % w/dw	Average % w/dw	SD	p-value	Ash % w/ww	Average % w/ww	SD
B1	1	3,9933	1,1335	28,3850	28,4002	2,8522	0,0130	0,5479	48,3370	50,27	4,6604	***	13,7205	14,3778	2,7443
	2	5,7353	1,612	28,1066				0,8001	49,6340				13,9504		
	3	8,1889	2,7151	33,1559				1,5394	56,6977				18,7986		
	4	7,6469	2,2433	29,3361				1,2367	55,1286				16,1726		
	5	5,2196	1,4015	26,8507				0,6555	46,7713				12,5584		
	6	6,5316	1,6046	24,5667				0,7228	45,0455				11,0662		
B2	1	5,3609	1,6414	30,6180	32,7067	1,7720		0,5209	31,7351	32,4892	2,8250		9,7167	10,6441	1,3000
	2	5,9667	1,9135	32,0697				0,5344	27,9279				8,9564		
	3	3,7927	1,359	35,8320				0,4823	35,4893				12,7165		
	4	5,1847	1,7293	33,3539				0,5363	31,0125				10,3439		
	5	3,7886	1,2312	32,4975				0,4199	34,1049				11,0832		
	6	8,4081	2,6796	31,8693				0,9289	34,6656				11,0477		

3. Total lipid content

Total lipid content of the RRM, batch 1 and 2, was determined by Bligh and Dyer (1959) described in chapter 2. **Table D.3** presents the raw material data from the analysis. Equation III was used to calculate amount of lipid in the samples (g/100 g, w/ww), were *a* represents gram evaporated fat, *b* added chloroform, *c* mL evaporated chloroform and *v* is the weight of final sample. The results was converted to total lipid content % (w/dw) based on average dry weight content of 28,79±3,83 and 32,71±1,77% in batch 1 and 2 respectively.

$$\text{Total lipid (\%, w/ww)} = a \times b \times \frac{100}{c \times v} \qquad \text{III}$$

The final results are based on the average of 4 and 6 parallels for batch 1 and 2, respectively. A Students T-test was performed, with p-value = 0,00164.

Table D.3: Results from Bligh and Dyer extraction of lipid from snow crab RRM, batch 1 and 2.

Batch	Nr	Sample (g, w/ww)	Chloroform (mL)	Evaporated chloroform (mL)	Lipid (g)	Total lipid % (g/100 g, w/dw)	Average % w/dw	SD	p-value	Total lipid % (w/ww)	Average % w/ww	SD
B1	1a	9,572	40	2	0,0175	12,7006	14,6231	1,7386	0,0014	3,6565	4,21	0,50
	1b	9,572	40	2	0,0195	14,1521				4,0744		
	2a	9,7485	40	2	0,0207	14,7510				4,2468		
	2b	9,7485	40	2	0,0237	16,8889				4,8623		
B2	1a	8,5716	40	2	0,0076	5,4213	5,6054	0,4309		1,7733	1,83	0,14
	1b	8,5716	40	2	0,0085	6,0633				1,9833		
	2a	7,3948	40	2	0,006	4,9612				1,6228		
	2b	7,3948	40	2	NA					NA		
	3a	10,4002	40	2	0,0097	5,7025				1,8653		
	3b	10,4002	40	2	0,01	5,8789				1,9230		

4. Total amino group composition

Determination of the total amino group composition was used to estimate the total amount of protein in the rest raw material in batch 2. Batch 1 was not analysed as this batch was used up by the time of hydrolysis. The RRM was analysed with 4 parallels, and each was analysed once (**Table D.5 - Table D.8**). Dried samples (0,1 g) were hydrolysed with HCl (6 M) for 24 hours, and separated and detected using HPLC. Extract volume was 20 mL, dilution 1:500. Calculations are shown for Sample A, Asp.

$$\text{Asparagine } (\mu g/mL) = g/mol \times nmol/L = \frac{115{,}00 \times 3{,}2111}{1000} = 0{,}3693\ \mu g/mL$$

$$mg/g\ RRM = \frac{\frac{\mu g}{mL} \times 1{,}25 \times extract\ volume\ (mL) \times dilution}{1 \times 1000 \times sample\ size(g)} = \frac{0{,}3692 \times 1{,}25 \times 20 \times 500}{1 \times 1000 \times 0{,}0950} = 48{,}5895\ mg/g\ RRM\ (w/dw)$$

Final calculated results are presented in **Table D.4**, and raw data for each parallel that table is based on are presented in **Table D.5**– **Table D.8**. The data in the tables are based on chromatograms generated with HPLC. Total amino acid composition as % w/ww is calculated based on the dry weight of batch 2 RRM (32,71% w/ww).

Table D.4: Total amino acid composition determined in batch 2 of snow crab rest raw material. Analysis is performed with four parallels, and each is analysed once. Abbreviations area a = amino groups.

Sample	Sample (g)	Total aa mg/g sample	Total aa g/g sample w/dw	Total aa g/100 g w/dw (%)	Average % w/dw	SD	Total aa (%) g/g w/ww	Total aa g/100 g w/ww	Average g/100 g w/ww	SD
A	0,0950	400,3900	0,4004	40,0390	36,6188	9,6294	0,1310	13,0968	11,9780	3,1498
B	0,0975	451,3600	0,4514	45,1360			0,1476	14,7640		
C	0,0978	228,1800	0,2282	22,8180			0,0746	7,4638		
D	0,1001	384,8200	0,3848	38,4820			0,1259	12,5875		

Table D.5: Raw data material for determination of total amino acid composition in parallel sample A, batch 2. Peak names present the amino acids.

Sample A Peak Name	Retention Time min	Area mV*min	Height mV	Relative Area %	Relative Height %	Amount umol/l	Mw as bound in protein g/mol	 nmol/ml	 ug/ml	 mg/g sample
Asp	1,5167	8,5793	76,9422	11,2689	16,4060	3,2111	115,0000	3,2111	0,3693	48,5895
Glu	2,3233	9,1738	41,0389	12,0497	8,7505	2,9890	129,0000	2,9890	0,3856	50,7338
Asn	3,2817	0,0267	0,1542	0,0350	0,0329	0,0145	114,0000	0,0145	0,0017	0,2182
His	4,0817	1,5718	7,4787	2,0646	1,5946	0,7922	137,0000	0,7922	0,1085	14,2809
Ser	4,4350	5,2848	30,8800	6,9416	6,5844	2,3099	87,0000	2,3099	0,2010	26,4426
Gln	4,6217	0,1896	2,7836	0,2490	0,5935	0,1039	128,0000	0,1039	0,0133	1,7504
Gly/Arg	7,5050	13,7519	43,1568	18,0630	9,2021	2,8528	98,0000	2,8528	0,2796	36,7860
Thr	8,1300	3,6574	15,0992	4,8040	3,2195	1,7153	101,0000	1,7153	0,1732	22,7960
Ala	12,6767	5,8833	28,1983	7,7276	6,0126	2,8362	71,0000	2,8362	0,2014	26,4960
Tyr	14,3283	1,9212	12,7331	2,5235	2,7150	0,8095	163,0000	0,8095	0,1320	17,3620
Aba	16,1500	0,1681	1,0727	0,2207	0,2287	0,0662	85,0000	0,0662	0,0056	0,7409
Met	17,9967	1,7013	13,3595	2,2346	2,8486	0,6494	131,0000	0,6494	0,0851	11,1937
Val	18,2967	6,8357	54,3046	8,9786	11,5791	2,4035	99,0000	2,4035	0,2380	31,3092
Phe	18,8217	3,3860	26,5906	4,4475	5,6698	1,3921	147,0000	1,3921	0,2046	26,9259
Ile	19,8000	4,2325	34,2445	5,5594	7,3018	1,4903	113,0000	1,4903	0,1684	22,1591
Leu	20,1533	6,0666	49,5646	7,9685	10,5684	2,3771	113,0000	2,3771	0,2686	35,3435
Lys	21,9050	3,7028	31,3878	4,8636	6,6926	1,6188	128,0000	1,6188	0,2072	27,2640
TOTAL:		76,1328	468,9893	100,0000	100,0000			27,6321	3,0430	400,3918

Table D.6: Raw data material for determination of total amino acid composition in parallel sample B, batch 2. Peak names present the amino acids.

Sample B	Retention Time	Area	Height	Relative Area	Relative Height	Amount	Mw as bound in protein			
Peak Name	min	mV*min	mV	%	%	umol/l	g/mol	nmol/ml	ug/ml	mg/g sample
Asp	1,5067	10,7588	95,7389	11,8484	17,1160	4,0269	115,0000	4,0269	0,4631	59,3709
Glu	2,3050	11,1910	48,6775	12,3243	8,7025	3,6462	129,0000	3,6462	0,4704	60,3025
Asn	3,1983	0,0615	0,3894	0,0678	0,0696	0,0335	114,0000	0,0335	0,0038	0,4902
His	4,0717	0,3244	1,4259	0,3572	0,2549	0,1635	137,0000	0,1635	0,0224	2,8714
Ser	4,4250	6,4417	36,7832	7,0941	6,5760	2,8156	87,0000	2,8156	0,2450	31,4045
Gln	4,6183	0,1714	3,0992	0,1887	0,5541	0,0939	128,0000	0,0939	0,0120	1,5415
Gly/Arg	7,4900	16,1937	52,7897	17,8337	9,4376	3,3593	98,0000	3,3593	0,3292	42,2071
Thr	8,1250	5,0219	19,2544	5,5305	3,4423	2,3553	101,0000	2,3553	0,2379	30,4983
Ala	12,6733	7,5218	36,0351	8,2836	6,4423	3,6261	71,0000	3,6261	0,2575	33,0067
Tyr	14,3250	0,1345	0,7515	0,1481	0,1343	0,0567	163,0000	0,0567	0,0092	1,1843
Aba	16,4167	0,2900	0,0000	0,3194	0,0000	0,1143	85,0000	0,1143	0,0097	1,2460
Met	17,9917	1,9005	13,8463	2,0930	2,4754	0,7255	131,0000	0,7255	0,0950	12,1839
Val	18,2900	9,1280	72,9157	10,0525	13,0357	3,2096	99,0000	3,2096	0,3177	40,7367
Phe	18,8167	3,7970	29,6478	4,1816	5,3004	1,5611	147,0000	1,5611	0,2295	29,4200
Ile	19,7950	5,6888	46,4144	6,2649	8,2979	2,0031	113,0000	2,0031	0,2264	29,0196
Leu	20,1450	7,6754	63,3148	8,4527	11,3193	3,0075	113,0000	3,0075	0,3398	43,5695
Lys	21,8967	4,5034	38,2701	4,9595	6,8418	1,9688	128,0000	1,9688	0,2520	32,3092
TOTAL:		90,8039	559,3536	100,0000	100,0000			32,7669	3,5206	451,3624

Table D.7: Raw data material for determination of total amino acid composition in parallel sample C, batch 2. Peak names present the amino acids.

Sample C	Retention Time	Area	Height	Relative Area	Relative Height	Amount	Amino acid	Mw (as bound in protein)			
Peak Name	min	mV*min	mV	%	%	umol/l		g/mol	nmol/ml	ug/ml	mg/g innv.
Asp	1,5217	5,6292	50,3745	12,3541	17,8820	2,1070	Asp	115,0000	2,1070	0,2423	30,9687
Glu	2,3200	5,8306	25,7400	12,7961	9,1372	1,8997	Glu	129,0000	1,8997	0,2451	31,3218
Asn	3,1933	0,0425	0,2991	0,0933	0,1062	0,0232	Asn	114,0000	0,0232	0,0026	0,3377
His	3,9467	0,3220	1,1601	0,7068	0,4118	0,1623	His	137,0000	0,1623	0,0222	2,8423
Ser	4,4383	2,9270	16,5628	6,4238	5,8795	1,2794	Ser	87,0000	1,2794	0,1113	14,2260
Gln	4,6283	0,0761	1,4993	0,1671	0,5322	0,0417	Gln	128,0000	0,0417	0,0053	0,6827
Gly/Arg	7,5117	7,3072	23,7942	16,0367	8,4465	1,5159	Gly/Arg	98,0000	1,5159	0,1486	18,9870
Thr	8,1383	2,4901	9,9600	5,4649	3,5356	1,1679	Thr	101,0000	1,1679	0,1180	15,0762
Ala	12,6867	3,8716	18,5570	8,4967	6,5874	1,8664	Ala	71,0000	1,8664	0,1325	16,9368
Tyr	14,3467	0,0406	0,2096	0,0890	0,0744	0,0171	Tyr	163,0000	0,0171	0,0028	0,3561
Aba	16,3200	0,1035	0,0000	0,2271	0,0000	0,0408	Aba	85,0000	0,0408	0,0035	0,4432
Met	18,0017	1,0394	6,7030	2,2812	2,3794	0,3968	Met	131,0000	0,3968	0,0520	6,6431
Val	18,3000	4,7661	38,1345	10,4599	13,5370	1,6758	Val	99,0000	1,6758	0,1659	21,2051
Phe	18,8200	1,9480	12,8003	4,2752	4,5439	0,8009	Phe	147,0000	0,8009	0,1177	15,0471
Ile	19,8017	3,1226	25,8797	6,8531	9,1868	1,0995	Ile	113,0000	1,0995	0,1242	15,8804
Leu	20,1500	4,0464	33,0898	8,8804	11,7463	1,5855	Leu	113,0000	1,5855	0,1792	22,8989
Lys	21,9000	2,0025	16,9406	4,3947	6,0136	0,8754	Lys	128,0000	0,8754	0,1121	14,3222
TOTAL:		45,5655	281,7046	100,0000	100,0000		Total:		16,5552	1,7852	228,1752

Table D.8: Raw data material for determination of total amino acid composition in parallel sample D, batch 2. Peak names present the amino acids.

Sample D	Retention Time	Area	Height	Relative Area	Relative Height	Amount	Amino acid	Mw (as bound in protein)			
Peak Name	min	mV*min	mV	%	%	umol/l		g/mol	nmol/ml	ug/ml	mg/g innv.
Asp	1,5167	9,4363	85,4730	11,9980	17,5961	3,5319	Asp	115,0000	3,5319	0,4062	50,7204
Glu	2,3117	9,8294	43,9977	12,4977	9,0577	3,2026	Glu	129,0000	3,2026	0,4131	51,5899
Asn	3,1933	0,1176	1,0488	0,1495	0,2159	0,0641	Asn	114,0000	0,0641	0,0073	0,9125
His	4,0517	0,3541	1,3260	0,4503	0,2730	0,1785	His	137,0000	0,1785	0,0245	3,0536
Ser	4,4300	4,8598	27,3647	6,1791	5,6335	2,1242	Ser	87,0000	2,1242	0,1848	23,0772
Gln	4,6417	0,0868	1,6608	0,1103	0,3419	0,0476	Gln	128,0000	0,0476	0,0061	0,7602
Gly/Arg	7,5000	13,0183	42,9654	16,5523	8,8451	2,7006	Gly/Arg	98,0000	2,7006	0,2647	33,0495
Thr	8,1283	4,2626	16,8850	5,4197	3,4761	1,9992	Thr	101,0000	1,9992	0,2019	25,2145
Ala	12,6750	6,5962	31,5096	8,3869	6,4868	3,1799	Ala	71,0000	3,1799	0,2258	28,1934
Tyr	14,3350	0,0506	0,2485	0,0643	0,0512	0,0213	Tyr	163,0000	0,0213	0,0035	0,4336
Aba	16,3133	0,2201	0,0000	0,2799	0,0000	0,0868	Aba	85,0000	0,0868	0,0074	0,9212
Met	17,9917	1,5807	11,1368	2,0098	2,2927	0,6034	Met	131,0000	0,6034	0,0790	9,8704
Val	18,2933	8,0800	63,4584	10,2735	13,0640	2,8411	Val	99,0000	2,8411	0,2813	35,1231
Phe	18,8167	3,6042	22,1835	4,5826	4,5668	1,4818	Phe	147,0000	1,4818	0,2178	27,2006
Ile	19,7917	5,2694	42,7898	6,6999	8,8090	1,8555	Ile	113,0000	1,8555	0,2097	26,1822
Leu	20,1433	7,0803	58,1867	9,0024	11,9787	2,7743	Leu	113,0000	2,7743	0,3135	39,1474
Lys	21,8967	4,2029	35,5163	5,3438	7,3116	1,8374	Lys	128,0000	1,8374	0,2352	29,3697
TOTAL:		78,6494	485,7511	100,0000	100,0000		Total:		28,5300	3,0816	384,8194

5. Soluble protein

Soluble protein in the RRM was determined according to Lowry (1951) (**Table D.9**). Samples from batch 1 and 2 were properly diluted, centrifuged and filtrated. The filtrate was mixed with reagents and absorbance read at 750 nm, in triplettes. A standard relation between absorbance and concentration of soluble protein was determined. The standards curve for BSA samples made for batch 1 was $y=0,0021x+0,0242$, $R^2 \approx 0,98$. The standards curve made for batch 2 was $y = 0,0018x + 0,0273$, $R^2 \approx 0,99$. These were used to calculate soluble protein x (µg/mL) based on absorbance values y. The concentration was corrected for the given dilution of the samples, and converted to mg/mL sample. The available amount of moisture in the RRM was calculated for each sample in order to know the total amount of soluble protein (**Table D.2**). The amount of water in the samples (mL) was multiplied with concentration of soluble protein (mg/mL). The amount of soluble protein (mg) in the sample was then divided on the total sample (g, w/ww), which gave the amount of soluble protein per gram RRM (**Table D.10**). A students t-test was performed, with p-value = 0,094.

Table D.9: Estimated content of soluble protein in snow crab rest raw material batch 1 and 2 by Lowry method.

Batch	Sample	Sample (g, w/ww)	Dilution	Absorbance			Soluble protein µg /mL			Average protein µg/mL	µg /mL corr dilution	mg/mL
				I	II	III	I	II	III			
B1	A	2,0537	100	0,511	0,507	0,525	231,8095	229,9048	238,4762	233,3968	23339,6825	23,3397
	B	2,0537	1000	0,069	0,071	0,070	21,3333	22,2857	21,8095	21,8095	21809,5238	21,8095
B2	A	2,8256	100	0,392	0,389	0,398	202,6111	200,9444	205,9444	203,1667	20316,6667	20,3167
	B	2,2758	100	0,406	0,392	0,395	210,3889	202,6111	204,2778	205,7593	20575,9259	20,5759
	C	2,0379	100	NA	0,376	0,382	NA	193,7222	197,0556	195,3889	19538,8889	19,5389
	D	1,9965	100	0,417	0,430	0,412	216,5000	223,7222	213,7222	217,9815	21798,1481	21,7981
	E	2,6573	100	0,391	0,383	0,396	202,0556	197,6111	204,8333	201,5000	20150,0000	20,1500
	F	2,1778	100	0,378	0,368	0,386	194,8333	189,2778	199,2778	194,4630	19446,2963	19,4463

Table D.10: Calculations of the constituent soluble protein in RRM (batch 1 and 2). Total soluble protein in the rest raw material is basde on total amount of water in the batches. Students T-test gave p-value 0,0942, indicating no statistically significant difference between the batches.

Batch	Sample	Water w/ww (%)	Water in sample w/ww (g)	Soluble protein (mg)	Total soluble protein mg/g RRM (w/ww)	Average (mg/g, w/ww)	SD	% Soluble protein (g/100g RRM) w/ww	Av. % soluble protein	SD	Soluble protein (%, w/dw)	Average % w/dw	SD	B1 vs B2, p-value
B1	9	71,5998	1,4704	34,3197	16,7112	16,1634	0,7747	1,6711	1,6163	0,0775	5,1089	4,9414	0,2368	0,0941
	10	71,5998	1,4704	32,0697	15,6156			1,5616			4,7739			
B2	A	67,2932	1,9014	38,6309	13,6718	13,6634	0,5751	1,3672	1,3663	0,0575	4,1797	4,1771	0,1758	
	B	67,2932	1,5315	31,5112	13,8462			1,3846			4,2330			
	C	67,2932	1,3714	26,7950	13,1484			1,3148			4,0197			
	D	67,2932	1,3435	29,2860	14,6687			1,4669			4,4845			
	E	67,2932	1,7882	36,0319	13,5596			1,3560			4,1454			
	F	67,2932	1,4655	28,4988	13,0861			1,3086			4,0006			

6. Free amino groups and degree of hydrolysis

Free amino groups and degree of hydrolysis was performed after Taylor (1957) as an indirect titration, see chapter 2.3.6. Results are presented in **Table D.11**. Average results are based on analysis of three parallels for each batch. After titration the volume (mL) of NaOH used was included in equation IV to calculate % free amino groups. *A* is mL NaOH used, *B* is concentration of NaOH (0,1 M), 14,007 is molecular mass of nitrogen, C is mass of sample in grams, and 100 and 1000 are scaling factors to acquire the result in % free amino acids (w/ww). Calculation are demonstrated for Sample A, batch 2. Results of free amino groups (%) was converted to w/dw with batch 1 and 2 containing 28,79 and 32,71 % dry weight (w/ww), respectively. Degree of hydrolysis in CM was found by equation V were D is % free amino acids (g/100 g RRM) and E is total protein of 36,62 % (g/100g RRM w/dw) divided by factor 6,25. The calculation is shown for sample A, batch 2. Total protein was not estimated for batch 1, and DH is thus only calculated for batch 2.

$$\% \text{ Free amino groups} = \frac{A \times B \times 14{,}007 \times 100}{C \times 1000} \qquad \text{IV}$$

$$\% \text{ free amino groups (g/100 g RRM) Batch 1, sample A} = \frac{3{,}6 \times 0{,}1 \times 14{,}007 \times 100}{1{,}61 \times 1000} \left(\frac{mL \times \frac{mol}{L} \times \frac{g}{mol} \times 100\%}{g \times 1000} \right) = 0{,}3132 \text{ g/100 g RRM (w/ww)}$$

$$\text{free amino groups \% (w/dw)} = \frac{0{,}3132}{32{,}71} \times 100 \ \% \left(\frac{\frac{g}{100g\ ww}}{\frac{g\ dw}{100\ g\ ww}} \times 100\% \right) = \underline{0{,}9575\ \%\ \text{(w/dw)}}$$

$$\% \text{ Degree of hydrolysis} = \frac{D \times 100}{E} \qquad \text{V} \qquad \Rightarrow \qquad \text{DH (\%)} = \frac{0{,}9575 \times 100}{\frac{36{,}62}{6{,}25}} = \underline{16{,}78\ \%}$$

Table D.11: Results from formol titration of snow crab CM, batch 1 and 2. Abbreviations are a.gr = amino groups, Av. = average, DH = degree of hydrolysis, NA = not available.

Batch	Nr	Sample (g)	NaOH used (mL)	Free a.gr % (g/100 g RRM, w/ww)	Free a.gr % (w/dw)	Av.	SD	p-value	DH	Av.	SD
B1	A	2,0076	9,6840	0,6757	2,3468	2,2138	0,1814	0,0014	NA		
	B	1,8010	7,4300	0,5779	2,0071				NA		
	C	1,7420	8,1900	0,6585	2,2874				NA		
B2	A	1,6100	3,6000	0,3132	0,9575	0,9836	0,1084		16,3419	16,7875	1,8504
	B	1,8209	4,6890	0,3607	1,1027				18,8200		
	C	1,6905	3,5160	0,2913	0,8906				15,2006		

7. Astaxanthin content

The astaxanthin content in the crude material was determined after Tolasa et al. (2005), on lipid samples extracted using Bligh and Dyer (1959), methods are described in chapter 2. Results are presented in **Table D.12**. The absorbance of lipid samples diluted in n-hexane was used in equation VII to calculate µg astaxanthin per gram lipid. *A* is absorbance of the sample, *E* is a factor 2100 - the standard absorbance of 1% (w/v) astaxanthin solution at 472 nm in a 1 cm cyvette, *10000* is scaling factor to obtain result in µg/g lipid dissolved in solute, and *C* is the concentration of lipid in n-hexane (Tolasa, Cakli et al. 2005)

$$\text{Astaxanthin } (\mu g/g \text{ lipid}) = \frac{A}{E} \times 10000 \times \frac{1}{C} \qquad \text{VII}$$

Amount of astaxanthin as µg per gram RRM was found by multiplying µg/g lipid with the amount of lipid per 100 g RRM (batch 1=4,21%; batch 2=1,83%, w/ww), and dividing on 100 g, as shown for sample A from batch 1 below.

$$\text{Astaxanthin } (\mu g/g \text{ RRM}) = \frac{386{,}6748 \times 4{,}21}{100} \quad \left(\frac{\frac{\mu g}{g\text{ lipid}} \times \frac{g\text{ lipid}}{100\text{ g RRM}}}{100\text{ g RRM}}\right) = 16{,}2790\ \mu g/g \text{ RRM (w/ww)}$$

A t-test was performed in Excel to determine whether the amount of astaxanthin was statistically significantly different between batch 1 and 2. .

Table D.12: Data from determination of astaxanthin in rest raw material of batch 1 and 2 by spectrophotometry.

Batch	Nr	Lipid sample (g)	n-hexane (mL)	Abs	Astaxanthin µg/g lipid	µg/100 g RRM (w/ww)	µg/g (w/ww)	Average µg/g (w/ww)	SD	µg/g w/dw	Average µg/g (w/dw)	SD	p-value
B1	A	0,0699	3	1,892	386,6748	1627,9011	16,2790	16,36	0,12	56,5440	56,8288	0,4028	0,0021
	B	0,0203	3	0,555	390,5700	1644,2998	16,4430			57,1136			
B2	A	0,1908	5	4,505	562,1693	1028,7698	10,2877	11,79	0,88	31,4512	33,5243	2,2122	
	B	0,1815	5	4,533	594,6478	1088,2054	10,8821			33,2683			
	C	0,1699	5	4,573	640,8532	1172,7613	11,7276			35,8533			

E. Enzymatic hydrolysis data

Fractions obtained
This appendix includes quantitative and qualitative results from fractions obtained in experiments with enzymatic hydrolysis using the proteases Alcalase® 2.4 L, Protamex®, Papain GSM80 and Corolase® 2TS (**Table E.1 – Table E.5**). Equation VIII were used to calculate fractions (%).

Enzymatic hydrolysis using Alcalase ® 2.4 L as proteolytic agent (EH^{AL}) was performed on batch 1 and 2, as described in chapter 2.4. The fractions obtained were sludge fraction (SF), water fraction (WF). The mass of these was measured (g), and the fraction (%, g/100 g, w/ww) was calculated based on total hydrolysis mixture, equation VIII. The average % SF and % WF are based on 6 parallels for enzyme treated samples, and 3 parallels for control samples. In the experiment with AL on batch 1 the hydrolysis mixture was centrifuged twice, with separation of sludge and water in between. This generated 2 sludge fractions, SF1 and SF2. These are merged in the table as SF1+2, and SF2 is described in % of the total sludge fraction obtained. This is further discussed in chapter 3.3.1. Values of total SF and WF fractions in grams are included as these were merged after the experiment. Finally, loss of hydrolysis mixture material is calculated based on total hydrolyse mixture and the resulting SF and WF fractions obtained.

$$\text{Fraction (\%)} = \frac{fraction\ (g)}{total\ hydrolysis\ sample\ (g)} \qquad \text{VIII}$$

Table E.1: Results from enzymatic hydrolysis of snow crab RRM from batch 1. The table describes sludge- and water fractions obtained using Alcalase as proteolytic agent. The hydrolysis mixture was centrifuged twice after end of hydrolysis, and the second sludge fraction (SF2) obtained is by convenience described as % of the total sludge fraction obtained. Loss of material during the experiment is described as negative values (g), and for replica 1 oil fractions described below is included in this value. Abbreviations are rep. = replica, EZ = enzyme, NO = control, SF = sludge fraction, WF = water fraction.

	Rep.	Nr	Sample (g)	EZ (g)	Water (mL)	Tot. hydrolysis mix (g)	SF1+2 (g)	SF 2 (%)of tot. hydr.mix	Tot. SF (g)	SF (%)	Av. SF (%)	SD	WF (g)	Tot. WF (g)	WF (%)of tot. hydr.mix	Av. WF (%)	SD	Loss (g)
BATCH 1 | ALCALASE® 2.4 L	1	A	30,0011	0,0300	15	270,4885	21,8737	4,3875	125,7636	48,5747	46,4942	2,7092	22,4594	142,6106	49,8753	52,7239	2,7747	-2,1143
		B	30,0151	0,0300	15,01		19,4042	3,6121		43,0677			25,2017		55,9353			
		C	30,1429	0,0301	15,07		21,4740	2,2939		47,4637			23,5563		52,0661			
		D	30,025	0,0300	15,0125		22,1390	2,8222		49,1241			22,6070		50,1625			
		E	30,0077	0,0300	15		19,4136	3,7577		43,1052			25,3440		56,2728			
		F	30,00402	0,0300	15,02		21,4591	8,9612		47,6297			23,4422		52,0313			
	1 NO	A	30,0041	-	15	135,0611	20,7157	4,6805	62,7512	46,0307	46,4612	0,4698	23,9290	71,6616	53,1707	53,0588	0,2260	-0,6483
		B	30,0351	-	15,02		21,1589	2,4765		46,9623			23,7885		52,7987			
		C	30,0019	-	15		20,8766	5,5521		46,3905			23,9441		53,2069			

Table E.2: Results from enzymatic hydrolysis of batch 2 using Alcalase® 2.4 L as proteolytic agent. Abbreviations are EZ = enzyme, rep. = replica, NO = control, SF = sludge fraction, WF = water fraction, av. = average, tot. = total.

	Rep.	Nr	Sample (g)	EZ (g)	Water (mL)	Total hydrolysis mix (g)	SF (g)	Total SF	SF (%) of tot. hydr.mix	Av. SF (%)	SD	WF (g)	Total WF (g)	WF (%)	Av. WF (%) of tot. hydr.mix	SD	Tot. WF +tot. SF (g)	Loss (g)
BATCH 2 | ALCALASE® 2.4	**1**	A	30,1343	0,0301	15,0672	275,2381	25,1060	151,1779	55,5055	52,2388	3,6403	19,9419	121,6619	44,0884	46,7274	3,9029	272,8398	-2,3983
		B	30,8174	0,0308	15,4087		25,0571		54,1694			20,6976		44,7449				
		C	30,1896	0,0302	15,0948		22,4685		49,5834			22,4999		49,6527				
		D	30,4911	0,0305	15,2456		26,5453		58,0008			18,7279		40,9200				
		E	31,0324	0,0310	15,5162		26,4722		56,8321			19,6829		42,2565				
		F	30,7050	0,0307	15,3525		25,5288		55,3912			20,1117		43,6374				
	2	A	30,9823	0,0310	15,4912	274,5863	25,5944	148,9546	55,0365			20,0543	121,5270	43,1234			270,4816	-4,1047
		B	30,1830	0,0302	15,0915		23,5613		52,0063			20,7560		45,8142				

	C	30,6778	0,0307	15,3389		24,9909		54,2721			20,6989		44,9513				
	D	30,8898	0,0309	15,4449		24,6019		53,0607			21,0433		45,3856				
	E	30,1359	0,0301	15,0680		25,3850		56,1193			19,3247		42,7216				
	F	30,0668	0,0301	15,0334		24,8211		54,9988			19,6498		43,5402				
3	A	30,5173	0,0305	15,2587	272,8497	22,6123	129,4508	49,3649			23,1227	141,5361	50,4791			270,9869	-1,8628
	B	30,0576	0,0301	15,0288		22,3742		49,5921			22,2920		49,4099				
	C	30,1588	0,0302	15,0794		22,0004		48,6000			23,0267		50,8671				
	D	30,7097	0,0307	15,3549		22,1153		47,9774			23,6323		51,2684				
	E	30,1055	0,0301	15,0528		20,5626		45,5042			24,3542		53,8949				
	F	30,2297	0,0302	15,1149		19,7860		43,6057			25,1082		55,3351				
3	A	29,2387	0,0292	14,6190	221,1119	23,0582	115,8143	52,5400			18,7251	102,8846	42,6667			218,6989	-2,4130
	B	29,8797	0,0299	14,9340		23,6338		52,7028			21,0918		47,0342				
	C	29,2654	0,0293	14,6364		22,4211		51,0370			21,4272		48,7746				
	D	29,5994	0,0296	14,7817		23,8402		53,6812			20,4759		46,1058				
	E	29,3159	0,0293	14,6944		22,8610		51,9101			21,1646		48,0581				
1 NO	A	31,5519	-	15,7760	140,0627	22,2686	68,2794	47,0518	47,0297	4,1062	24,7800	71,6411	52,3582	52,4122	4,0695	139,9205	-0,1421
	B	30,1887	-	15,0944		23,9711		52,9361			21,8694		48,2949				
	C	31,6345	-	15,8173		22,0397		46,4465			24,9917		52,6676				
2 NO	A	29,9962	-	14,9981	137,2298	23,0973	70,0045	51,3338			21,2519	65,3111	47,2324			135,3156	-1,9142
	B	30,1798	-	15,0899		24,2029		53,4638			20,5601		45,4169				
	C	31,3105	-	15,6553		22,7043		48,3422			23,4991		50,0345				
3 NO	A	30,5758	-	15,2879	138,2295	19,1235	58,0826	41,6964			26,4731	79,3005	57,7212			137,3831	-0,8464
	B	30,8164	-	15,4082		19,7987		42,8315			26,1363		56,5420				
	C	30,7608	-	15,3804		19,1604		41,5256			26,6911		57,8466				
4 NO	A	28,5713	-	14,3786	130,6700	19,1241	60,3975	44,5265			23,4192	70,0774	54,5268			130,4749	-0,1951
	B	30,0656	-	15,0566		20,4740		45,3746			24,5711		54,4546				
	C	28,3177	-	14,2802		20,7994		48,8273			22,0871		51,8502				

Table E.3: Results from enzymatic hydrolysis of batch 2 using Protamex® as proteolytic agent. Abbreviations are rep. = replica, EZ = enzyme, NO = control, SF = sludge fraction, WF = water fraction, av. = average, tot. = total.

PROTAMEX®

Rep.	Nr	Sample (g)	EZ (g)	Water (mL)	Total hydrolysis mix (g)	SF (g)	Total SF	SF (%)	Av. SF (%)of tot. hydr.mix	SD	WF (g)	Total WF (g)	WF (%)of tot. hydr.mix	Av. WF (%)	SD	Tot WF + tot SF (g)	Loss (g)
1	A	32,3290	0,0323	16,1645	281,0505	25,6378	149,5584	52,8333	52,3880	1,9942	22,4729	132,6311	46,3112	46,8529	1,4131	282,1895	1,1390
	B	31,9055	0,0319	15,9528		25,2273		52,6774			22,3890		46,7507				
	C	29,4321	0,0294	14,7161		23,1330		52,3637			20,8308		47,1524				
	D	31,2614	0,0313	15,6307		25,1930		53,6897			22,0985		47,0949				
	E	31,3383	0,0313	15,6692		26,9041		57,1956			22,6064		48,0591				
	F	30,9759	0,0310	15,4880		23,4632		50,4641			22,2335		47,8193				
2	A	29,7682	0,0298	14,8841	269,8032	23,5725	143,8645	52,7561			20,6321	122,8624	46,1753			266,7269	-3,0763
	B	29,9084	0,0299	14,9542		24,3749		54,2961			19,5994		43,6585				
	C	29,4441	0,0294	14,7221		23,0810		52,2247			20,6056		46,6237				
	D	29,8846	0,0299	14,9423		23,3829		52,1279			20,9765		46,7633				
	E	30,1455	0,0301	15,0728		24,8012		54,8112			20,1681		44,5720				
	F	30,5982	0,0306	15,2991		24,6520		53,6754			20,8807		45,4641				
3	A	29,9976	0,0300	14,9988	276,7389	22,7854	140,0856	50,6045			21,7682	132,3803	48,3454			272,4659	-4,2730
	B	31,8134	0,0318	15,9067		24,1546		50,5835			22,8264		47,8021				
	C	30,0442	0,0300	15,0221		23,8987		52,9948			20,6570		45,8064				
	D	31,1894	0,0312	15,5947		23,1005		49,3439			22,2682		47,5661				
	E	29,3003	0,0293	14,6502		22,4476		51,0408			21,0058		47,7624				
	F	32,0248	0,0320	16,0124		23,6988		49,3014			23,8547		49,6257				
1 NO	A	29,6168	-	14,8084	137,0478	22,2298	69,4441	50,0387	50,6547	1,6266	21,8517	65,7892	49,1876	48,2825	1,6908	135,2333	-1,8145
	B	29,7492	-	14,8746		23,8679		53,4869			20,1516		45,1589				
	C	31,9992	-	15,9996		23,3464		48,6395			23,7859		49,5552				
2 NO	A	30,5125	-	15,2563	137,0717	23,6398	69,2457	51,6505			21,7985	66,7070	47,6275			135,9527	-1,1189
	B	30,4522	-	15,2261		22,4017		49,0423			22,9135		50,1628				
	C	30,4164	-	15,2082		23,2042		50,8590			21,9950		48,2086				
3 NO	A	30,2381	-	15,1191	135,1857	22,2069	68,5585	48,9601			22,7652	65,1969	50,1910			133,7554	-1,4303

	B	29,4284	-	14,7142	22,9742	52,0454	20,5626	46,5822
	C	30,4573	-	15,2287	23,3774	51,1698	21,8691	47,8683

Table E.4: Results from enzymatic hydrolysis of batch 2 using Papain GSM80 as proteolytic agent. Abbreviations are rep. = replica, EZ = enzyme, NO = control, SF = sludge fraction, WF = water fraction, av. = average, tot. = total.

PAPAIN GSM80

Rep.	Nr	Sample (g)	EZ (g)	Water (mL)	Tot. hydrolysis mix (g)	SF (g)	Tot. SF	SF (%)of tot. hydr.mix	Av. SF (%)	SD	WF (g)	Tot. WF (g)	WF (%)of tot. hydr.mix	Av. WF (%)	SD	Tot. WF +tot SF (g)	Loss (g)
1	A	29,3806	0,0294	14,6903	269,3621	25,0309	149,9556	56,7590	53,8021	2,2675	18,4656	115,4385	41,8718	45,0047	2,5519	265,3941	-3,9680
	B	30,5943	0,0306	15,2972		26,4104		57,5114			18,8538		41,0561				
	C	29,7599	0,0298	14,8800		23,8941		53,4907			20,0762		44,9438				
	D	30,1475	0,0301	15,0738		25,1487		55,5755			19,4431		42,9668				
	E	30,0349	0,0300	15,0175		24,7460		54,8906			19,7720		43,8575				
	F	29,5379	0,0295	14,7690		24,7255		55,7680			18,8278		42,4658				
2	A	30,0112	0,0300	15,0056	268,4511	23,4146	146,5145	51,9784			21,2211	119,7922	47,1090			266,3067	-2,1444
	B	28,8312	0,0288	14,4156		22,5275		52,0559			21,3351		49,3005				
	C	30,4595	0,0305	15,2298		25,7661		56,3567			19,4358		42,5108				
	D	29,2213	0,0292	14,6107		24,4265		55,6905			18,8289		42,9284				
	E	29,2147	0,0292	14,6074		24,3371		55,4992			18,8594		43,0077				
	F	31,1103	0,0311	15,5552		26,0427		55,7701			20,1119		43,0693				
3	A	30,1576	0,0302	15,0788	271,1927	25,0205	146,8438	55,2737			19,5481	120,3103	43,1844			267,1541	-4,0386
	B	29,9437	0,0299	14,9719		24,5708		54,6680			19,7883		44,0273				
	C	29,5102	0,0295	14,7551		23,7247		53,5609			20,1002		45,3782				
	D	30,8168	0,0308	15,4084		24,9186		53,8710			20,6886		44,7263				
	E	30,6513	0,0307	15,3257		24,6852		53,6546			20,3501		44,2320				
	F	29,5951	0,0296	14,7976		23,9240		53,8559			19,8350		44,6511				
4	A	29,8340	0,0298	14,9144	134,7151	22,2886	68,2211	49,7755			21,7852	65,0988	48,6513			133,3199	-1,3952
	B	29,9960	0,0300	15,2421	138,4100	22,4721	70,6820	49,6422			22,2842	66,3240	49,2272			137,0060	-1,4040
	C	29,2802	0,0293	15,0772		22,1267		49,8499			21,9145		49,3718				
	D	30,5196	0,0305	15,1753		23,2957		50,9469			22,0219		48,1612				
	E	30,1003	0,0301	15,4198		23,8058		52,2628			21,3991		46,9792				
	F	31,7212	0,0317	15,6636		24,9142		52,5433			22,0179		46,4351				
1 NO	A	29,9788	-	14,9894	136,7543	23,6285	73,0068	52,5449	53,1751	1,4499	20,6929	62,1329	46,0167	45,7524	1,4968	135,1397	-1,6146
	B	30,8585	-	15,4293		24,5335		53,0021			21,2468		45,9016				

	C	30,3322	-	15,1661		24,8448		54,6060	20,1932		44,3823		
2 NO	A	30,4206	-	15,2103	135,3251	24,4902	73,0121	53,6702	20,7789	61,1348	45,5369	135,1397	-0,1854
	B	30,3248	-	15,1624		24,2602		53,3341	20,7787		45,6803		
	C	29,4713	-	14,7357		24,2617		54,8821	19,5772		44,2853		
3 NO	A	30,0441	-	15,0221	133,8402	24,0160	71,7439	53,2906	20,4459	60,1813	45,3686	134,1469	0,3067
	B	29,9139	-	14,9570		23,4571		52,2769	20,8836		46,5416		
	C	29,2688	-	14,6344		24,2708		55,2825	18,8518		42,9395		
4 NO	A	30,2023	-	15,5550	134,0486	23,2140	69,3219	50,7329	22,0414	63,6397	48,1702	131,9252	-2,1234
	B	29,5272	-	14,1227		23,4564		53,7376	20,0926		46,0313		
	C	29,9331	-	14,7083		22,6515		50,7410	21,5057		48,1743		

Table E.5: Results from enzymatic hydrolysis of batch 2 using Corolase® 2TS as proteolytic agent. Abbreviations are rep. = replica, EZ = enzyme, NO = control, SF = sludge fraction, WF = water fraction, av. = average, tot. = total.

COROLASE® 2TS

Rep.	Nr	Sample (g)	EZ g	Water (mL)	Tot. hydrolysis mix (g)	SF (g)	Tot. SF	SF (%) of tot. hydr.mix	Av. SF (%)	SD	WF (g)	Tot. WF (g)	WF (%) of tot. hydr.mix	Av. WF (%)	SD	Tot. WF +tot SF (g)	Loss (g)
1	A	29,7325	0,0297	14,7695	268,0707	22,5023	133,5164	50,5309	49,7297	1,3644	21,8784	134,1012	49,1299	49,7944	2,0144	267,6176	-0,4531
	B	30,0085	0,0300	15,1342		22,2380		49,2288			22,9183		50,7348				
	C	30,4241	0,0304	15,3268		22,4630		49,0659			23,1742		50,6193				
	D	29,5165	0,0295	14,8857		21,9242		49,3436			22,3732		50,3541				
	E	29,2521	0,0293	14,7749		21,7840		49,4459			22,3799		50,7985				
	F	29,4078	0,0294	14,6598		22,6049		51,2618			21,3772		48,4777				
2	A	29,1519	0,0292	15,2471	264,9277	22,2086	131,9555	49,9877			22,1086	130,2524	49,7626			262,2079	-2,7198
	B	29,8397	0,0298	15,1007		22,1274		49,2045			22,6734		50,4187				
	C	28,7017	0,0287	14,2847		21,4779		49,9311			21,4362		49,8341				
	D	29,5722	0,0296	14,5397		22,5844		51,1637			19,4659		44,0989				
	E	29,2761	0,0293	14,6952		21,3908		48,6148			22,5054		51,1480				
	F	29,8145	0,0298	14,5278		22,1664		49,9557			22,0629		49,7224				
3	A	20,2018	0,0202	10,2883	161,9602	16,2665	79,6127	53,3148			14,2145	82,1578	46,5892			161,7705	-0,1897
	B	28,1005	0,0281	14,8502		20,8133		48,4269			22,1549		51,5484				
	C	27,3565	0,0274	14,4964		20,3351		48,5553			21,5208		51,3865				
	D	30,6420	0,0306	15,9182		22,1978		47,6441			24,2676		52,0866				
4	A	29,7510	0,0298	14,9687	272,0186	22,5163	133,1476	50,3164	49,6720	2,1276	21,8510	130,6667	48,8296	47,9732	4,3444	263,8143	-8,2043
	B	29,7908	0,0298	14,6953		22,7034		51,0007			22,3525		50,2124				
	C	31,0197	0,0310	15,2000		21,6919		46,9007			21,2804		46,0110				
	D	29,8983	0,0299	14,8306		21,7080		48,5000			24,5804		54,9175				
	E	30,1532	0,0302	15,5271		22,6827		49,6226			16,7091		36,5542				
	F	30,2920	0,0303	15,7110		21,8453		47,4554			23,8933		51,9044				
5	A	28,9310	0,0289	14,8964	132,1244	20,4337	63,7256	46,5924			22,8307	66,8442	52,0579			130,5698	-1,5546
	B	28,2530	0,0283	14,3452		21,6297		50,7424			20,4172		47,8980				
	C	30,4112	0,0304	15,2000		21,6622		47,4615			23,5963		51,6991				
6	A	20,1908	0,0202	10,2027	179,8217	15,3975	91,7426	50,6269			14,5195	83,8544	47,7400			175,5970	-4,2247

	B	19,3906	0,0194	9,2625		15,2656		53,2413			12,7885		44,6020				
	C	19,9194	0,0199	9,1734		15,5280		53,3375			12,8133		44,0127				
	D	19,1610	0,0192	9,8547		14,9477		51,4819			13,6399		46,9777				
	E	20,0187	0,0200	10,9504		15,2392		49,1760			15,2029		49,0588				
	F	20,3084	0,0203	11,2701		15,3646		48,6240			14,8903		47,1230				
1 NO	A	29,6238	-	14,7515	134,5475	21,9736	66,1629	49,5176	49,6082	1,2178	22,2790	68,0601	50,2059	50,1434	1,2602	134,2230	-0,3245
	B	29,4569	-	14,4631		21,4152		48,7596			22,4767		51,1765				
	C	30,8323	-	15,4199		22,7741		49,2390			23,3044		50,3855				
2 NO	A	31,9767	-	16,2892	136,3299	23,6235	67,9849	48,9445			24,4497	67,9423	50,6563			135,9272	-0,4027
	B	29,4098	-	14,9324		21,9381		49,4745			22,2497		50,1773				
	C	28,5850	-	15,1368		22,4233		51,2863			21,2429		48,5865				
3 NO	A	30,6897	-	15,3663	93,0598	23,7537	46,3476	51,5757			22,1421	46,5291	48,0765			92,8767	-0,1831
	B	31,8712	-	15,1326		22,5939		48,0682			24,3870		51,8830				
4 NO	A	30,8723	-	15,7203	135,3267	24,9224	73,1144	53,4900	53,8725	0,6178	21,6117	61,6076	46,3844	44,6480	1,2548	134,7220	-0,6047
	B	29,6322	-	14,5868		24,0261		54,3343			20,0416		45,3235				
	C	29,3320	-	15,1831		24,1659		54,2870			19,9543		44,8259				
5 NO	A	28,7199	-	14,2842	129,6284	22,9831	69,6083	53,4440			19,5214	58,4248	45,3943			128,0331	-1,5953
	B	28,0579	-	14,5772		22,6689		53,1696			19,2956		45,2575				
	C	29,3261	-	14,6631		23,9563		54,4596			19,6078		44,5742				
6 NO	A	20,3799	-	10,4302	93,5263	16,8942	50,3993	54,8333			13,3413	40,5676	43,3017			90,9669	-2,5594
	B	20,9729	-	9,7148		16,3051		53,1324			12,9284		42,1289				
	C	20,5940	-	11,4345		17,2000		53,7022			14,2979		44,6412				

F. Dry weight distribution and recovery of ash in hydrolytic fractions

Dry weight and ash content of the fractions obatined by proteolytic treatment was determined by heating at 105°C and 550°C for 24 hours, respectively. The results are presented in **Table F.1- Table F.4**, for each protease. Tables are organized after fraction and treated/control samples within every table. Based on average fraction constituents SF and WF of total hydrolysis mixture, the amount (%) of ashrecovered from the total ash in RRM sample has been calculated. As several of the fractions are analysed for dry weight and ash after merging of parallels, the average recovery of ash in each fraction was based on known average size of fractions (%) and the general starting mass of RRM (30g). Before enzymatic treatment, the RRM sample was added 15 g water, thus the hydrolysis mix had a total mass of 45 g. The mass of added enzyme (0,03 g) is neglected here. The amount of ash in such a sample was 4,31 g for batch 1, and 3,19 g for batch 2 (14,38 % and 10,64% (w/ww), respectively). Example calculations are shown for Alcalase (batch 1), SF1, replica 1, sample nr A, where fraction (%) = 46,49 %, and fraction (g) = 0,4649 × 45 g = 20,92 g

Ash in fraction (g) = (11,0024/100) × 20,92 g = 2,30 g ⟹ Ash recovered from RRM (%) = $\frac{2,30\ g}{4,31\ g} \times 100\%$ = 53,36 %

Table F.1: Dry weight and ash determination results for fractions obtained by enzymatic hydrolysis of snow crab RRM using Alcalase as proteolytic agent. Results for batch 1 and 2 are separated by bold line. Water fraction from AL(B1) was not analyzed. Samples designated with letters are individual parallels, while samples designated I, II etc. are samples from merged parallels from the given replica. Abbreviations are SF1 = sludge fraction 1, SF2 = sludge fraction 2, WF = water fraction, Fr. = fraction, Av. = average, NA = not analysed.

	Fr.	Rep	Nr	Ww (g)	Dw (g)	dw/ww %	Av. Dw/ww %	SD	Ash (g)	Ash % w/ww	Av. Ash % w/ww)	SD	Recovery ash (%) from RRM	Av. Recovery (%)	SD recovery
BATCH 1 | ALCALASE	SF 1	1	A	0,9507	0,3205	33,7120	31,1426	4,3115	0,1046	11,0024	9,1962	2,1989	53,3604	44,6003	10,6642
			B	0,6967	0,2406	34,5342			0,0608	8,7269			42,3242		
			C	0,9517	0,2471	25,9641			0,0628	6,5987			32,0030		
			D	1,3193	0,3545	26,8703			0,1039	7,8754			38,1947		
			E	1,4834	0,5389	36,3287			0,1868	12,5927			61,0730		
			F	1,0154	0,299	29,4465			0,0851	8,3809			40,6465		
	SF 1	1 NO	A	0,6231	0,1968	31,5840	32,0938	1,4316	0,0519	8,3293	9,2561	1,3107	40,3675	44,8591	6,3521
			B	0,7955	0,2465	30,9868			NA	NA					
			C	0,6452	0,2175	33,7105			0,0657	10,1828			49,3507		
	SF 2 Control, no data	1	A	22,4	4,5	20,0893	23,8001	3,6713	NA	NA	NA	NA	NA		
			D	13,4	3,2	23,8806			NA	NA			NA		
			F	57,6	15,8	27,4306			NA	NA			NA		

SF	1	A	2,7665	0,7791	28,1619	29,9539	2,2366	0,2247	8,1222	9,2041	0,9579	59,7926	67,7570	7,0521
		B	2,5431	0,7646	30,0657			0,2313	9,0952			66,9557		
		C	2,7336	0,7505	27,4546			0,1968	7,1993			52,9987		
		D	3,4255	0,9218	26,9099			0,2736	7,9872			58,7986		
		E	3,1015	0,8428	27,1739			0,2751	8,8699			65,2971		
		F	3,4121	1,0095	29,5859			0,3180	9,3198			68,6089		
	2	A	2,6444	0,7274	27,5072			0,2355	8,9056			65,5600		
		B	2,6501	0,8269	31,2026			0,2649	9,9958			73,5859		
		C	3,6158	1,0689	29,5619			0,3377	9,3396			68,7546		
		D	3,2478	0,945	29,0966			0,2956	9,1015			67,0024		
		E	5,4593	1,6354	29,9562			0,5725	10,4867			77,1994		
		F	3,5085	1,0077	28,7217			0,3142	8,9554			65,9265		
	3	A	2,774	0,8654	31,1968			0,2402	8,6590			63,7443		
		B	2,7838	0,9411	33,8063			0,3030	10,8844			80,1272		
		C	2,3277	0,7057	30,3175			0,2117	9,0948			66,9528		
		D	2,657	0,8148	30,6662			0,2398	9,0252			66,4405		
		E	2,3686	0,7917	33,4248			0,2584	10,9094			80,3112		
		F	2,4141	0,8295	34,3606			0,2347	9,7220			71,5703		
	1 NO	A	2,7148	0,8744	32,2086	31,7593	2,0708	0,2509	9,2419	9,7323	1,8145	61,2516	64,5018	12,0254
		B	2,7884	0,9221	33,0691			0,2773	9,9448			65,9097		
		C	2,9493	0,9266	31,4176			0,2645	8,9682			59,4376		
	2 NO	A	4,0191	1,3695	34,0748			0,5402	13,4408			89,0800		
		B	4,1666	1,322	31,7285			0,4658	11,1794			74,0921		
		C	3,0623	0,8434	27,5414			0,2304	7,5238			49,8642		
	3 NO	A	2,0818	0,6571	31,5640			0,1783	8,5647			56,7632		
		B	3,2352	0,971	30,0136			0,2626	8,1170			53,7958		
		C	2,7765	0,95	34,2157			0,2946	10,6105			70,3217		
WF	4	I	2,5447	0,1587	6,2365	6,7065	0,6647	0,0345	1,3558	1,4688	0,1598	8,9276	9,6719	1,0526
		II	2,2253	0,1597	7,1766			0,0352	1,5818			10,4162		
	4 NO	I	2,0529	0,1172	5,7090	5,7049	0,0058	0,0301	1,4662	1,4740	0,0110	10,8296	10,8873	0,0816
		II	2,6926	0,1535	5,7008			0,0399	1,4818			10,9450		

Table F.2: Dry weight and ash determination (w/ww) results for fractions obtained by enzymatic hydrolysis of snow crab RRM using Protamex as proteolytic agent. Samples designated with letters are individual parallels, while samples designated I, II etc. are samples from merged parallels from the given replica. Abbreviations are SF = sludge fraction, WF = water fraction, Fr. = fraction, Av. = average.

PROTAMEX

Fr.	Rep	Nr	Ww (g)	Dw (g)	dw/ww %	Av. Dw/ww %	SD	Ash (g)	Ash % w/ww	Av. Ash % w/ww)	SD	Recovery ash (%)	Av. Recovery (%)	SD recovery
SF	1	A	2,7755	0,8377	30,1819	30,3014	1,8088	0,2672	9,6271	10,3858	1,1233	71,0738	76,6752	8,2928
		B	5,3275	1,6716	31,3768			0,6061	11,3768			83,9914		
		C	2,5688	0,7789	30,3216			0,2401	9,3468			69,0043		
	2	A	5,7129	1,6872	29,5332			0,6165	10,7914			79,6692		
		B	4,9160	1,3781	28,0330			NA	NA			NA		
		C	3,4533	0,9880	28,6103			0,3299	9,5532			70,5281		
	3	A	4,9025	1,4919	30,4314			0,4763	9,7155			71,7261		
		B	5,0300	NA	NA			NA	NA			NA		
		C	3,6550	1,2399	33,9234			0,4492	12,2900			90,7333		
	1 NO	A	3,1327	0,9987	31,8798	32,2200	0,9056	0,3371	10,7607	10,9345	0,6053	76,8143	78,0551	4,3206
		B	2,8935	0,9174	31,7055			0,2984	10,3128			73,6169		
		C	3,1441	NA	NA			NA	NA			NA		
	2 NO	A	3,8811	1,1972	30,8469			0,4110	10,5898			75,5943		
		B	2,6262	0,8288	31,5589			0,2761	10,5133			75,0483		
		C	4,3745	1,4587	33,3455			0,5352	12,2345			87,3353		
	3 NO	A	3,2022	1,0295	32,1498			0,3461	10,8082			77,1534		
		B	2,6094	0,8593	32,9309			0,2855	10,9412			78,1030		
		C	2,4347	0,8118	33,3429			0,2755	11,3156			80,7752		
WF	1	A	1,9473	0,1375	7,0611	6,9313	0,0771	0,0326	1,6741	1,5783	0,0496	11,0536	10,4210	0,3275
		B	2,3108	0,1595	6,9024			0,0357	1,5449			10,2006		
	2	A	2,6544	0,1811	6,8226			0,0418	1,5747			10,3975		
		B	2,1230	0,1471	6,9289			0,0328	1,5450			10,2010		
	3	A	2,8062	0,1949	6,9453			0,0444	1,5822			10,4468		
		B	2,4728	0,1713	6,9274			0,0383	1,5489			10,2265		
	1 NO	A	2,4267	0,1506	6,2060	6,5500	0,5450	0,0363	1,4959	1,5275	0,1119	10,1780	10,3932	0,7612
		B	1,9939	0,1243	6,2340			0,0301	1,5096			10,2715		

2 NO	A	2,4371	0,1844	7,5664	0,0423	1,7357	11,8097
	B	2,1824	0,1406	6,4424	0,0310	1,4205	9,6649
3 NO	A	2,3578	0,1588	6,7351	0,0366	1,5523	10,5620
	B	2,4328	0,1488	6,1164	0,0353	1,4510	9,8728

Table F.3: Dry weight and ash determination (w/ww) results for fractions obtained by enzymatic hydrolysis of snow crab RRM using Papain GSM80 as proteolytic agent. Number designation nr I, II etc. are samples from merged parallels from the given replica. Replicas treated at different temperatures are marked with the given temperature in superscript. Abbreviations are SF = sludge fraction, WF = water fraction, Fr. = fraction, Av. = average, ND = no data.

PAPAIN

Fr.	Rep	Nr	Ww (g)	Dw (g)	dw/ww %	Av. Dw/ww %	SD	Ash (g)	Ash % w/ww	Av. Ash % w/ww)	SD	Recovery ash (%)	Av. Recovery (%)	SD
SF	1	A	5,7468	1,6768	29,1780	26,7649	2,3484	0,5217	9,0781	8,2055	1,0816	68,8298	62,2135	8,2005
		B	5,3614	1,452	27,0825			0,4647	8,6675			65,7168		
		C	5,7248	1,6966	29,6360			0,5315	9,2842			70,3922		
	2	A	4,6404	1,2998	28,0105			ND	ND			ND		
		B	5,7653	1,7659	30,6298			0,5771	10,0099			75,8946		
		C	4,6345	1,39	29,9924			0,4530	9,7745			74,1100		
	3	A	5,4788	3,4048	ND			ND	ND			ND		
		B	5,2477	1,2666	24,1363			0,3723	7,0945			53,7905		
		C	5,6193	1,4231	25,3252			0,4268	7,5953			57,5869		
	4	A	3,8695	1,0282	26,5719			0,2988	7,7219			58,5474		
		B	2,9261	0,7476	25,5494			0,2378	8,1269			61,6175		
		C	3,1264	0,7637	24,4275			0,2480	7,9324			60,1435		
		D	2,5833	0,6077	23,5242			0,1715	6,6388			50,3351		
		E	3,8085	0,9638	25,3066			0,2983	7,8325			59,3856		
		F	3,928	0,9953	25,3386			0,2716	6,9145			52,4252		
	1 NO	A	5,0161	1,5369	30,6393	29,0203	3,6179	0,4483	8,9372	9,1780	2,0467	66,9720	68,7761	15,3375
		B	2,7314	0,8634	31,6102			ND	ND			ND		
		C	3,8473	1,1301	29,3738			0,3374	8,7698			65,7173		
	2 NO	A	5,7289	1,7136	29,9115			0,5404	9,4329			70,6862		
		B	2,7406	0,8186	29,8694			0,2484	9,0637			67,9198		
		C	3,7608	1,1122	29,5735			0,3400	9,0406			67,7469		
	3 NO	A	4,9889	1,489	29,8463			0,4934	9,8900			74,1114		
		B	2,8626	0,8813	30,7867			0,2740	9,5717			71,7266		
		C	3,1202	1,0575	33,8921			0,4311	13,8164			103,5347		
	4 NO	A	3,8131	1,0965	28,7561			0,3785	9,9263			74,3838		

		B	3,616	0,8595	23,7694			0,2599	7,1875			53,8602		
		C	2,4917	0,5037	20,2151			0,1326	5,3217			39,8784		
WF	1	I	4,5854	0,3156	6,8827	6,9417	0,1367	0,0679	1,4808	1,4642	0,0412	9,3914	9,2864	0,2612
		II	4,1961	0,2882	6,8683			0,0619	1,4752			9,3559		
	2	I	5,1399	0,3531	6,8698			0,0774	1,5059			9,5505		
		II	4,418	0,3038	6,8764			0,0647	1,4645			9,2879		
	3	I	4,7209	0,3261	6,9076			0,0695	1,4722			9,3368		
		II	5,1677	0,356	6,8889			ND	ND			ND		
		B	6,0068	0,4315	7,1835			0,0883	1,4700			9,3230		
		C	6,2058	0,4454	7,1772			0,0921	1,4841			9,4124		
		D	6,2289	0,4217	6,7701			0,0907	1,4561			9,2350		
		E	5,8822	ND	ND			0,0796	1,3532			8,5825		
		F	5,4245	0,3793	6,9923			0,0803	1,4803			9,3885		
	1 NO	I	5,173	0,3379	6,5320	6,5171	0,1478	0,0768	1,4846	1,4728	0,0262	9,5723	9,4962	0,1688
		II	4,3847	0,286	6,5227			0,0637	1,4528			9,3669		
	2 NO	I	4,6373	0,2959	6,3809			ND	ND			ND		
		II	2,7867	0,1812	6,5023			0,0404	1,4497			9,3473		
	3 NO	I	5,3574	0,3419	6,3818			ND	ND			ND		
		II	4,1686	0,2701	6,4794			ND	ND			ND		
		B	5,385	0,3673	6,8208			0,081	1,5042			9,6983		

Table F.4: Dry weight and ash determination (w/ww) results for fractions obtained by enzymatic hydrolysis of snow crab RRM using Corolase as proteolytic agent. Number designation nr I, II etc. are samples from merged parallels from the given replica. Replicas treated at different temperatures are marked with the given temperature in superscript. Abbreviations are SF = sludge fraction, WF = water fraction, Fr. = fraction, Av. = average, NA = not analysed, ND = no data.

COROLASE

Fr.	Rep	Nr	Ww (g)	Dw (g)	dw/ww %	Av. Dw/ww %	SD	Ash (g)	Ash % w/ww	Av. Ash % w/ww)	SD	Recovery ash (%)	Av. Recovery (%)	SD recovery
SF	1	I	5,6978	1,4868	26,0943	33,2601	6,1509	0,2527	NA	11,2588	2,4519	NA	78,9026	4,3428
		II	3,1429	1,4062	44,7421			0,7126	NA			NA		
	2	I	4,8879	1,6151	33,0428			0,5506	11,2646			78,9427		
		II	4,9373	1,5719	31,8372			0,5786	11,7190			82,1272		
	3	I	5,1074	1,6097	31,5170			0,5302	10,3810			72,7509		
		II	4,6475	1,5024	32,3271			0,5424	11,6708			81,7897		
	4	I	4,884	1,79	36,6503	32,5453	3,0158	0,8578	17,5635	13,7588	3,4788	122,9431	96,3109	24,3511
		II	3,7661	1,1268	29,9195			0,4045	10,7406			75,1831		
	5	I	2,1561	0,7096	32,9113			0,2797	12,9725			90,8066		
		II	2,2101	0,6785	30,7000			ND	ND			NA		
	1 NO	I	2,968	1,076	36,2534	33,6044	1,7547	0,3342	11,2601	10,5330	1,0533	78,7187	73,6359	7,3637
		II	2,8364	0,9844	34,7060			0,3474	12,2479			85,6245		
	2 NO	I	2,0515	0,6676	32,5420			0,1919	9,3541			65,3942		
		II	2,3966	0,7674	32,0204			0,2440	10,1811			71,1754		
	3 NO	I	1,4106	0,4492	31,8446			0,1383	9,8043			68,5415		
		II	1,7081	0,5852	34,2603			0,1768	10,3507			72,3610		
	4 NO	I	3,3572	0,9954	29,6497	29,8381	1,0241	0,3042	9,0611	10,4141	1,3562	68,7909	79,0625	10,2962
		II	4,6542	1,4569	31,3029			0,5692	12,2298			92,8473		
	5 NO	I	4,0041	1,1582	28,9254			0,4228	10,5592			80,1640		
		II	3,045	0,8975	29,4745			0,2986	9,8062			74,4478		
WF	1		4,7463	0,3664	7,7197	7,6652	0,0699	0,0717	1,5107	1,4978	0,0284	10,6005	10,5103	0,1034
	2		5,2304	0,3968	7,5864			0,0775	1,4817			10,3975		
	3		5,1498	0,396	7,6896			0,0773	1,5010			10,5330		
	4	I	2,2931	0,1849	8,0633	7,8788	0,1678	0,0354	1,5438	1,4958	0,0424	10,4367	10,1123	0,2866

	II	2,6078	0,2044	7,8380			0,0386	1,4802			10,0068		
5	I	2,1943	ND	ND			ND	ND			NA		
	II	2,583	0,1998	7,7352			0,0378	1,4634			9,8935		
1 NO		6,0839	0,3561	5,8532	5,8796	0,0850	0,0875	1,4382	1,4462	0,0793	10,1630	10,2193	0,1626
2 NO		5,3003	0,308	5,8110			0,0757	1,4282			10,0923		
3 NO		5,4547	0,3259	5,9747			0,0803	1,4721			10,4026		
4 NO	I	2,3919	0,1667	6,9694	6,7330	0,2188	0,0386	1,6138	1,4667	0,1095	10,1538	9,2281	0,6888
	II	2,265	0,1555	6,8653			0,0311	1,3731			8,6393		
5 NO	I	2,545	0,1675	6,5815			0,0378	1,4853			9,3452		
	II	2,56	0,1668	6,5156			0,0357	1,3945			8,7743		

G. Recovery of proteins in hydrolytic fractions

An estimate of soluble protein in fractions obtained by enzymatic hydrolysis was determined by Lowry (1957). Diluted samples were mixed with reagents was absorbance were read at 750 nm. A standard curve was made by determining the relation between absorbance and known concentrations of soluble protein (μg/mL). The equation made from the standards curve is given for respective samples. The results are presented with two tables per protease (**Table G.1 - Table G.10**). The first presents raw material data and average soluble protein concentration (mg/mL). The following tables presents the results as average soluble protein in mg/g RRM and by how much the amount of soluble protein has increased from the RRM to the fraction (%). There are two table for each protease, one for raw data from analysis, and one for the calculated values of concentration of soluble protein in fraction (mg/g RRM) and increase of soluble protein from RRM to fraction (%). For sludge samples, the amount of water in the samples was used to calculate the total amount of soluble protein in the fraction, as it was assumed that the amount of water in the fraction would give a measure of the available volume for soluble protein.

The increase from RRM (%) is presented in a chart for all water fractions in chapter 3.3.6. The calculations used for these values are shown below, and results can be viewed in figure 3.d. The increase of soluble protein in each fraction was based on known size of fractions (%) and the known amount of soluble protein (16,16 mg/g in batch 1, 13,66 mg/g in batch 2) in the general starting mass of RRM (30g, appendix D). Example calculations are shown for sample Alcalase® 2.4 L (B2), WF, replica 4, A for mg/g RRM, and for Alcalase® 2.4 L (TEST 2), sample A for % increase. All p-values are results from Students T-test performed as described in chapter 2.8.

Standards curve: μg/mL soluble protein $= \frac{(abs - 0{,}0241)}{0{,}0019}$

soluble protein ($\boldsymbol{\mu}$g/mL) $= \frac{\boldsymbol{\sim 0{,}307 - 0{,}0241}}{\boldsymbol{0{,}0019}}$ = 142,4035 $\boldsymbol{\mu}$g/mL (average for sample, based on 3 parallel absorbance values)

soluble protein correlated for dilution ($\boldsymbol{\mu}$g/mL) = 14240,35

soluble protein in fraction (mg/mL) = 14,24035

fraction (g) = 20,9140

available water in fraction (based on dry weight, appendix F) = 13,7469 g = 13,7469 mL

soluble protein in fraction (mg) = 14,24035 × 13,7469 $\frac{mg}{mL} \times mL$ = 195,7604 mg original RRM sample for hydrolysis = 30,001 g

soluble protein per RRM (mg/g RRM) = $\frac{195{,}7604\ mg}{30{,}0011\ g\ RRM} = \underline{\underline{6{,}5251\ \text{mg/g RRM}}}$

Increase in amount of soluble protein from RRM to fraction:

Soluble protein in RRM sample = 13,66 mg/g RRM × 29,2387 g RRM = 399,55 mg

Soluble protein in fraction = 657,1 mg

Change in soluble protein from RRM to fraction = 657,1 mg – 399,55 mg = 257,59 mg

Increase in soluble protein (%) = $\frac{257{,}59}{399{,}55} \times 100\% = \underline{\underline{64{,}47\%}}$

The most efficient protease was determined based on the % increase of soluble protein. Corolase® 2TS at temperature 65°C had the highest increase (143,5±6,7%), and further, the degree of proteinisation was calculated for this protease. Example calculations are shown for Corolase (65°C), water fraction, sample nr A. The total amount of protein in the starting RRM sample for hydrolysis was based on the total amount of protein (11,97% w/ww). The RRM sample (g) is the mass of the total RRM samples for the replica. The deproteinisation represent how much of the total protein content that has been extracted in the water fraction.

Total amount of protein in RRM sample = 178,34 g × 0,1197 = 21,3475 g protein in total sample

Amount of protein in fraction (g) = 5238,88 mg = 5,238 g

Deproteinisation (%) = $\frac{5{,}238\ g}{21{,}3475\ g} \times 100\% = 24{,}5410\ \%$

Table G.1: Raw data for soluble protein in fractions obtained by enzymatic hydrolysis using Alcalase® 2.4 L as proteolytic agent on snow crab RRM, batch 1. Concentration of soluble protein (mg/mL) is determined using Lowry method. Abbreviations are SF1 = sludge fraction 1, SF 2 = sludge fraction 2, WF = water fraction, rep. = replica.

Rep.	Fr.	Nr	Original sample for hydrolysis	Parallell for Lowry (g / mL)	Ratio sample: water	Absorbance I	Absorbance II	Absorbance III	Standard curve (x = μg/mL, y = abs.)	Av. (I-III) (mg/mL, corr. dil.)	Av. (replica) (mg/mL)	SD
TEST 2	SF 1	A	30,0011	2,0067	100	0,307	0,288	0,289	x = (y - 0,0241)/0,0019	14,2404	13,9421	1,9259
		B	30,0151	2,0025	100	0,286	0,274	0,26		13,1175		
		C	30,1429	2,0982	100	0,232	0,22	0,257		11,1702		
		D	30,025	2,0146	100	0,285	0,278	0,306		13,9772		
		E	30,0077	2,0437	100	0,355	0,348	0,345		17,1175		
		F	30,00402	2,0173	100	0,297	0,286	0,289		14,0298		
	SF 2	A	30,0011	0,8118	100	0,524	0,538	0,564	x = (y - 0,0371)/0,0016	31,5563	29,0736	1,8304
		B	30,0151	0,5772	100	0,481	0,469	0,486		27,5979		
		C	30,1429	0,3466	100	0,511	0,534	0,534		30,5771		
		D	30,025	0,5053	100	0,509	0,466	0,499		28,3896		
		E	30,0077	0,5302	100	0,51	0,503	0,518		29,5771		
		F	30,00402	1,6765	100	0,465	0,462	0,468		26,7438		
	SF1 NO	A	30,0041	2,0531	100	0,275	0,272	0,361		14,6614	11,8953	2,4584
		B	30,0351	2,0556	100	0,246	0,229	0,228		11,0649		
		C	30,0019	2,0977	100	0,213	0,213	0,214		9,9596		
	SF2 NO	A	30,0041	0,5754	100	0,452	0,447	0,44		25,5771	25,6951	0,8086
		B	30,0351	0,4377	100	0,446	0,44	0,423		24,9521		
		C	30,0019	0,9855	100	0,469	0,465	0,452		26,5563		
	WF	A	30,0011	7,4594	333,33	0,192	0,19	0,19	x = (y-0,0299)/0,0017	31,5226	30,9394	1,5991
		B	30,0151	10,1917	400	0,156	0,16	0,157		30,0627		
		C	30,1429	8,4863	357	0,184	0,179	0,184		32,0110		
		D	30,025	7,5945	333,33	0,201	0,2	0,198		33,2872		
		E	30,0077	10,344	400	0,155	0,154	0,152		29,1216		
		F	30,00402	8,4222	357	0,172	0,171	0,17		29,6310		
	WF NO	A	30,0041	8,929	370,4	0,163	0,163	0,165		29,1454	29,3149	1,5684
		B	30,0351	8,7685	370,4	0,165	0,188	0,163		30,9611		
		C	30,0019	8,9441	370,4	0,159	0,155	0,159		27,8381		

Table G.2: Results from of soluble protein by Lowry method in proteolytic fractions using Alcalase® 2.4 L on snow crab RRM, batch 1. Calculations are shown at the top of this appendix. Abbreviations are SF1 = sludge fraction 1, SF 2 = sludge fraction 2, WF = water fraction, rep. = replica, fr. = fraction, av. = average, ez. = enzyme treated, no = control.

Rep.	Fr.	Sample	WF (mL) / SF (g)	Available liquid (g=mL)	Soluble protein in fraction mg	Total (mg)/(g RRM)	Average protein per rrm (mg/g)	SD mg/g rrm	p-value: Ez vs no	p-value Ez vs RRM	% increase from RRM	Av. % increase from RRM	SD
TEST 2	SF 1	A	20,9140	13,7469	195,7604	6,5251	6,3761	0,6175	0,2638	0,0128	-59,6304	-60,5524	3,8205
		B	18,7033	12,0993	158,7130	5,2878					-67,2855		
		C	20,9814	16,9822	189,6943	6,2932					-61,0653		
		D	21,5142	15,3256	214,2087	7,1343					-55,8611		
		E	18,6841	11,7667	201,4166	6,7122					-58,4731		
		F	19,5361	13,4813	189,1407	6,3038					-60,9993		
	SF 2	A	0,9597	0,6834	21,5658	0,7188	0,6145	0,3217	0,6949	0,0160	-95,5527	-96,1983	1,9902
		B	0,7009	0,4991	13,7745	0,4589					-97,1607		
		C	0,4926	0,3508	10,7259	0,3558					-97,7985		
		D	0,6248	0,4449	12,6312	0,4207					-97,3973		
		E	0,7295	0,5195	15,3647	0,5120					-96,8322		
		F	1,9230	1,3694	36,6223	1,2206					-92,4485		
	SF1 NO	A	19,7461	13,4683	197,4641	6,5812	2,9385	1,1838			-59,2831	-66,9754	7,3238
		B	20,6349	14,1370	156,4252	5,2081					-67,7786		
		C	19,7175	12,7253	126,7396	4,2244					-73,8645		
	SF2NO	A	0,9696	0,6905	17,6599	0,5886	0,5391	0,2204			-96,3586	-96,6646	1,3637
		B	0,5040	0,3589	8,9553	0,2982					-98,1553		
		C	1,1591	0,8254	21,9195	0,7306					-95,4799		
	WF	A	22,4594	22,4594	707,9778	23,5984	24,4443	0,8669	0,2305	0,0069	45,9990	51,2325	5,3635
		B	25,2017	25,2017	757,6323	25,2417					56,1658		
		C	23,5563	23,5563	754,0607	25,0162					54,7706		
		D	22,6070	22,6070	752,5248	25,0633					55,0619		
		E	25,3440	25,3440	738,0570	24,5956					52,1684		
		F	23,4422	23,4422	694,6158	23,1508					43,2295		
	WF NO	A	23,9290	23,9290	697,4202	23,2442	23,3278	1,1546			43,8074	44,3246	7,1434
		B	23,7885	23,7885	736,5177	24,5219					51,7125		
		C	23,9441	23,9441	666,5583	22,2172					37,4538		

Table G.3: Raw data for soluble protein in fractions obtained by enzymatic hydrolysis using Alcalase® 2.4 L as proteolytic agent on snow crab RRM, batch 2. Concentration of soluble protein (mg/mL) is determined using Lowry method. Samples designated with letters are individual parallels, while samples designated I, II etc. are samples from merged parallels from the given replica. Abbreviations are SF = sludge fraction , WF = water fraction, fr. = fraction, rep. = replica.

Fr.	Replica	Sample	Original sample for hydrolysis	Parallell for Lowry (g / mL)	Dilution	Absorbance I	Absorbance II	Absorbance III	Standard curve (x = μg/mL, y = abs.)	Av. (I-III) soluble protein (mg/mL, corr. dilution)	Av. (replica) soluble protein (mg/mL)	SD
SF	1	I	183,3698	2,1399	100	0,262	0,266	0,275	x = (y - 0,0273)/0,0018	13,3537	12,7385	1,2216
		II	183,3698	2,098	100	0,246	0,246	0,249		12,2056		
		III	183,3698	2,0525	100	0,271	0,271	0,278		13,6685		
	2	I	182,9356	2,1144	100	0,272	0,269	0,275		13,5944		
		II	182,9356	2,027	100	0,26	0,258	0,266		13,0019		
		III	182,9356	2,0781	100	0,23	0,236	0,237		11,5019		
	3	I	181,7786	2,0554	100	0,258	0,264	0,266		13,0759		
		II	181,7786	2,0299	100	0,211	0,21	0,213		10,2241		
		III	181,7786	2,124	100	0,278	0,279	0,282		14,0204		
	1 NO	I	93,3751	2,2271	100	0,199	0,203	0,205		9,7241	9,1747	0,6163
		II	93,3751	2,3476	100	0,204	0,214	0,203		9,9833		
		III	93,3751	2,027	100	0,197	0,203	0,204		9,6685		
	2 NO	I	91,4865	2,0143	100	0,194	0,198	0,196		9,3722		
		II	91,4865	2,101	100	0,186	0,198	0,194		9,1870		
		III	91,4865	2,0695	100	0,197	0,197	0,194		9,3722		
	3 NO	I	92,153	2,0454	100	0,174	0,178	0,182		8,3722		
		II	92,153	2,0334	100	0,18	0,182	0,185		8,6130		
		III	92,153	2,0224	100	0,173	0,177	0,179		8,2796		
WF	1	I	183,3698	0,1	200	0,278	0,284	0,282	x = (y - 0,0267)/0,0019	13,3537	12,7385	1,2216
		II	183,3698	0,1	200	0,289	0,286	0,29		12,2056		
		III	183,3698	0,1	200	0,298	0,298	0,293		13,6685		
	2	I	182,9356	0,08	250	0,241	0,245	0,246		13,5944		
		II	182,9356	0,08	250	0,26	0,256	0,265		13,0019		
		III	182,9356	0,08	250	0,25	0,257	0,253		11,5019		
	3	I	181,7786	0,05	400	0,165	0,166	0,168		13,0759		
		II	181,7786	0,05	400	0,166	0,169	0,171		10,2241		
		III	181,7786	0,05	400	0,179	0,18	0,18		14,0204		

4	A	29,2387	0,08	250	0,296	0,3	0,32	x = (y - 0,0246)/0,002	35,0917	35,9333	3,3488
	B	29,8797	0,08	250	0,322	0,322	0,323		37,2167		
	C	29,2654	0,08	250	0,343	0,343	0,345		39,8833		
	D	29,5994	0,08	250	0,277	0,258	0,278		30,8000		
	E	29,3159	0,08	250	0,314	0,321	0,319		36,6750		
1 NO	I	93,3751	0,05	400	0,153	0,127	0,128		9,7241	9,1747	0,6163
	II	93,3751	0,05	400	0,133	0,131	0,13		9,9833		
	III	93,3751	0,05	400	0,131	0,134	0,133		9,6685		
2 NO	I	91,4865	0,06	333,33	0,147	0,15	0,144		9,3722		
	II	91,4865	0,06	333,33	0,151	0,149	0,152		9,1870		
	III	91,4865	0,06	333,33	0,151	0,147	0,149		9,3722		
3 NO	I	92,153	0,05	400	0,12	0,12	0,122		8,3722		
	II	92,153	0,05	400	0,13	0,129	0,131		8,6130		
	III	92,153	0,05	400	0,128	0,129	0,129		8,2796		
4 NO	NO A	28,5713	0,08	250	0,201	0,197	0,198		21,7583	22,5500	0,7535
	NO B	30,0656	0,08	250	0,204	0,203	0,21		22,6333		
	NO C	28,3177	0,08	250	0,216	0,205	0,211		23,2583		

Table G.4: Results from estimation of soluble protein by Lowry method in proteolytic fractions using Alcalase® 2.4 L on snow crab RRM, batch 2. Calculations are shown at the top of this appendix. Samples designated with letters are individual parallels, while samples designated I, II etc. are samples from merged parallels from the given replica. Abbreviations are SF = sludge fraction 2, WF = water fraction, rep. = replica, fr. = fraction, av. = average, ez. = enzyme treated, no = control.

	Rep.	Nr	WF (mL) / SF (g)	Available liquid (g=mL)	Soluble protein in fraction mg	Total soluble protein/rrm (mg)/(g)	Average protein per rrm (mg/g)	SD mg/g rrm	p-value: Ez vs no	p-value Ez vs RRM	% increase from RRM	Av. % increase from RRM	SD
SF	1	I	151,1779	108,5074	1448,9761	7,9019	7,0265	0,9785	0,0000	0,0000	-42,1671	-48,5740	7,1617
		II	151,1779	108,5074	1324,3935	7,2225					-47,1396		
		III	151,1779	108,5074	1483,1358	8,0882					-40,8037		
	2	I	148,9546	105,1263	1429,1343	7,8122					-42,8237		
		II	148,9546	105,1263	1366,8372	7,4717					-45,3161		
		III	148,9546	105,1263	1209,1477	6,6097					-51,6248		
	3	I	129,4508	88,3197	1154,8619	6,3531					-53,5026		
		II	129,4508	88,3197	902,9872	4,9675					-63,6437		
		III	129,4508	88,3197	1238,2749	6,8120					-50,1442		
	1 NO	I	68,2794	46,2717	449,9496	4,8187	4,4577	0,6370			-64,7326	-67,3750	4,6617
		II	68,2794	46,2717	461,9460	4,9472					-63,7923		
		III	68,2794	46,2717	447,3790	4,7912					-64,9340		
	2 NO	I	70,0045	48,2227	451,9536	4,9401					-63,8442		
		II	70,0045	48,2227	443,0235	4,8425					-64,5586		
		III	70,0045	48,2227	451,9536	4,9401					-63,8442		
	3 NO	I	58,0826	39,5362	331,0056	3,5919					-73,7114		
		II	58,0826	39,5362	340,5236	3,6952					-72,9555		
		III	58,0826	39,5362	327,3449	3,5522					-74,0022		
WF	1	I	121,6619	121,6619	1624,6370	8,8599	8,9327	1,0562	0,0007	0,0000	-35,1560	-34,6232	7,7304
		II	121,6619	121,6619	1484,9511	8,0981					-40,7313		
		III	121,6619	121,6619	1662,9379	9,0688					-33,6273		
	2	I	121,5270	121,5270	1652,0921	9,0310					-33,9037		
		II	121,5270	121,5270	1580,0761	8,6373					-36,7849		
		III	121,5270	121,5270	1397,7856	7,6409					-44,0779		
	3	I	141,5361	141,5361	1850,7156	10,1812					-25,4860		

	II	141,5361	141,5361	1447,0756	7,9606					-41,7374		
	III	141,5361	141,5361	1984,3885	10,9165					-20,1040		
4	A	18,7251	18,7251	657,0950	22,4735	25,1459	3,2159	0,0078	0,0011	64,4793	84,0385	23,5367
	B	21,0918	21,0918	784,9665	26,2709					92,2720		
	C	21,4272	21,4272	854,5882	29,2013					113,7192		
	D	20,4759	20,4759	630,6577	21,3064					55,9380		
	E	21,1646	21,1646	776,2117	26,4775					93,7841		
1 NO	I	71,6411	71,6411	696,6434	7,4607	7,1355	0,3984			-45,3965	-47,7766	2,9161
	II	71,6411	71,6411	715,2170	7,6596					-43,9407		
	III	71,6411	71,6411	692,6633	7,4181					-45,7084		
2 NO	I	65,3111	65,3111	612,1101	6,6907					-51,0318		
	II	65,3111	65,3111	600,0155	6,5585					-51,9994		
	III	65,3111	65,3111	612,1101	6,6907					-51,0318		
3 NO	I	79,3005	79,3005	663,9214	7,2046					-47,2711		
	II	79,3005	79,3005	683,0123	7,4117					-45,7549		
	III	79,3005	79,3005	656,5788	7,1249					-47,8543		
4 NO	A	23,4192	23,4192	509,5628	17,8348	18,1576	0,3315			30,5296	32,8922	2,4260
	B	24,5711	24,5711	556,1259	18,4971					35,3769		
	C	22,0871	22,0871	513,7091	18,1409					32,7702		

Table G.5: Raw data for soluble protein in fractions obtained by enzymatic hydrolysis using Protamex® as proteolytic agent on snow crab RRM, batch 2. Concentration of soluble protein (mg/mL) is determined using Lowry method. Samples designated I, II etc. are samples from merged parallels from the given replica. Abbreviations are SF = sludge fraction , WF = water fraction, rep. = replica, fr. = fraction, av. = average.

PROTAMEX

Fr.	Rep.	Nr	Original sample for hydrolysis	Parallell for Lowry (g / mL)	Dilution	Absorbance I	Absorbance II	Absorbance III	Standard curve (x = μg/mL, y = abs.)	Av. (I-III) soluble protein (mg/mL, corr. dilution)	Av. (replica) soluble protein (mg/mL)	SD
SF	1	I	187,2422	4,3444	100	0,33	0,339	0,333	x =(y - 0,0281)/0,0018	16,9944	16,0109	1,3684
		II	187,2422	2,1286	100	0,328	0,338	0,334		16,9574		
		III	187,2422	1,9779	100	0,291	0,294	0,298		14,7907		
	2	I	164,749	3,39	100	0,265	0,268	0,275		13,4019		
		II	164,749	2,3051	100	0,313	0,321	0,323		16,1611		
		III	164,749	2,2569	100	0,292	0,293	0,296		14,7537		
	3	I	184,3697	2,3199	100	0,332	0,339	0,323		16,8463		
		II	184,3697	2,6254	100	0,325	0,339	0,328		16,8093		
		III	184,3697	2,7734	100	0,342	0,342	0,339		17,3833		
	1 NO	I	91,3652	3,4721	100	0,269	0,28	0,276		13,7167	13,2763	0,9735
		II	91,3652	2,5853	100	0,276	0,273	0,276		13,7167		
		III	91,3652	2,5649	100	0,271	0,269	0,269		13,4204		
	2 NO	I	91,3811	2,8759	100	0,279	0,283	0,286		14,1426		
		II	91,3811	2,1597	100	0,238	0,232	0,236		11,5130		
		III	91,3811	2,0848	100	0,239	0,24	0,242		11,7907		
	3 NO	I	90,1238	2,9053	100	0,26	0,267	0,269		13,1796		
		II	90,1238	2,966	100	0,271	0,281	0,281		13,8648		
		III	90,1238	3,3753	100	0,278	0,287	0,283		14,1426		
WF	1	I	187,2422	0,06666	300	0,281	0,268	0,273	x = (y - 0,0281)/0,002	36,8850	36,6683	0,8983
		II	187,2422	0,06666	300	0,274	0,268	0,28		36,8850		
		III	187,2422	0,06666	300	0,268	0,274	0,27		36,3850		
	2	I	164,749	0,06666	300	0,266	0,268	0,27		35,9850		
		II	164,749	0,06666	300	0,267	0,265	0,271		35,9350		
		III	164,749	0,06666	300	0,276	0,275	0,284		37,5350		

	3	I	184,3697	0,06666	300	0,26	0,26	0,268	35,1850		
		II	184,3697	0,06666	300	0,273	0,277	0,276	37,0850		
		III	184,3697	0,06666	300	0,28	0,282	0,285	38,1350		
	1 NO	I	91,3652	0,06666	300	0,235	0,232	0,238	31,0350	32,2183	1,4553
		II	91,3652	0,06666	300	0,231	0,234	0,23	30,5350		
		III	91,3652	0,06666	300	0,239	0,241	0,244	31,9850		
	2 NO	I	91,3811	0,06666	300	0,236	0,235	0,237	31,1850		
		II	91,3811	0,06666	300	0,232	0,239	0,24	31,3350		
		III	91,3811	0,06666	300	0,243	0,245	0,245	32,4350		
	3 NO	I	90,1238	0,06666	300	0,244	0,242	0,247	32,4350		
		II	90,1238	0,06666	300	0,257	0,257	0,267	34,8350		
		III	90,1238	0,06666	300	0,248	0,256	0,264	34,1850		

Table G.6: Results from estimation of soluble protein by Lowry method in proteolytic fractions using Protamex® on snow crab RRM, batch 2. Calculations are shown at the top of this appendix. Abbreviations are SF = sludge fraction 2, WF = water fraction, rep. = replica, fr. = fraction, av. = average, ez. = enzyme treated, no = control.

PROTAMEX

Fr.	Rep.	Nr	WF (mL) / SF (g)	Available liquid (g=mL)	Soluble protein in fraction (mg)	Total soluble protein/RRM (mg)/(g)	Average protein per rrm (mg/g)	SD mg/g rrm	p-value: Ez vs no	p-value Ez vs RRM	% increase from RRM	Av. % increase form RRM	SD
SF	1	I	149,5584	103,7535	1763,2329	9,4169	8,9881	0,5897	0,0000	0,0000	-31,0797	-34,2178	4,3158
		II	149,5584	103,7535	1759,3902	9,3963					-31,2299		
		III	149,5584	103,7535	1534,5910	8,1958					-40,0167		
	2	I	143,8645	102,5387	1374,2090	8,3412					-38,9520		
		II	143,8645	102,5387	1657,1399	10,0586					-26,3831		
		III	143,8645	102,5387	1512,8261	9,1826					-32,7941		
	3	I	140,0856	95,0097	1600,5614	8,6813					-36,4634		
		II	140,0856	95,0097	1597,0426	8,6622					-36,6031		
		III	140,0856	95,0097	1651,5852	8,9580					-34,4379		
	1 NO	I	69,4441	31,5773	433,1353	4,7407	6,0546	1,1138			-65,3036	-55,6878	8,1516
		II	69,4441	31,5773	433,1353	4,7407					-65,3036		
		III	69,4441	31,5773	423,7790	4,6383					-66,0531		
	2 NO	I	69,2457	47,1445	666,7450	7,2963					-46,5996		
		II	69,2457	47,1445	542,7725	5,9397					-56,5287		
		III	69,2457	47,1445	555,8682	6,0830					-55,4798		
	3 NO	I	68,5585	46,0659	607,1317	6,7366					-50,6957		
		II	68,5585	46,0659	638,6953	7,0869					-48,1325		
		III	68,5585	46,0659	651,4914	7,2288					-47,0933		
WF	1	I	132,6311	132,6311	4892,0981	26,1271	26,5474	0,8319	0,0000032	0,0000	91,2197	94,2955	6,0889
		II	132,6311	132,6311	4892,0981	26,1271					91,2197		
		III	132,6311	132,6311	4825,7826	25,7729					88,6276		
	2	I	122,8624	122,8624	4421,2035	26,8360					96,4079		
		II	122,8624	122,8624	4415,0603	26,7987					96,1350		
		III	122,8624	122,8624	4611,6402	27,9919					104,8679		
	3	I	132,3803	132,3803	4657,8009	25,2634					84,8982		
		II	132,3803	132,3803	4909,3234	26,6276					94,8827		

	III	132,3803	132,3803	5048,3227	27,3815			100,4005		
1 NO	I	65,7892	65,7892	2041,7678	22,3473	23,3418	1,0595	63,5561	70,8343	7,7540
	II	65,7892	65,7892	2008,8732	21,9873			60,9211		
	III	65,7892	65,7892	2104,2676	23,0314			68,5627		
2 NO	I	66,7070	66,7070	2080,2578	22,7646			66,6104		
	II	66,7070	66,7070	2090,2638	22,8741			67,4118		
	III	66,7070	66,7070	2163,6415	23,6771			73,2887		
3 NO	I	65,1969	65,1969	2114,6615	23,4640			71,7286		
	II	65,1969	65,1969	2271,1340	25,2002			84,4355		
	III	65,1969	65,1969	2228,7560	24,7299			80,9940		

Table G.7: Raw data for soluble protein in fractions obtained by enzymatic hydrolysis using Papain GSM80 as proteolytic agent on snow crab RRM, batch 2. Concentration of soluble protein (mg/mL) is determined using Lowry method. Samples designated with letters are individual parallels, while samples designated I, II etc. are samples from merged parallels from the given replica. Abbreviations are SF = sludge fraction , WF = water fraction, rep. = replica, fr. = fraction, av. = average, no = control, NA = not analysed.

PAPAIN

Fr.	Rep.	Nr	Original sample for hydrolysis	Parallell for Lowry (g / mL)	Dilution	Absorbance I	II	III	Standard curve (x = µg/mL, y = abs.)	Av. (I-III) soluble protein (mg/mL, corr. dilution)	Av. (replica) soluble protein (mg/mL)	SD
SF	1	I	179,4551	4,4147	100	0,378	0,388	0,393	x = (y-0,0329)/0,0019	18,6018	17,0101	1,1401
		II	179,4551	4,1145	100	0,368	0,374	0,38		17,9526		
		III	179,4551	1,9978	100	0,349	0,353	0,356		16,8298		
	2	I	178,8482	2,0849	100	0,318	0,323	0,321		15,1456		
		II	178,8482	4,7549	100	0,359	0,365	0,371		17,4789		
		III	178,8482	2,1082	100	0,342	0,334	0,344		16,1632		
	3	I	180,6747	2,929	100	0,343	0,306	0,348		15,7596		
		II	180,6747	2,6047	100	0,358	0,363	0,357		17,1807		
		III	180,6747	2,7934	100	0,325	NA	0,424		17,9789		
	1 NO	I	91,1695	2,7124	100	0,352	0,361	0,356		17,0228	15,9194	1,0128
		II	91,1695	2,9531	100	0,352	0,361	0,356		17,0228		
		III	91,1695	2,6293	100	0,321	0,33	0,336		15,5842		
	2 NO	I	90,2167	2,4433	100	0,309	0,325	0,342		15,3912		
		II	90,2167	2,6398	100	0,34	0,338	0,348		16,2684		
		III	90,2167	2,6998	100	0,314	0,322	0,331		15,2333		
	3 NO	I	89,2268	3,0602	100	0,33	0,336	0,332		15,7772		
		II	89,2268	2,7505	100	0,287	0,303	0,307		14,0053		
		III	89,2268	2,3497	100	0,345	0,361	0,36		16,9702		
WF	1	I	179,4551	0,1	200	0,402	0,419	0,423	x = (y -0,0363)/0,0019	39,8281	40,7392	3,7125
		II	179,4551	0,1	200	0,415	0,43	0,424		40,7053		
		III	179,4551	0,1	200	0,518	0,524	0,524		51,1263		
	2	I	178,8482	0,08	250	0,342	0,337	0,341		39,9605		
		II	178,8482	0,08	250	0,339	0,34	0,342		40,0044		
		III	178,8482	0,08	250	0,338	0,343	0,344		40,1798		
	3	I	180,6747	0,08	250	0,333	0,34	0,336		39,4781		

	II	180,6747	0,08	250	0,344	0,343	0,351		40,7500		
	III	180,6747	0,08	250	0,342	0,351	0,366		41,6711		
4	I	89,2145	0,08	250	NA	0,354	0,355	x = (y-0,0306)/0,0022	36,8068		
	II	92,2368	0,08	250	0,361	0,362	0,362		37,6212		
1 NO	I	91,1695	0,066	300	0,272	0,271	0,282		37,6895	37,3417	2,2230
	II	91,1695	0,066	300	0,29	0,294	0,298		40,6895		
	III	91,1695	0,066	300	0,282	0,28	0,293		39,2684		
2 NO	I	90,2167	0,066	300	0,259	0,256	0,262		35,1632		
	II	90,2167	0,066	300	0,273	0,275	0,278		37,7421		
	III	90,2167	0,066	300	0,273	0,279	0,277		37,9000		
3 NO	I	89,2268	0,08	250	0,316	0,313	0,311		36,4518		
	II	89,2268	0,08	250	0,316	0,329	0,328		37,8991		
	III	89,2268	0,08	250	0,319	0,324	0,333		38,0307		
4 NO	I	89,6626	0,08	250	0,316	0,315	0,321		32,5833		

Table G.8: Results from estimation of soluble proteinby Lowry method in proteolytic fractions using Papain GSM80 on snow crab RRM, batch 2. Calculations are shown at the top of this appendix. Samples designated with letters are individual parallels, while samples designated I, II etc. are samples from merged parallels from the given replica. Abbreviations are SF = sludge fraction 2, WF = water fraction, Rep. = replica, Av.= average.

	Fr.	**Rep.**	**Nr**	**WF (mL) SF (g)**	**Available liquid (g=mL)**	**Soluble protein in fraction mg**	**Total soluble protein/rrm (mg)/(g)**	**Average protein per rrm (mg/g)**	**SD mg/g rrm**	**p-value: Ez vs no**	**p-value Ez vs RRM**	**% increase from RRM**	**Av. % increase from RRM**	**SD**
PAPAIN	SF	1	I	149,9556	107,0201	1990,7616	11,0934	10,1273	0,7888	0,0017	0,0000	-18,8096	-25,8801	5,7730
			II	149,9556	107,0201	1921,2924	10,7063					-21,6428		
			III	149,9556	107,0201	1801,1295	10,0367					-26,5435		
		2	I	146,5145	103,2279	1563,4497	8,7418					-36,0205		
			II	146,5145	103,2279	1804,3147	10,0885					-26,1639		
			III	146,5145	103,2279	1668,4886	9,3291					-31,7221		
		3	I	146,8438	110,5282	1741,8860	9,6410					-29,4392		
			II	146,8438	110,5282	1898,9524	10,5103					-23,0767		
			III	146,8438	110,5282	1987,1811	10,9987					-19,5027		
		1 NO	I	73,0068	50,7097	863,2216	9,4683	8,8888	0,5649			-30,7031	-34,9445	4,1344
			II	73,0068	50,7097	863,2216	9,4683					-30,7031		
			III	73,0068	50,7097	790,2708	8,6681					-36,5594		
		2 NO	I	73,0121	51,2656	789,0405	8,7461					-35,9891		
			II	73,0121	51,2656	834,0104	9,2445					-32,3410		
			III	73,0121	51,2656	780,9460	8,6563					-36,6458		
		3 NO	I	71,7439	49,1386	775,2690	8,6887					-36,4086		
			II	71,7439	49,1386	688,1989	7,7129					-43,5505		
			III	71,7439	49,1386	833,8905	9,3457					-31,6002		
	WF	1	I	115,4385	115,4385	4597,6927	25,6203	27,2952	1,9363	0,0179	0,0000	87,5104	99,7687	14,1715
			II	115,4385	115,4385	4698,9545	26,1846					91,6402		
			III	115,4385	115,4385	5901,9452	32,8881					140,7025		
		2	I	119,7922	119,7922	4786,9594	26,7655					95,8919		
			II	119,7922	119,7922	4792,2134	26,7949					96,1069		
			III	119,7922	119,7922	4813,2296	26,9124					96,9669		
		3	I	120,3103	120,3103	4749,6185	26,2882					92,3989		

	II	120,3103	120,3103	4902,6447	27,1352			98,5977		
	III	120,3103	120,3103	5013,4568	27,7485			103,0865		
4	I	65,0988	65,0988	2396,0797	26,8575			96,5654		
	II	66,3240	66,3240	2495,1893	27,0520			97,9887		
1 NO	I	62,1329	62,1329	2341,7563	25,6857	25,4190	1,3277	87,9894	86,0369	9,7171
	II	62,1329	62,1329	2528,1550	27,7303			102,9529		
	III	62,1329	62,1329	2439,8609	26,7618			95,8649		
2 NO	I	61,1348	61,1348	2149,6926	23,8281			74,3936		
	II	61,1348	61,1348	2307,3561	25,5757			87,1841		
	III	61,1348	61,1348	2317,0089	25,6827			87,9672		
3 NO	I	60,1813	60,1813	2193,7140	24,5858			79,9392		
	II	60,1813	60,1813	2280,8185	25,5620			87,0840		
	III	60,1813	60,1813	2288,7371	25,6508			87,7335		
4 NO	NO	63,6397	63,6397	2073,5936	23,1266			69,2597		

Table G.9: Raw data for soluble protein in fractions obtained by enzymatic hydrolysis using Corolase® 2TS as proteolytic agent on snow crab RRM, batch 2. Treatments with different temperatures are marked with given temperature in superscript. Concentration of soluble protein (mg/mL) is determined using Lowry method. Samples designated I, II etc. are samples from merged parallels from the given replica. Abbreviations are CO = Corolase® 2TS, SF = sludge fraction , WF = water fraction, rep. = replica, fr. = fraction, av. = average, no = control..

	Fr.	Rep.	Nr	Original sample for hydrolysis	Parallell for Lowry (g / mL)	Dilution	Absorbance I	II	III	Standard curve (x = µg/mL, y = abs.)	Av. (I-III) soluble protein (mg/mL, corr. dilution)	Av. (replica) soluble protein (mg/mL)	SD
COROLASE	WF CO^{50}	1	I	178,3415	0,08	250	0,334	0,338	0,337	x = (y-0,0238)/0,002	39,0667	40,1569	0,9655
			II	178,3415	0,08	250	0,342	0,333	0,336		39,1500		
		2	I	176,3561	0,08	250	0,34	0,342	0,347		39,9000		
			II	176,3561	0,08	250	0,349	0,344	0,349		40,4417		
		3	I	106,3008	0,08	250	0,348	0,351	0,354		40,9000		
			II	106,3008	0,08	250	0,356	0,36	0,351		41,4833		
	WF CO^{65}	4	I	180,905	0,08	250	0,361	0,366	0,373	x = (y -0,0302)/0,0019	44,2719	44,7982	0,9468
			II	180,905	0,08	250	0,379	0,383	0,381		46,1579		
		5	I	87,5952	0,08	250	0,368	0,37	0,372		44,7105		
			II	87,5952	0,08	250	0,363	0,364	0,368		44,0526		
		1 NO	I	89,913	0,08	250	0,2	0,204	0,21		22,6083	22,4277	0,2683
			II	89,913	0,08	250	0,207	0,204	0,206		22,7333		
		2 NO	I	89,9715	0,08	250	0,196	0,2	0,203		21,9833		
			II	89,9715	0,08	250	0,201	0,202	0,204		22,3167		
		3 NO	I	62,5609	0,08	250	0,201	0,204	0,203		22,3583		
			II	62,5609	0,08	250	0,204	0,201	0,208		22,5667		
		4 NO	I	89,8365	0,08	250	0,305	0,313	0,317		37,0351	35,8618	0,8348
			II	89,8365	0,08	250	0,299	0,303	0,301		35,6316		
		5 NO	I	86,1039	0,08	250	0,296	0,299	0,295		35,0614		
			II	86,1039	0,08	250	0,299	0,305	0,301		35,7193		

Table G.10: Results from estimation of soluble protein by Lowry method in proteolytic fractions using Corolase® 2TS on snow crab RRM, batch 2. Calculations are shown at the top of this appendix. Treatments with different temperatures are marked with given temperature in superscript. Samples designated I, II etc. are samples from merged parallels from the given replica. Abbreviations are SF = sludge fraction 2, WF = water fraction, Rep. = replica, Av.= average.

	Fr.	Rep.	Nr	WF (mL)	Soluble protein in fraction mg	Total soluble protein/rrm (mg/g)	Average protein per rrm (mg/g)	SD mg/g rrm	p-value: Enz vs no	p-value Ez vs RRM	% increase from RRM	Av. % increase from RRM	SD	Total protein in sample (g)	Deproteini-sation (%)	Average %	SD
COROLASE	WF CO^{50}	1	I	134,1012	5238,8869	29,3756	30,3041	1,2078	***	***	114,9947	121,7905	8,8400	21,3475	24,5410	25,3167	1,0091
			II	134,1012	5250,0620	29,4383					115,4533			21,3475	24,5934		
		2	I	130,2524	5197,0708	29,4692					115,6797			21,1098	24,6192		
			II	130,2524	5267,6241	29,8692					118,6077			21,1098	24,9534		
		3	I	82,1578	3360,2540	31,6108					131,3539			12,7242	26,4084		
			II	82,1578	3408,1794	32,0617					134,6536			12,7242	26,7850		
	WF CO^{65}	4	I	130,6667	5784,8670	31,9774	33,2631	0,9158	***	***	134,0367	143,4468	6,7027	21,6543	26,7146	27,7887	0,7651
			II	130,6667	6031,2998	33,3396					144,0066			21,6543	27,8526		
		5	I	66,8442	2988,6394	34,1188					149,7091			10,4851	28,5036		
			II	66,8442	2944,6629	33,6167					146,0348			10,4851	28,0841		
	CO^{50}	1 NO	I	68,0601	1538,7254	17,1135	16,8646	0,2497			25,2506	23,4289	1,8277	10,7626	14,2970	14,0890	0,2086
			II	68,0601	1547,2329	17,2081					25,9431			10,7626	14,3760		
		2 NO	I	67,9423	1493,5982	16,6008					21,4983			10,7696	13,8687		
			II	67,9423	1516,2457	16,8525					23,3405			10,7696	14,0790		
		3 NO	I	46,5291	1040,3131	16,6288					21,7033			7,4885	13,8921		
			II	46,5291	1050,0067	16,7838					22,8373			7,4885	14,0215		
	CO^{65}	4 NO	I	61,6076	2281,6429	25,3977	24,4651	0,6777			85,8815	79,0557	4,9600	10,7534	21,2178	20,4387	0,5662
			II	61,6076	2195,1761	24,4352					78,8372			10,7534	20,4137		
		5 NO	I	58,4248	2048,4555	23,7905					74,1185			10,3066	19,8751		
			II	58,4248	2086,8929	24,2369					77,3857			10,3066	20,2480		

H. Free amino groups in hydrolytic fractions

Free amino groups in the water fractions obtained by enzymatic hydrolysis of batch 2 was determined by formol titration (**Table H.1**). The amount of free amino acids (mg/mL) is calculated given that the density of the water fractions are 1g/mL. The water fraction parallels generated with enzymatic hydrolysis had been merged, and the formol titration is performed with 1-2 parallels per merged fraction. The proteases are Alcalase® 2.4 L (AL, batch 2), Protamex® (PR), Papain GSM80 (PA) and Corolase® 2TS (CO, at tempteratures 50 and 65°C). Calculations for amount of free amino groups are shown in appendix D for the RRM.

Table H.1: Concentration of of free amino groups (mg/mL) in water fractions obtained by enzymatic hydrolysis. Proteolytic agents and respective abbreviations are Alcalase® 2.4 L (AL, batch 2), Protamex® (PR), Papain GSM80 (PA) and Corolase® 2TS (CO, at tempteratures 50 and 65°C)

Enzyme	Replica	Nr	Sample (g)	NaOH brukt (mL)	Free aa. g/100 mL WF (%)	mg/mL WF	Av. mg/mL WF	SD
AL (B2)	4	I	1,6929	5,81	0,4807	4,8072	4,5321	0,3890
		II	1,6682	5,07	0,4257	4,2570		
	4 NO	NOI	1,5956	3,93	0,3450	3,4500	3,3237	0,1786
		NOII	1,6209	3,7	0,3197	3,1974		
PR	1	I	1,6058	3,46	0,3018	3,0181	3,2301	0,4646
		II	1,5	3,93	0,3670	3,6698		
	2	I	1,6567	3,73	0,3154	3,1536		
		II	1,5319	3,69	0,3374	3,3740		
	3	I	1,7127	3,01	0,2462	2,4617		
		II	1,842	4,87	0,3703	3,7033		
	1 NO	I	1,5747	3,07	0,2731	2,7308	2,8181	0,1195
		II	1,5224	3,27	0,3009	3,0086		
	2 NO	I	1,8072	3,49	0,2705	2,7050		
	3 NO	I	1,7402	3,49	0,2809	2,8091		
		II	1,6096	3,26	0,2837	2,8369		
PA	1	I	1,9913	4,62	0,3250	3,2498	4,2023	0,9803
		II	1,5408	5,11	0,4645	4,6454		
	2	I	1,5652	5,77	0,5164	5,1636		
		II	1,5214	6,22	0,5727	5,7265		
	3	I	1,7421	4,21	0,3385	3,3850		
		II	1,8725	6,23	0,4660	4,6603		

	4	I	1,672	4,42	0,3703	3,7028		
		II	1,7071	3,76	0,3085	3,0851		
	1 NO	I	1,5732	3,08	0,2742	2,7423	2,9789	0,3759
		II	1,5682	3,99	0,3564	3,5638		
	2 NO	I	1,7697	3,95	0,3126	3,1264		
		II	1,6197	3,26	0,2819	2,8192		
	3 NO	I	1,6676	3,96	0,3326	3,3262		
		II	1,721	3,73	0,3036	3,0358		
	4 NO	I	1,7126	3,52	0,2879	2,8789		
		II	1,8207	3,04	0,2339	2,3387		
CO 50°C	1	I	1,4671	3,52	0,3361	3,3607	3,4330	0,4272
		II	1,6344	4,88	0,4182	4,1822		
	2	I	1,5168	3,69	0,3408	3,4076		
		II	1,5437	3,94	0,3575	3,5750		
	3	I	1,4318	3,03	0,2964	2,9642		
		II	1,6043	3,56	0,3108	3,1082		
	1 NO	I	1,6532	3,28	0,2779	2,7790	3,0407	0,2245
		II	1,6164	3,69	0,3198	3,1976		
	2 NO	I	1,5682	3,26	0,2912	2,9118		
		II	1,4858	3,54	0,3337	3,3372		
	3 NO	I	1,5428	3,28	0,2978	2,9779		
CO 65°C	4	I	1,5397	5,08	0,4621	4,6214	4,0489	0,5625
		II	1,5737	4,68	0,4166	4,1655		
	5	I	1,7798	4,16	0,3274	3,2739		
		II	1,6531	4,88	0,4135	4,1349		
	4 NO	I	1,9752	3,73	0,2645	2,6451	2,8006	0,1713
		II	1,7584	3,5	0,2788	2,7880		
	5 NO	I	1,9312	3,76	0,2727	2,7271		
		II	1,8326	3,98	0,3042	3,0420		

I. Recovery of lipids in hydrolytic fractions

Extraction of total lipid content in fractions obtained by enzymatic hydrolysis was performed. Calculations are demonstrated in appendix D. **Table I.1 - Table I.5**. presents the results for all proteases individually. All analysis are performed with 2-6 parallels per replica, and each analysis parallel was performed in duplicate. Corolase® 2TS was used at proteolytic agent at temperatures 50°C and 65°C, individually. Alcalase® 2.4 L was used on both batch 1 and 2.

Table I.1: Total lipid content in sludge and water fractions from enzymatic hydrolysis using Alcalase® 2.4 L as proteolytic agent on snow crab rest raw material, batch 1. Capitalized letters are fraction parallel samples, and lowercase letter are parallels of the fraction sample. Abbreviations are Fr. = fraction, $CHCL_3$ = chloroform, SF = sludge fraction, WF = water fraction, Av. = average.

ALCALASE | BATCH 1

Fr.	Rep.	Nr.	Sample (g)	$CHCl_3$ (mL)	Evaporated $CHCl_3$ (mL)	Lipid (g)	Totalt lipid (%)	Av. total lipid (%)	SD
SF	TEST 2	Aa	5,2919	40	2	0,0226	8,5414	11,2185	1,7851
		Ab	5,2919	40	2	0,0294	11,1113		
		Ba	5,0403	40	2	0,0357	14,1658		
		Bb	5,0403	40	2	0,035	13,8881		
		Ca	5,1686	40	2	0,032	12,3825		
		Cb	5,1686	40	2	0,0258	9,9834		
		Da	5,7045	40	2	0,0309	10,8336		
		Db	5,7045	40	2	0,0313	10,9738		
		Ea	5,897	40	2	0,0283	9,5981		
		Eb	5,897	40	2	0,0373	12,6505		
		Fa	5,3773	40	2	0,0246	9,1496		
		Fb	5,3773	40	2	0,0305	11,3440		
	2 NO	Aa	5,24	40	2	0,0256	9,7710	10,3466	0,8543
		Ab	5,24	40	2	0,0302	11,5267		
		Ba	5,3164	40	2	0,0257	9,6682		
		Bb	5,3164	40	2	0,0277	10,4206		
WF	2	A a	5,0055	40	2	0,0064	2,5572	1,7910	0,5079
		Ab	5,0055	40	2	0,004	1,5982		
		Ba	5,0109	40	2	0,0049	1,9557		
		Bb	5,0109	40	2	0,0036	1,4369		

	Ca	4,9906	40	2	0,0067	2,6850		
	Cb	4,9906	40	2	0,004	1,6030		
	Da	5,0137	40	2	0,0047	1,8749		
	Db	5,0137	40	2	0,0036	1,4361		
	Ea	5,0221	40	2	0,0045	1,7921		
	Eb	5,0221	40	2	0,0019	0,7567		
	Fa	5,0046	40	2	0,0048	1,9182		
	Fb	5,0046	40	2	0,0047	1,8783		
2 NO	Aa	5,0204	40	2	0,0035	1,3943	1,5406	0,2890
	Ab	5,0204	40	2	0,0051	2,0317		
	Ba	5,1117	40	2	0,003	1,1738		
	Bb	5,1117	40	2	0,0039	1,5259		
	Ca	5,0678	40	2	0,0037	1,4602		
	Cb	5,0678	40	2	0,0042	1,6575		

Table I.2: Total lipid content in sludge and water fractions from enzymatic hydrolysis using Alcalase® 2.4 L as proteolytic agent on snow crab rest raw material, batch 2. Capitalized letters are fraction parallel samples, and lowercase letter are parallels of the fraction sample. Abbreviations are Fr. = fraction, $CHCL_3$ = chloroform, SF = sludge fraction, WF = water fraction, Av. = average, ND = no data.

Fr.	Rep.	Nr.	Sample (g)	$CHCl_3$ (mL)	Evaporated $CHCl_3$ (mL)	Lipid (g)	Totalt lipid (%)	Av. total lipid (%)	SD
SF	1	A a	6,1961	40	2	0,0047	1,5171	2,0332	0,3368
		Ab	6,1961	40	2	0,0062	2,0013		
		Ba	6,5648	40	2	0,0049	1,4928		
		Bb	6,5648	40	2	0,0059	1,7975		
	2	A a	6,4966	40	2	0,0062	1,9087		
		Ab	6,4966	40	2	0,0069	2,1242		
		Ba	6,6693	40	2	0,006	1,7993		
		Bb	6,6693	40	2	0,0073	2,1891		
	3	A a	7,4173	40	2	0,0087	2,3459		
		Ab	7,4173	40	2	0,0092	2,4807		
		Ba	9,0252	40	2	0,0105	2,3268		
		Bb	9,0252	40	2	0,0109	2,4155		
	1 NO	Aa	6,1514	40	2	0,0067	2,1784	2,3458	0,2034
		Ab	6,1514	40	2	0,0075	2,4385		
		Ba	5,7894	40	2	0,006	2,0728		
		Bb	5,7894	40	2	0,0066	2,2800		
	2 NO	Aa	5,9576	40	2	0,0062	2,0814		
		Ab	5,9576	40	2	0,0066	2,2157		
		Ba	5,6482	40	2	0,0062	2,1954		
		Bb	5,6482	40	2	0,0067	2,3724		
	3 NO	Aa	6,6531	40	2	0,0086	2,5853		
		Ab	6,6531	40	2	0,0088	2,6454		
		Ba	5,8615	40	2	0,0076	2,5932		
		Bb	5,8615	40	2	0,0073	2,4908		
WF	1	A a	15,0091	40	2	0,0001	0,0133	0,1024	0,0517
		Ab	15,0091	40	2	0,0011	0,1466		

	Ba	15,2817	40	2	0,0007	0,0916		
	Bb	15,2817	40	2	0,0007	0,0916		
2	A a	15,0845	40	2	0,0002	0,0265		
	Ba	14,8564	40	2	0,0074	ND		
	Bb	14,8564	40	2	0,001	0,1346		
3	A a	15,098	40	2	0,0007	0,0927		
	Ab	15,098	40	2	0,0011	0,1457		
	Ba	14,9472	40	2	0,0013	0,1739		
	Bb	14,9472	40	2	0,0008	0,1070		
1 NO	Aa	15,0055	40	2	0,001	0,1333	0,0906	0,0390
	Ab	15,0055	40	2	0,0004	0,0533		
	Ba	15,067	40	2	0,0006	0,0796		
	Bb	15,067	40	2	0,0009	0,1195		
2 NO	Aa	15,072	40	2	0,0004	0,0531		
	Ab	15,072	40	2	0,0011	0,1460		
	Ba	14,8956	40	2	0,0003	0,0403		
	Bb	14,8956	40	2	0,0008	0,1074		
3 NO	Aa	15,0511	40	2	ND	ND		
	Ab	15,0511	40	2	0,0009	0,1196		
	Ba	14,9144	40	2	0,0004	0,0536		
	Bb	14,9144	40	2	ND	ND		

Table I.3: Total lipid content in sludge and water fractions from enzymatic hydrolysis using Protamex® as proteolytic agent on snow crab rest raw material, batch 2. Capitalized letters are fraction parallel samples, and lowercase letter are parallels of the fraction sample. Abbreviations are Fr. = fraction, $CHCL_3$ = chloroform, SF = sludge fraction, WF = water fraction, Av. = average.

PROTAMEX

Fr.	Rep.	Nr.	Sample (g)	$CHCl_3$ (mL)	Evaporated $CHCl_3$ (mL)	Lipid (g)	Totalt lipid (%)	Av. total lipid (%)	SD
SF	1	A a	7,476	40	2	0,0083	2,2204	2,0268	0,2603
		Ab	7,476	40	2	0,0087	2,3274		
		Ba	6,006	40	2	0,0054	1,7982		
		Bb	6,006	40	2	0,0063	2,0979		
	2	A a	8,3919	40	2	0,0083	1,9781		
		Ab	8,3919	40	2	0,0089	2,1211		
		Ba	6,7809	40	2	0,0076	2,2416		
		Bb	6,7809	40	2	0,0078	2,3006		
	3	A a	6,2573	40	2	0,0044	1,4064		
		Ab	6,2573	40	2	0,0064	2,0456		
		Ba	7,0819	40	2	0,0065	1,8357		
		Bb	7,0819	40	2	0,0069	1,9486		
	1 NO	Aa	6,817	40	2	0,0071	2,0830	2,2332	0,2330
		Ab	6,817	40	2	0,0084	2,4644		
		Ba	4,4884	40	2	0,0047	2,0943		
		Bb	4,4884	40	2	0,006	2,6736		
	2 NO	Aa	5,3402	40	2	0,0061	2,2846		
		Ab	5,3402	40	2	0,0063	2,3595		
		Ba	5,7619	40	2	0,0057	1,9785		
		Bb	5,7619	40	2	0,0072	2,4992		
	3 NO	Aa	7,3747	40	2	0,0075	2,0340		
		Ab	7,3747	40	2	0,0084	2,2781		
		Ba	8,0513	40	2	0,0086	2,1363		
		Bb	8,0513	40	2	0,0077	1,9127		
WF	1	A a	14,9538	40	2	0,0016	0,2140	0,1163	0,0706
		Ab	14,9538	40	2	0,0006	0,0802		

	Ba	15,0357	40	2	0,0017	0,2261		
	Bb	15,0357	40	2	0,0009	0,1197		
2	A a	15,2372	40	2	0,0011	0,1444		
	Ab	15,2372	40	2	0,0011	0,1444		
	Ba	15,4333	40	2	0,0015	0,1944		
	Bb	15,4333	40	2	0,0003	0,0389		
3	A a	15,3809	40	2	0,0006	0,0780		
	Ab	15,3809	40	2	0,0003	0,0390		
	Ba	15,4888	40	2	0,0001	0,0129		
	Bb	15,4888	40	2	0,0008	0,1033		
1 NO	Aa	15,0708	40	2	0,0007	0,0929	0,0905	0,0571
	Ab	15,0708	40	2	0,0013	0,1725		
	Ba	15,4228	40	2	0,0012	0,1556		
	Bb	15,4228	40	2	0,0006	0,0778		
2 NO	Aa	15,395	40	2	0,0009	0,1169		
	Ab	15,395	40	2	0,0004	0,0520		
	Ba	15,3297	40	2	0,0004	0,0522		
3 NO	Aa	15,123	40	2	0,0011	0,1455		
	Ab	15,123	40	2	0,0001	0,0132		
	Ba	15,4519	40	2	0	0,0000		
	Bb	15,4519	40	2	0,0009	0,1165		

Table I.4: Total lipid content in sludge and water fractions from enzymatic hydrolysis using Papain GSM80 as proteolytic agent on snow crab rest raw material, batch 2. Capitalized letters are fraction parallel samples, and lowercase letter are parallels of the fraction sample. Abbreviations are Fr. = fraction, $CHCL_3$ = chloroform, SF = sludge fraction, WF = water fraction, Av. = average, ND = no data.

PAPAIN

Fr.	Rep.	Nr.	Sample (g)	$CHCl_3$ (mL)	Evaporated $CHCl_3$ (mL)	Lipid (g)	Totalt lipid (%)	Av. total lipid (%)	SD
SF	1	A a	6,6785	40	2	0,0056	1,6770	1,9185	0,1659
		Ab	6,6785	40	2	0,0061	1,8268		
		Ba	8,1683	40	2	0,0078	1,9098		
		Bb	8,1683	40	2	0,0074	1,8119		
	2	A a	8,3857	40	2	0,0078	1,8603		
		Ab	8,3857	40	2	0,009	2,1465		
		Ba	9,0038	40	2	0,0086	1,9103		
		Bb	9,0038	40	2	0,0102	2,2657		
	3	A a	6,6914	40	2	0,006	1,7933		
		Ab	6,6914	40	2	0,0069	2,0623		
		Ba	8,8357	40	2	0,008	1,8108		
		Bb	8,8357	40	2	0,0086	1,9466		
	1 NO	Aa	6,9313	40	2	0,0078	2,2507	2,0879	0,1282
		Ab	6,9313	40	2	0,0078	2,2507		
		Ba	8,127	40	2	0,0083	2,0426		
		Bb	8,127	40	2	0,0094	2,3133		
	2 NO	Aa	6,6406	40	2	0,0068	2,0480		
		Ab	6,6406	40	2	0,0072	2,1685		
		Ba	7,9709	40	2	0,0079	1,9822		
		Bb	7,9709	40	2	0,0081	2,0324		
	3 NO	Aa	9,4515	40	2	0,009	1,9045		
		Ab	9,4515	40	2	0,0095	2,0103		
		Ba	7,8984	40	2	0,0078	1,9751		
		Bb	7,8984	40	2	0,0082	2,0764		
WF	1	A a	15,408	40	2	ND	ND	0,0741	0,0373
		Ab	15,408	40	2	0	0,0000		
		Ba	15,4761	40	2	0,0007	0,0905		

	Bb	15,4761	40	2	0,0007	0,0905		
2	A a	15,35	40	2	0,0001	0,0130		
	Ab	15,35	40	2	0,0007	0,0912		
	Ba	15,4358	40	2	0,0007	0,0907		
	Bb	15,4358	40	2	0,0006	0,0777		
3	A a	15,2761	40	2	0,0009	0,1178		
	Ab	15,2761	40	2	0,0006	0,0786		
	Ba	15,385	40	2	ND	ND		
	Bb	15,385	40	2	0,0007	0,0910		
1 NO	Aa	15,3064	40	2	0,0003	0,0392	0,0459	0,0592
	Ab	15,3064	40	2	ND	ND		
	Ba	15,3959	40	2	0	0,0000		
	Bb	15,3959	40	2	ND	ND		
2 NO	Aa	15,2877	40	2	0,0001	0,0131		
	Ab	15,2877	40	2	ND	ND		
	Ba	15,2475	40	2	ND	ND		
	Bb	15,2475	40	2	0,001	0,1312		
3 NO	Aa	15,1981	40	2	ND	ND		
	Ab	15,1981	40	2	ND	ND		
	Ba	15,2875	40	2	ND	ND		
	Bb	15,2875	40	2	ND	ND		

Table I.5: Total lipid content in sludge and water fractions from enzymatic hydrolysis using Corolase® 2TS as proteolytic agent on snow crab rest raw material, batch 2. Samples designated with I, II etc. are parallels from same fraction sample or merged replica, capitalized letters are fraction parallel samples, and lowercase letter are parallels of the fraction sample. Replica 1-3 and 6 are treated with different temperatures during enxymatic hydrolysis, 50° and 65°C, respectively. Abbreviations are Fr. = fraction, $CHCL_3$ = chloroform, SF = sludge fraction, WF = water fraction, Av. = average.

COROLASE

Fr.	Rep.	Nr.	Sample (g)	$CHCl_3$ (mL)	Evaporated $CHCl_3$ (mL)	Lipid (g)	Totalt lipid (%)	Av. total lipid (%)	SD
SF	1	Ia	8,496	40	2	0,0091	2,1422	2,2308	0,1670
	CO^{50}	Ib	8,496	40	2	0,0098	2,3070		
		IIa	7,2505	40	2	0,0079	2,1792		
		IIb	7,2505	40	2	0,0078	2,1516		
	2	Ia	8,6146	40	2	0,0095	2,2056		
		Ib	8,6146	40	2	0,0112	2,6002		
		IIa	8,5512	40	2	0,0089	2,0816		
		IIb	8,5512	40	2	0,0087	2,0348		
	3	Ia	5,3789	40	2	0,0055	2,0450		
		Ib	5,3789	40	2	0,0061	2,2681		
		IIa	6,5616	40	2	0,008	2,4384		
		IIb	6,5616	40	2	0,0076	2,3165		
	6	DIa	7,5571	40	2	0,005	1,3233	1,7393	0,2163
	CO^{65}	DIb	7,5571	40	2	0,006	1,5879		
		DIIa	5,0528	40	2	0,0048	1,8999		
		DIIb	5,0528	40	2	0,0043	1,7020		
		EIa	6,3416	40	2	0,0062	1,9553		
		EIb	6,3416	40	2	0,0067	2,1130		
		EIIa	6,7979	40	2	0,0049	1,4416		
		EIIb	6,7979	40	2	0,006	1,7653		
		FIa	7,0176	40	2	0,0062	1,7670		
		FIb	7,0176	40	2	0,0065	1,8525		
		FIIa	6,3507	40	2	0,0056	1,7636		
		FIIb	6,3507	40	2	0,0054	1,7006		
	1 NO	Ia	5,8541	40	2	0,0048	1,6399	2,1421	0,2922

CO^{50}	Ib	5,8541	40	2		0,0059	2,0157		
	IIa	5,1316	40	2		0,0055	2,1436		
	IIb	5,1316	40	2		ND	ND		
2 NO	Ia	8,0642	40	2		0,009	2,2321		
	Ib	8,0642	40	2		0,0078	1,9345		
	IIa	5,1481	40	2		0,0054	2,0979		
	IIb	5,1481	40	2		0,0064	2,4864		
3 NO	Ia	3,722	40	2		0,0039	2,0956		
	Ib	3,722	40	2		0,0049	2,6330		
6 NO	CIa	7,3217	40	2		0,0069	1,8848	1,7009	0,1684
CO^{65}	CIb	7,3217	40	2		0,0066	1,8029		
	CIIa	8,9859	40	2		0,007	1,5580		
	CIIb	8,9859	40	2		0,007	1,5580		

As most of the fractions are analysed for lipid after merging of parallels, the average recovery of lipids in each fraction was based on known average size of fractions (%) and the general starting mass of RRM (30g). Before enzymatic treatment, the RRM sample was added 15 g water, thus the hydrolysis mix had a total mass of 45 g. The mass of added enzyme (0,03 g) is neglected here. The amount of lipid in such a sample was 1,263 g for batch 1, and 0,549 g for batch 2 (4,21% and 1,83%, respectively). Based on average fraction constituents SF and WF of total hydrolysis mixture, the amount (%) of lipid from the total lipid in RRM sample has been calculated (**Table I.6**).

Table I.6: Average recovery of lipid in sludge (SF) and water (WF) fractions obtained by enzymatic hydrolysis. Proteases used were Alcalase® 2.4 L (AL, batch 1 and 2), Protamex® (PR), Papain GSM80 (PA) and Corolase® 2TS (CO).

Fraction	Enzyme	Av. Total lipid in fraction (%)	SD	Average fraction (%)	Average fraction (g)	Average lipid in fraction (g)	Recovery of lipid % from RRM
SF	AL (B1)	11,2185	1,7851	46,49	20,922	2,347	185,84
	AL (B2)	2,0332	0,3368	52,24	23,507	0,478	87,06
	PR	2,0268	0,2603	52,39	23,575	0,478	87,03
	PA	1,9185	0,1659	53,80	24,211	0,464	84,60
	CO50	2,2308	0,1670	49,73	22,378	0,499	90,93
	CO65	1,7393	0,2163	49,67	22,352	0,389	70,82
Control	AL (B1)	10,3466	0,8543	46,46	20,908	2,163	171,28
	AL (B2)	2,3458	0,2034	47,03	21,163	0,496	90,43
	PR	2,2332	0,2330	50,65	22,795	0,509	92,72
	PA	2,0879	0,1282	53,18	23,929	0,500	91,00
	CO50	2,1421	0,2922	49,61	22,324	0,478	87,10
	CO65	1,7009	0,1684	53,87	24,243	0,412	75,11
WF	AL (B1)	1,7910	0,5079	52,72	23,726	0,425	33,64
	AL (B2)	0,1024	0,0517	46,73	21,027	0,022	3,92
	PR	0,1163	0,0706	46,85	21,084	0,025	4,47
	PA	0,0741	0,0373	45,00	20,252	0,015	2,73
Control	AL (B1)	1,5406	0,2890	53,06	23,876	0,368	29,12
	AL (B2)	0,0906	0,0390	52,41	23,585	0,021	3,89
	PR	0,0905	0,0571	48,28	21,727	0,020	3,58
	PA	0,0459	0,0592	45,75	20,589	0,009	1,72

J. Molecular weight distributions in Corolase® 2TS hydrolysates

Molecular weight distribution in hydrolysates obtained by Corolase® 2TS at 50°C and 65°C was analysed by FPLC. In analysis of proteins using fast protein liquid chromatography the molecules in the solution are separated by gel filtration, and detected by absorbance as they elute from the column according to decreasing size. The results generate chromatograms with retention time as a function of absorbance (chapter 3.3.6). Elution volume was found for all data points given the flow of 0,5 mL/min. Peaks in chromatograms was found by graphic reading. This highest detected absorbance represent main peptide size groups.
A relation between elution volume and molecular weight must be established using standard samples with known molecular weights. In this project, standard samples of Vitamin B12 (M_w = 1356,57), Aprotinin (M_w = 6512) and Cytochrome C (M_w = 12384) (1 mg/mL) was used (**Table J.1**). Partition coefficient K_{av} was calculated for peaks in chromatograms for each standard sample and water fractions obtained by enzymatic hydrolysis using Corolase® 2TS as proteolytic agent at 50°C and 65°C.
In equation VII, V_e is elution volume, V_0 is void volume (8 mL) and V_t is total bed volume (24 mL). Calculation is shown for Vitamin B12 values. K_{av} for standard samples was plotted against known log(Mw) to generate a standard curve (**Figure J.1**, $\log(M_w) = -2{,}9816K_{av} + 5{,}3643$). This relation was used to find molecular weights of main peptide groups in hydrolysates from enzymatic hydorlysis. The partition coefficient was calculated for all tops and molecular weight of main peptide size groups was found by the standard curve (**Table J.2**). The second highest apex in analysis of water fractions showed a molecular weight of about 40 Da, and was excluded from the results as the detection range of the instrument is 100-7000 Da.

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \qquad \text{VIII}$$

$$K_{av}(\text{VitB12}) = \frac{18{,}52-8}{24-8} = 0{,}66$$

Table J.1: Standards used for determination of relation between elution volume and molecular weight. Kav values are calculated as above.

Standard	Peak mAU	Da (g/mol)	logDa	V_e	V_t	V_0	Kav
Vitamin B12	61,15	1356,57	3,13	18,52	24,00	8,00	0,66
Aprotinin	13,95	6512,00	3,81	18,00	24,00	8,00	0,63
CytC	70,24	12384,00	4,09	14,60	24,00	8,00	0,41

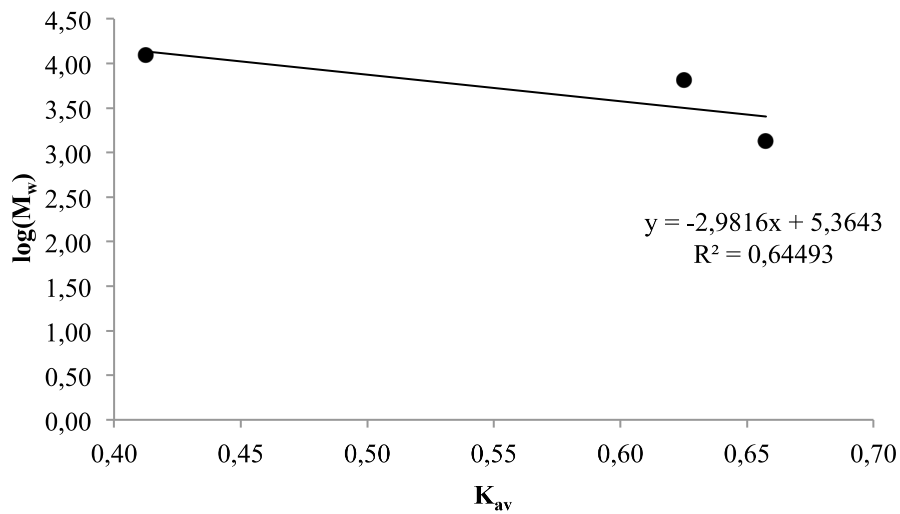

Figure J.1: Plot of partition coefficient against $\log(M_w)$ of standard samples Vitamin B12, Aprotinin and Cytochrome C to determine relation between the factors. Linear regression was used to determine the equation of the line.

Table J.2: Determination of molecular weight (M_w, g/mol) of main peptide size groups (top 1,2 and 3) in hydrolysates from enzymatic hydrolysis using Corolase® 2TS at temperature 50°C (CO50) and 65°C (CO65). Control samples for each treatment was also analysed. M_w was found by standard curve $\log(M_w) = -2{,}9816K_{av} + 5{,}3643$.

Water fraction	Top nr	Absorbance (mAU)	Elution volume (Ve)	Void volume (V0, mL)	Total bed volume (Vt, mL)	K_{av}	log(Mw)	Mw (g/mol)
CO50°C	1	287	18,32	8,00	24,00	0,65	3,44	2761,65
	2	86	20,72	8,00	24,00	0,80	2,99	986,12
control	1	275	18,32	8,00	24,00	0,65	3,44	2761,65
	2	61	20,60	8,00	24,00	0,79	3,02	1038,22
CO65°C	1	277	18,32	8,00	24,00	0,65	3,44	2761,65
	2	90	20,80	8,00	24,00	0,80	2,98	952,84
control	1	267	18,32	8,00	24,00	0,65	3,44	2761,65
	2	59	20,68	8,00	24,00	0,79	3,00	1003,19

K. Heat treatment

1. Fractions obtained

Heat treatment was performed on snow crab rest raw material, batch 2, according to method described in chapter 2.5. The fractions obtained after centrifugation and separation by decantation was measured and the constituent sludge and water fractions are presented in **Table K.1**. Constituents are based on total treatment mix, which means water is included for replica 4. Final values are average and standard deviation of parallel samples from replica 1-3 and 6.

Table K.1: Fractions obtained by heat treatment of snow crab rest raw material, batch 2. Replica 1-3 was not added water, and total mixture is therefor only the crude material. Abbreviations are Rep. = replica, SF = sludge fraction, Av. = average, WF = water fraction.

Rep.	Nr	Crude material (g)	Water added (g)	Total mixture (g)	SF (g)	SF (%)	Av. SF (%)	SD	WF (g)	WF (%)	Av. WF (%)	SD
1	A	50,2551	-	50,2551	38,2012	76,0146	71,3217	3,6697	8,8295	17,5694	20,8227	2,1426
	B	49,5968	-	49,5968	38,5423	77,7113			8,4638	17,0652		
	C	49,9821	-	49,9821	37,4553	74,9374			9,3990	18,8047		
	D	50,4282	-	50,4282	38,2932	75,9361			9,1941	18,2321		
	E	51,4828	-	51,4828	38,3884	74,5655			9,8592	19,1505		
	F	51,422	-	51,422	37,4684	72,8645			10,3203	20,0698		
2	A	39,8409	-	39,8409	26,655	66,9036			8,7836	22,0467		
	B	39,1945	-	39,1945	26,5122	67,6427			8,8631	22,6131		
	C	40,7784	-	40,7784	27,3542	67,0801			9,3149	22,8427		
	D	43,0234	-	43,0234	31,5756	73,3917			9,5174	22,1215		
	E	39,9111	-	39,9111	27,1047	67,9127			9,4791	23,7505		
	F	39,455	-	39,455	29,0501	73,6284			7,2682	18,4215		
3	A	39,8494	-	39,8494	26,9979	67,7498			8,8363	22,1742		
	B	40,7218	-	40,7218	28,4758	69,9277			9,1707	22,5204		
	C	40,1345	-	40,1345	28,1279	70,0841			8,5681	21,3485		
	D	43,8052	-	43,8052	30,5605	69,7645			9,2459	21,1069		
	E	39,2522	-	39,2522	28,1148	71,6260			8,2862	21,1102		
	F	35,1191	-	35,1191	23,196	66,0495			8,3800	23,8617		

4	A	29,342	14,6763	44,0183	22,4854	51,0819	49,8458	1,4360	21,4256	48,6743	49,6981	1,4903
	B	29,79	15,0929	44,8829	21,4296	47,7456			23,2202	51,7351		
	C	29,741	14,8696	44,6106	22,14	49,6295			22,2689	49,9184		
	D	30,8347	15,6393	46,474	22,887	49,2469			23,4419	50,4409		
	E	29,9419	15,2184	45,1603	22,3792	49,5550			22,5824	50,0050		
	F	30,686	15,2994	45,9854	23,8278	51,8160			21,8039	47,4148		

2. Increase of soluble protein by heat treatment

Estimation of soluble protein content in water fractions obtained by heat treatment of snow crab rest raw material, batch 2 was performed (Table K.2). Method is described in chapter 2.3.5, calculations are demonstrated in appendix G, and results presented in. Replica 1-3 and 4 are separated as the mixtures differ by addition of water in replica 4 (**Table K.1**).

Table K.2: Raw data for soluble protein in fractions obtained by heat treatment (70°C) of snow crab RRM, batch 1. Concentration of soluble protein (mg/mL) is determined using Lowry method. Abbreviations are WF = water fraction, rep. = replica, Av. = average.

Fr.	Rep.	Nr	Original sample (g)	Parallel for Lowry (mL)	Ratio sample: water	Absorbance			Standard curve (x = μg/mL, y = abs.)	Av. (a-c) soluble protein (mg/mL, corr. dilution)	Av. (replica) soluble protein (mg/mL)	SD	WF (mL)	Soluble protein in fraction (mg)	Total soluble protein/ RRM (mg/g, w/ww)	Av. (mg/g RRM, w/ww)	SD	p-value WF (HT) vs RRM	% increase from RRM	Av. % increase from RRM	SD
						a	b	c													
WF	1	I	151,72	0,08	250	0,433	0,442	0,447	$x = \frac{y-0{,}0283}{0{,}002}$	51,5458	49,4208	4,3842	28,0877	1447,8039	9,5426	10,2796	1,2712	**	-30,1594	-24,7653	9,3038
		II	151,447	0,08	250	0,437	0,439	0,437		51,1708			27,9782	1431,6678	9,4533				-30,8133		
	2	I	120,5304	0,08	250	0,433	0,441	0,439		51,1708			27,5776	1411,1688	11,7080				-14,3113		
		II	121,6729	0,08	250	0,353	0,351	0,353		40,5042			25,6487	1038,8792	8,5383				-37,5097		
	3	I	119,2361	0,08	250	0,44	0,442	0,441		51,5875			25,6906	1325,3138	11,1150				-18,6510		
		II	119,6461	0,08	250	0,43	0,432	0,436		50,5458			26,7966	1354,4565	11,3205				-17,1471		
	4	I	89,0249	0,08	250	0,319	0,317	0,316	$x = \frac{y-0{,}0306}{0{,}0022}$	32,5833	32,1951	0,5491	66,2769	2159,5223	24,2575	24,0533	0,2887	***	77,5364	76,0422	2,1132
		II	91,3107	0,08	250	0,309	ND	0,312		31,8068			68,4660	2177,6856	23,8492				74,5479		

3. Free amino groups in heat treatment water fractions

Free amino groups in the water fractions obtained by heat treatment (70°C) of batch 2 was determined by formol titration (**Table K.3**). The amount of free amino acids (mg/mL) is calculated given that the density of the water fractions are 1g/mL. The water fraction parallels generated with enzymatic hydrolysis had been merged, and the formol titration is performed with 2-4 parallels per merged fraction. Calculations for amount of free amino groups are shown in appendix D for the RRM. The results from heat treatment at 70°C has been compared to control samples from enzymatic treatment as there are simple heat treatments as well. Data for free amino groups in all heat treatments except the HT 70°C can be viewed in appendix H.

Table K.3: Concentration of of free amino groups (mg/mL) in water fractions obtained by heat treatment (70°C).

Fraction	Replica	Nr	Sample (g)	NaOH brukt (mL)	Free aa. g/100 mL WF	mg/mL WF	Av. mg/mL WF	SD
WF	1	A	1,511	4,416	0,4094	4,0936	4,5610	1,7065
		B	1,5146	5,11	0,4726	4,7257		
	2	A	1,5603	4,61	0,4138	4,1385		
		B	1,5335	3,701	0,3380	3,3805		
	3	A	1,6658	5,134	0,4317	4,3170		
		B	1,523	9,998	0,9195	9,1951		
	4	A	1,5239	4,61	0,4237	4,2373		
		B	1,5483	4,42	0,3999	3,9986		
		C	1,8671	4	0,3001	3,0008		
		D	1,5703	5,07	0,4522	4,5224		

L. Pigment extraction

1. Yield of fractions

The rest raw material samples were heated at 65°C for 1 hour, added rapeseed oil in oil to RRM ratio 1:2. The mixture was centrifuged and separated by decantation. Sludge and water fractions obtained were measured, and the yield based on starting samples was calculated (**Table L.1**). Water fractions were discarded because of small volume.

Table L.1: Sludge fraction (SF) and oil fraction (OF) obtained after heat treatment of crude material added rapeseed oil. The yield of fractions (%) is based on the original crudematerial for SF, and oil added for OF. Two replicas of this experiment was performed, with six parallel samples in each.

Treatment	Replica	Nr	Crude material (g)	Oil added (g)	SF (g)	SF (%)	Av. SF (%)	SD	OF (g)	OF (%) yield	Av. OF yield (%)	SD
$HT^{65°C}_{oil\ 1:2}$	1	A	20,1837	10,1011	15,9628	79,0876	74,8406	13,3600	8,3202	82,36925	81,6834	1,8889
		B	20,252	10,1078	15,2119	75,1131			8,1905	81,03148		
		C	20,0643	10,0296	15,2734	76,1223			8,5706	85,45306		
		D	20,1796	10,3482	15,1512	75,0818			8,3216	80,41592		
		E	20,5179	10,34	15,6999	76,5181			8,5266	82,46228		
		F	20,1874	10,4819	15,5309	76,9336			8,6279	82,31237		
	2	A	20,4543	10,3263	16,4878	80,6080			8,5939	83,22342		
		B	19,9554	9,9499	16,1222	80,7912			8,0016	80,41890		
		C	19,8229	9,9862	16,489	83,1816			7,7869	77,97661		
		D	20,2403	10,9416	16,1262	79,6737			8,9177	81,50271		
		E	20,4413	10,2812	16,7009	81,7018			8,5214	82,88332		
		F	20,4238	10,4641	6,796	33,2749			8,3871	80,15118		

Combination experiment

A combination of enzymatic hydrolysis and heat treatment with rapeseed oil was performed on snow crab rest raw material. Sludge samples from enzymatic hydrolysis with Corolase® 2TS as proteolytic agent was heated in water bath with rapeseed oil, as described in chapter 2.6. **Table L.2** presents the results for sludge fractions and oil fractions obtained after the heat treatment. Water fractions were discarted because of small volume. Constituents of fractions are based on starting mass of enzyme sludge and rapeseed oil for SF (%) and OF (%), respectively. OF was not measured for $CO^{65°C}HT^{65°C}_{oil\ 2:1}$.

Table L.2: Sludge fraction (SF) and oil fraction (OF) obtained after heat treatment of sludge fractions from enzymatic hydrolysis added rapeseed oil. The yield of fractions (%) is based on the original enzymatic sludge (g) for SF, and rapeseed oil added (g) for OF. Samples designated NO are heat treated control sludge samples from enzymatic hydrolysis. Replica are replicas of the heat treatment, and number indicate which replica of enzymatic hydrolysis sludge that is used. Letters i-ii indicate parallels of the enzyme replica made for heat treatment.

Treatment	Replica	Nr	Ez. SF sample (g)	Oil added (g)	SF (g)	SF (%)	Av. SF (%)	SD	OF (g)	OF (%) yield	Av. OF yield (%)	SD
$CO^{50°C}HT^{65°C}_{oil\ 1:2}$	1	A	19,9052	9,9229	18,646	93,6740	95,8941	4,0531	7,7646	78,2493	69,7840	6,7924
		B	20,0251	10,7135	20,4569	102,1563			7,6073	71,0067		
		C	19,6775	9,8645	18,2981	92,9900			8,0247	81,3493		
	2	A	11,2843	5,6329	10,7367	95,1472			3,6483	64,7677		
		B	10,5189	5,2654	9,9437	94,5317			3,3053	62,7740		
		C	10,5294	5,2851	10,3598	98,3893			3,4383	65,0565		
		D	10,264	5,1913	10,2908	100,2611			3,6177	69,6877		
		E	9,2426	4,6512	8,3186	90,0028			3,041	65,3810		
	NO 1	A	20,5313	10,5166	19,1257	93,1539	99,5894	5,6130	8,3417	79,3194	72,2287	9,3749
		B	20,0267	10,113	19,2239	95,9914			8,191	80,9948		
		C	18,913	9,401	18,5788	98,2330			7,1828	76,4046		
	NO 2	A	6,0854	3,0903	6,3112	103,7105			1,8675	60,4310		
		B	4,5434	2,2835	4,855	106,8583			1,4613	63,9939		
$CO^{50°C}HT^{65°C}_{oil\ 1:2}$	1	A	19,9704	9,9826	19,4176	97,2319	105,7193	7,9022	8,3152	83,29694	78,2404	7,6963
		B	20,5233	10,2027	21,9726	107,0617			8,3704	82,04103		
(stored at -20 for 12 hours after centrifugation, with subsequent additional centrifugation)		C	18,2995	9,2413	20,6536	112,8643			6,4119	69,38310		
	NO 1	A	19,1217	9,648	21,5931	112,9246	113,4032	0,4643	6,9395	71,92682	69,1962	2,5371
		B	19,4099	9,7033	22,0985	113,8517			6,671	68,74981		
		C	15,0425	7,5187	17,0632	113,4333			5,0309	66,91183		

$CO^{65°C}HT^{65°C}_{oil\ 1:2}$	1	A	10,2063	5,1083	9,9107	97,1037	98,1764	2,9556	3,152	61,70350	71,8342	6,0003
		B	10,9306	5,492	10,628	97,2316			3,9781	72,43445		
		C	10,026	5,1251	10,228	102,0148			3,5679	69,61620		
		D	11,0437	5,5244	10,7408	97,2573			4,2525	76,97669		
	2	A	10,0799	5,056	9,9882	99,0903			3,2887	65,04549		
		B	10,1031	5,0573	9,8001	97,0009			3,9997	79,08766		
		C	10,0721	5,0793	10,3128	102,3898			3,7925	74,66580		
		D	9,9231	4,2678	9,2605	93,3227			3,207	75,14410		
	NO 1	A	10,4589	5,2389	10,7432	102,7183	97,8394	2,9892	4,0039	76,42635	76,6869	3,2742
		B	10,3851	5,2476	9,8904	95,2364			4,0955	78,04520		
		C	10,431	5,239	10,3728	99,4420			3,9883	76,12712		
		D	9,7708	4,887	9,8502	100,8126			3,456	70,71823		
	NO 2	A	10,2908	5,4261	9,9706	96,8885			4,291	79,08074		
		B	10,354	5,2387	9,86	95,2289			4,0983	78,23124		
		C	9,7283	4,8907	9,5455	98,1209			3,9699	81,17243		
		D	11,0384	5,6132	10,4056	94,2673			4,1366	73,69415		
$CO^{65°C}HT^{65°C}_{oil\ 2:1}$	1	A	10,7632	21,5189	11,7268	108,9527	103,3949	9,0310	ND	ND	ND	ND
		B	10,0724	20,2908	9,7391	96,6910			ND	ND		
		C	7,6976	15,0812	8,246	107,1243			ND	ND		
		D	3,8047	7,6094	4,557	119,7729			ND	ND		
	2	A	13,5725	27,0207	13,1012	96,5275			ND	ND		
		B	14,1722	29,4181	14,13	99,7022			ND	ND		
		C	14,7691	29,4448	14,0297	94,9936			ND	ND		
	NO 1	A	8,9566	18,0342	7,9724	89,0115	100,9146	17,3570	ND	ND		
		B	8,0528	16,1528	7,3749	91,5818			ND	ND		
		C	6,638	13,381	9,0128	135,7758			ND	ND		
		D	6,0977	12,2468	5,8808	96,4429			ND	ND		
	NO 2	A	16,8942	31,6266	16,205	95,9205			ND	ND		
		B	16,3045	34,6293	15,7754	96,7549			ND	ND		

2. Astaxanthin recovery with rapeseed oil

Oil fractions from experiments aiming on extracting astaxanthin in rapeseed oil was analysed for astaxanthin using spectrophotometry (chapter 2.3.7). Calculating concentration of astaxanthin (µg/g lipid) in the samples were performed after Tolasa et al. (2005). As rapeseed oil was not successfully separated from the sludge after heating together. Total separation is assumed to be possible, so the amount of oil added before start was used to calculate astaxanthin recovery. In order to compare the recovery of astaxanthin in rapeseed oil from the RRM, the yield of astaxanthin was calculated for all experiments (**Table L.3- Table L.7**). For combination experiments, the recovery of astaxanthin was calculated based on total amount of astaxanthin in RRM, and for amount of astaxanthin in SF used for heat treatment with oil. Calculations for the latter are presented above **Table L.4**.

Astaxanthin in the oil fraction (µg/OF) = 3,2489 × 10,1011 $\left(\frac{\mu g}{g\ lipid} \times g\ OF\right)$ = 32,8171 µg/OF

Astaxanthin per RRM (µg/g) = $\frac{32,8171}{20,1837}$ $\left(\frac{\frac{\mu g}{OF}}{\frac{g\ SF}{OF}}\right)$ =1,6259 µg/g SF

1 g RRM ≈ 11,7927 µg astaxanthin (appendix D)

Recovery of astaxanthin (%) = $\frac{1,6259\ \frac{\mu g}{g\ SF}}{11,7927\frac{\mu g}{g\ SF}}$ ×100% = 13,7875% astaxanthin recovered from RRM

Table L.3: Raw data from astaxanthin analysis of rapeseed oil from heat treatment (65°C, 1 h) of snow crab rest raw material.

$HT^{65°C}_{oil\ 1:2}$	Rep.	Sample	Nr	RRM sample (g)	Oil added/lipid extracted (g)	Lipid sample (g)	n-hexane (mL)	Absorbance	Astaxanthin ug/g lipid	µg/ OF	Astaxanthin per RRM (µg/g, w/ww)	Recovery of Astaxanthin (%) from RRM	Average recovery (%)	SD
	1	A	I	20,1837	10,1011	0,3078	5	0,042	3,2489	32,8171	1,6259	13,7875	11,5686	3,2299
			II	20,1837	10,1011	0,305	5	0,042	3,2787	33,1184	1,6408	13,9141		
			III	20,1837	10,1011	0,2124	5	0,047	5,2686	53,2185	2,6367	22,3588		
		B	I	20,252	10,1078	0,3052	5	0,041	3,1985	32,3301	1,5964	13,5371		
			II	20,252	10,1078	0,3078	5	0,035	2,7074	27,3657	1,3513	11,4584		
			III	20,252	10,1078	0,3214	5	0,044	3,2595	32,9469	1,6268	13,7954		
		C	I	20,0643	10,0296	0,3169	5	0,04	3,0053	30,1420	1,5023	12,7390		
			II	20,0643	10,0296	0,3169	5	0,033	2,4794	24,8672	1,2394	10,5097		
			III	20,0643	10,0296	0,3176	5	0,042	3,1486	31,5793	1,5739	13,3465		

	D	I	20,1796	10,3482	0,3099	5	0,026	1,9976	20,6713	1,0244	8,6864
		II	20,1796	10,3482	0,3125	5	0,021	1,6000	16,5571	0,8205	6,9576
		III	20,1796	10,3482	0,2952	5	0,02	1,6131	16,6928	0,8272	7,0146
	E	I	20,5179	10,34	0,2919	5	0,031	2,5286	26,1456	1,2743	10,8057
		II	20,5179	10,34	0,3252	5	0,031	2,2697	23,4683	1,1438	9,6992
		III	20,5179	10,34	0,3258	5	0,028	2,0462	21,1582	1,0312	8,7444
	F	I	20,1874	10,4819	0,289	5	0,028	2,3068	24,1797	1,1978	10,1568
		II	20,1874	10,4819	0,2977	5	0,03	2,3993	25,1497	1,2458	10,5643
		III	20,1874	10,4819	0,3081	5	0,031	2,3956	25,1108	1,2439	10,5479
2	A	I	20,4543	10,3263	0,301	5	0,035	2,7685	28,5889	1,3977	11,8522
		II	20,4543	10,3263	0,3046	5	0,032	2,5013	25,8295	1,2628	10,7082
		III	20,4543	10,3263	0,2858	5	0,032	2,6659	27,5285	1,3459	11,4126
	B	I	19,9554	9,9499	0,3291	5	0,027	1,9534	19,4359	0,9740	8,2591
		II	19,9554	9,9499	0,3018	5	0,033	2,6034	25,9038	1,2981	11,0075
		III	19,9554	9,9499	0,2734	5	0,029	2,5255	25,1286	1,2592	10,6781
	C	I	19,8229	9,9862	0,3287	5	0,062	4,4910	44,8480	2,2624	19,1850
		II	19,8229	9,9862	0,3045	5	0,047	3,6750	36,6996	1,8514	15,6993
		III	19,8229	9,9862	0,277	5	0,048	4,1258	41,2014	2,0785	17,6251
	D	I	20,2403	10,9416	0,281	5	0,025	2,1183	23,1774	1,1451	9,7104
		II	20,2403	10,9416	0,3309	5	0,035	2,5184	27,5552	1,3614	11,5444
		III	20,2403	10,9416	0,3016	5	0,026	2,0525	22,4581	1,1096	9,4090
	E	I	20,4413	10,2812	0,3062	5	0,032	2,4883	25,5823	1,2515	10,6125
		II	20,4413	10,2812	0,2882	5	0,029	2,3958	24,6319	1,2050	10,2183
		III	20,4413	10,2812	0,2808	5	0,03	2,5438	26,1528	1,2794	10,8492
	F	I	20,4238	10,4641	0,2915	5	0,023	1,8786	19,6581	0,9625	8,1619
		II	20,4238	10,4641	0,2782	5	0,023	1,9684	20,5979	1,0085	8,5521
		III	20,4238	10,4641	0,2929	5	0,035	2,8451	29,7715	1,4577	12,3609

Calculations for recovery of astaxanthin after combination experiment enzymatic hydrolysis with subsequent heat treatment including rapeseed oil are presented below. Calculations are demonstrated for sample I, CO1 a, CO50HT65 oil 1:2 (**Table L.4**). The values for concentration of astaxanthin in oil analysed after Tolasa et al. (2005) can be viewed in appendix D.

Concentration of astaxanthin in rapeseed oil: 2,9761 μg/ g oil

Total amount of rapeseed oil added 9,9229 g

Total amount astaxanthin in OF = 2,9761 × 9,9229 ($\frac{\mu g}{g\ oil} \times g\ oil$) = <u>29,5315 μg astaxanthin per SF</u>

Astaxanthin available in SF before heating with oil:

Total SF (g) = 19,9052 g, total lipid = 2,2308% (appendix I)

Total amount of lipid in SF = 0,4441 g

Astaxanthin in batch 2: 644,41 μg/g lipid (appendix D)

Astaxanthin in SF (μg) = 644,41 × 0,4441 ($\frac{\mu\text{g astaxanthin}}{g\ lipid} \times g\ lipid$) = <u>286,18 μg astaxanthin per SF</u>

Recovered astaxanthin per SF (%) = $\frac{29,5315}{286,18} \times 100\%$ ($\frac{\mu\text{g astaxantin in OF}}{astaxatnhin\ in\ SF}$) = <u>10,32% recovered from SF to OF</u>

Astaxantin available in original RRM sample before combination experiment:

Yield of SF = 74,9719%

RRM sample before combination experiment (g) = $\frac{SF\ (g)}{0,749719}$ = 26,5502 g RRM

Total lipid in RRM sample = 2,2308% (appendix D)

Total amount of lipid in SF = 0,4859 g

Astaxanthin in RRM sample (µg) = 644,41 × 0,4859 ($\frac{\mu\text{g astaxanthin}}{g\ lipid} \times g\ lipid$) = <u>313,0986 µg per RRM sample</u>

Astaxanthin recovered from RRM sample = $\frac{29,5315}{313,0986}$ ×100% ($\frac{\mu\text{g astaxantin in OF}}{astaxatnhin\ in\ SF}$) = <u>9,4323% recovered from RRM to OF</u>

Table L.4: Raw data from astaxanthin analysis of rapeseed oil from combination experiment of snow crab rest raw material. Rest raw material was exposed to enzymatic hydrolysis using Corolase® 2TS as proteolytic agent, at 50°C for 1 hour. After centrifugation and separation of sludge from water fraction, the sludge fraction was heated together with rapeseed oil (oil:SF ratio 1:2) at 65°C for 1 hour. Concentration of astaxanthin is measured using spectrophotometry.

$CO^{50°C}HT^{65°C}_{oil\ 1:2}$

Rep.	Nr	SF (g)	Oil added/ (g)	Lipid in sample (%)	Lipid in SF (g)	SF (ug/g)	Lipid sample (g)	n-hexane (mL)	Abs.	Astaxanthin (ug/g lipid)	ug/OF	Astaxanthin recovery from SF (%)	Av. Ax extracted (%)	SD	SF % yield	Theoretical RRM sample for enz hydr	Lipid (g) in RRM sample	Astaxanthin (ug) in RRM sample	Astaxanthin recovery from RRM (%)	Av. Recovery from RRM	SD
CO1 a	I	19,9052	9,9229	2,2308	0,4440	286,1472	0,1200	3,0000	0,0250	2,9762	29,5324	10,3207	6,2636	2,3048	74,9719	26,5502	0,4859	313,0986	9,4323	5,7244	2,1064
	II	19,9052	9,9229	2,2308	0,4440	286,1472	0,1200	3,0000	0,0240	2,8571	28,3511	9,9079			74,9719	26,5502	0,4859	313,0986	9,0550		
	III	19,9052	9,9229	2,2308	0,4440	286,1472	0,1154	3,0000	0,0230	2,8472	28,2529	9,8735			74,9719	26,5502	0,4859	313,0986	9,0236		
CO2 a	I	20,0251	10,7135	2,2308	0,4467	287,8708	0,1218	3,0000	0,0180	2,1112	22,6182	7,8571			74,9719	26,7101	0,4888	314,9846	7,1807		
	II	20,0251	10,7135	2,2308	0,4467	287,8708	0,1093	3,0000	0,0160	2,0912	22,4044	7,7828			74,9719	26,7101	0,4888	314,9846	7,1129		
	III	20,0251	10,7135	2,2308	0,4467	287,8708	0,1552	3,0000	0,0240	2,2091	23,6675	8,2216			74,9719	26,7101	0,4888	314,9846	7,5139		
CO3 a	I	19,6775	9,8645	2,2308	0,4390	282,8739	0,1183	3,0000	0,0140	1,6906	16,6771	5,8956			74,9719	26,2465	0,4803	309,5170	5,3881		
	II	19,6775	9,8645	2,2308	0,4390	282,8739	0,1192	3,0000	0,0160	1,9175	18,9156	6,6869			74,9719	26,2465	0,4803	309,5170	6,1113		
	III	19,6775	9,8645	2,2308	0,4390	282,8739	0,1128	3,0000	0,0140	1,7730	17,4902	6,1831			74,9719	26,2465	0,4803	309,5170	5,6508		
CO1 a	I	11,2843	5,6329	2,2308	0,2517	162,2174	0,2152	5,0000	0,0100	1,1064	6,2322	3,8419			74,9719	15,0514	0,2754	177,4963	3,5112		
	II	11,2843	5,6329	2,2308	0,2517	162,2174	0,2185	5,0000	0,0120	1,3076	7,3657	4,5406			74,9719	15,0514	0,2754	177,4963	4,1498		
	III	11,2843	5,6329	2,2308	0,2517	162,2174	0,2169	5,0000	0,0070	0,7684	4,3283	2,6682			74,9719	15,0514	0,2754	177,4963	2,4386		
CO1 b	I	10,5189	5,2654	2,2308	0,2347	151,2144	0,2411	5,0000	0,0150	1,4813	7,7997	5,1580			74,9719	14,0304	0,2568	165,4569	4,7140		
	II	10,5189	5,2654	2,2308	0,2347	151,2144	0,2109	5,0000	0,0130	1,4676	7,7277	5,1104			74,9719	14,0304	0,2568	165,4569	4,6705		
	III	10,5189	5,2654	2,2308	0,2347	151,2144	0,2381	5,0000	0,0150	1,5000	7,8979	5,2230			74,9719	14,0304	0,2568	165,4569	4,7734		
CO2 a	I	10,5294	5,2851	2,2308	0,2349	151,3654	0,2204	5,0000	0,0130	1,4044	7,4223	4,9035			74,9719	14,0445	0,2570	165,6221	4,4814		
	II	10,5294	5,2851	2,2308	0,2349	151,3654	0,2048	5,0000	0,0080	0,9301	4,9155	3,2474			74,9719	14,0445	0,2570	165,6221	2,9679		
	III	10,5294	5,2851	2,2308	0,2349	151,3654	0,2082	5,0000	0,0120	1,3723	7,2528	4,7916			74,9719	14,0445	0,2570	165,6221	4,3791		
CO2 b	I	10,264	5,1913	2,2308	0,2290	147,5501	0,2190	5,0000	0,0110	1,1959	6,2083	4,2076			74,9719	13,6905	0,2505	161,4475	3,8454		
	II	10,264	5,1913	2,2308	0,2290	147,5501	0,1986	5,0000	0,0090	1,0790	5,6013	3,7962			74,9719	13,6905	0,2505	161,4475	3,4694		
	III	10,264	5,1913	2,2308	0,2290	147,5501	0,2086	5,0000	0,0110	1,2555	6,5179	4,4174			74,9719	13,6905	0,2505	161,4475	4,0371		
CO3	I	9,2426	4,6512	2,2308	0,2062	132,8670	0,2526	5,0000	0,0270	2,5450	11,8371	8,9090			74,9719	12,3281	0,2256	145,3814	8,1421		
	II	9,2426	4,6512	2,2308	0,2062	132,8670	0,2300	5,0000	0,0260	2,6915	12,5188	9,4220			74,9719	12,3281	0,2256	145,3814	8,6110		
	III	9,2426	4,6512	2,2308	0,2062	132,8670	0,2265	5,0000	0,0200	2,1024	9,7786	7,3597			74,9719	12,3281	0,2256	145,3814	6,7262		

CO1 Noa	I	20,5313	10,5166	2,1421	0,4398	283,4121	0,1350	3,0000	0,0110	1,1640	12,2415	4,3193	3,0492	1,1055	74,4934	27,5612	0,5044	325,0216	3,7664	2,6589	0,9640
	II	20,5313	10,5166	2,1421	0,4398	283,4121	0,1135	3,0000	0,0090	1,1328	11,9131	4,2034			74,4934	27,5612	0,5044	325,0216	3,6653		
	III	20,5313	10,5166	2,1421	0,4398	283,4121	0,1109	3,0000	0,0080	1,0305	10,8377	3,8240			74,4934	27,5612	0,5044	325,0216	3,3344		
CO2 Noa	I	20,0267	10,113	2,1421	0,4290	276,4467	0,1242	3,0000	0,0100	1,1502	11,6322	4,2077			74,4934	26,8839	0,4920	317,0335	3,6691		
	II	20,0267	10,113	2,1421	0,4290	276,4467	0,1150	3,0000	0,0090	1,1180	11,3065	4,0899			74,4934	26,8839	0,4920	317,0335	3,5663		
	III	20,0267	10,113	2,1421	0,4290	276,4467	0,1069	3,0000	0,0080	1,0691	10,8117	3,9110			74,4934	26,8839	0,4920	317,0335	3,4103		
CO3 Nob	I	18,913	9,401	2,1421	0,4051	261,0733	0,1585	3,0000	0,0100	0,9013	8,4732	3,2455			74,4934	25,3888	0,4646	299,4030	2,8300		
	II	18,913	9,401	2,1421	0,4051	261,0733	0,1637	3,0000	0,0110	0,9599	9,0244	3,4567			74,4934	25,3888	0,4646	299,4030	3,0141		
	III	18,913	9,401	2,1421	0,4051	261,0733	0,1037	3,0000	0,0050	0,6888	6,4754	2,4803			74,4934	25,3888	0,4646	299,4030	2,1628		
CO1 NO	I	4,5434	2,2835	2,1421	0,0973	62,7167	0,2220	5,0000	0,0060	0,6435	1,4694	2,3430			74,4934	6,0991	0,1116	71,9245	2,0430		
	II	4,5434	2,2835	2,1421	0,0973	62,7167	0,2528	5,0000	0,0070	0,6593	1,5055	2,4004			74,4934	6,0991	0,1116	71,9245	2,0931		
	III	4,5434	2,2835	2,1421	0,0973	62,7167	0,2105	5,0000	0,0060	0,6787	1,5497	2,4710			74,4934	6,0991	0,1116	71,9245	2,1546		
CO2 NO	I	6,0854	3,0903	2,1421	0,1304	84,0023	0,2303	5,0000	0,0010	0,1034	0,3195	0,3803			74,4934	8,1690	0,1495	96,3352	0,3316		
	II	6,0854	3,0903	2,1421	0,1304	84,0023	0,2214	5,0000	0,0060	0,6452	1,9940	2,3737			74,4934	8,1690	0,1495	96,3352	2,0699		
	III	6,0854	3,0903	2,1421	0,1304	84,0023	0,2155	5,0000	0,0050	0,5524	1,7072	2,0323			74,4934	8,1690	0,1495	96,3352	1,7721		

Table L.5: Raw data from astaxanthin analysis of rapeseed oil from combination experiment of snow crab rest raw material. Rest raw material was exposed to enzymatic hydrolysis using Corolase® 2TS as proteolytic agent, at 50°C for 1 hour. After centrifugation and separation of sludge from water fraction, the sludge fraction was heated together with rapeseed oil (oil:SF ratio 1:2) at 65°C for 1 hour. After centrifugation, heat treatment mixture was stored at -20°C for 12 hours before new centrifugation and separation of fractions. Concentration of astaxanthin is measured using spectrophotometry.

	Rep	Nr	SF (g)	Oil added/ (g)	Lipid in sample (%)	Lipid in SF (g)	SF (ug/g)	Lipid sample (g)	n-hexane (mL)	Abs.	Astaxanthin ug/g lipid	ug/OF	Extracted from available in SF (%)	Av. Ax extracted (%)	SD	SF % yield	Theoretical RRM sample for enz hydr	Lipid (g) in RRM sample	Astaxanthin (ug) in RRM sample	Astaxanthin recovery from RRM (%)	Av. Recovery from RRM	SD
$CO^{50°C}HT^{65°C}_{oil\ 1:2}$ (stored -20°C, 12 h)	1 b	I	19,9704	9,9826	2,2308	0,4455	287,0845	0,1127	3	0,0680	8,6196	86,0460	29,9724	28,3947	2,1074	74,9719	26,6372	0,4875	314,1242	27,3923	25,9505	1,9260
		II	19,9704	9,9826	2,2308	0,4455	287,0845	0,1193	3	0,0730	8,7415	87,2626	30,3961			74,9719	26,6372	0,4875	314,1242	27,7796		
		III	19,9704	9,9826	2,2308	0,4455	287,0845	0,1121	3	0,0670	8,5383	85,2344	29,6897			74,9719	26,6372	0,4875	314,1242	27,1340		
	2 b	I	20,5233	10,2027	2,2308	0,4578	295,0327	0,1121	3	0,0660	8,4109	85,8135	29,0861			74,9719	27,3746	0,5010	322,8210	26,5824		
		II	20,5233	10,2027	2,2308	0,4578	295,0327	0,1162	3	0,0700	8,6059	87,8029	29,7604			74,9719	27,3746	0,5010	322,8210	27,1986		
		III	20,5233	10,2027	2,2308	0,4578	295,0327	0,1150	3	0,0690	8,5714	87,4517	29,6414			74,9719	27,3746	0,5010	322,8210	27,0898		
	3 b	I	18,2995	9,2413	2,2308	0,4082	263,0644	0,1437	3	0,0760	7,5554	69,8219	26,5418			74,9719	24,4085	0,4467	287,8418	24,2571		
		II	18,2995	9,2413	2,2308	0,4082	263,0644	0,1267	3	0,0640	7,2161	66,6866	25,3499			74,9719	24,4085	0,4467	287,8418	23,1678		
		III	18,2995	9,2413	2,2308	0,4082	263,0644	0,1119	3	0,0560	7,1492	66,0683	25,1149			74,9719	24,4085	0,4467	287,8418	22,9530		
	1 Nob	I	19,1217	9,648	2,1421	0,4096	263,9542	0,1118	3	0,0720	9,2001	88,7626	33,6280	28,0475	5,0833	74,4934	25,6690	0,4697	302,7069	29,3230	24,4568	4,4326
		II	19,1217	9,648	2,1421	0,4096	263,9542	0,1321	3	0,0870	9,4085	90,7728	34,3896			74,4934	25,6690	0,4697	302,7069	29,9870		
		III	19,1217	9,648	2,1421	0,4096	263,9542	0,1153	3	0,0770	9,5403	92,0451	34,8716			74,4934	25,6690	0,4697	302,7069	30,4073		
	2 Nob	I	19,4099	9,7033	2,1421	0,4158	267,9324	0,1298	3	0,0630	6,9337	67,2802	25,1109			74,4934	26,0559	0,4768	307,2692	21,8962		
		II	19,4099	9,7033	2,1421	0,4158	267,9324	0,1246	3	0,0670	7,6817	74,5381	27,8197			74,4934	26,0559	0,4768	307,2692	24,2582		
		III	19,4099	9,7033	2,1421	0,4158	267,9324	0,1225	3	0,0660	7,6968	74,6843	27,8743			74,4934	26,0559	0,4768	307,2692	24,3058		
	3 Nob	I	15,0425	7,5187	2,1421	0,3222	207,6453	0,1059	3	0,0460	6,2053	46,6559	22,4690			74,4934	20,1931	0,3695	238,1309	19,5925		
		II	15,0425	7,5187	2,1421	0,3222	207,6453	0,1266	3	0,0580	6,5448	49,2084	23,6983			74,4934	20,1931	0,3695	238,1309	20,6644		
		III	15,0425	7,5187	2,1421	0,3222	207,6453	0,1192	3	0,0520	6,2320	46,8567	22,5657			74,4934	20,1931	0,3695	238,1309	19,6769		

Table L.6: Raw data from astaxanthin analysis of rapeseed oil from combination experiment of snow crab rest raw material. Rest raw material was exposed to enzymatic hydrolysis using Corolase® 2TS as proteolytic agent, at 65°C for 1 hour. After centrifugation and separation of sludge from water fraction, the sludge fraction was heated together with rapeseed oil (oil:SF ratio 1:2) at 65°C for 1 hour. Concentration of astaxanthin is measured using spectrophotometry.

$\mathbf{CO^{65°C}HT^{65°C}_{oil\ 1:2}}$

Rep	Nr	SF (g)	Oil added/ (g)	Lipid in sample (%)	Lipid in SF (g)	SF (ug/g)	Lipid sample (g)	n-hexane (mL)	Abs.	Astaxanthin ug/g lipid	ug/OF	Extracted from available in SF (%)	Av. Ax extracted (%)	SD	SF % yield	Theoretical RRM sample for enz hydr	Lipid (g) in RRM sample	Astaxanthin (ug) in RRM sample	Astaxanthin recovery from RRM (%)	Av. Recovery from RRM	SD
4I a	I	10,2063	5,1083	1,7393	0,1775	114,3945	0,1211	3	0,0480	5,6624	28,9251	25,2854	18,5412	5,5422	74,8612	13,6336	0,2495	160,7773	17,9908	13,1922	3,9433
	II	10,2063	5,1083	1,7393	0,1775	114,3945	0,1392	3	0,0540	5,5419	28,3095	24,7473			74,8612	13,6336	0,2495	160,7773	17,6079		
	III	10,2063	5,1083	1,7393	0,1775	114,3945	0,1213	3	0,0470	5,5353	28,2758	24,7178			74,8612	13,6336	0,2495	160,7773	17,5870		
4II a	I	10,9306	5,492	1,7393	0,1901	122,5126	0,1187	3	0,0350	4,2123	23,1340	18,8829			74,8612	14,6011	0,2672	172,1870	13,4354		
	II	10,9306	5,492	1,7393	0,1901	122,5126	0,1117	3	0,0320	4,0926	22,4765	18,3463			74,8612	14,6011	0,2672	172,1870	13,0536		
	III	10,9306	5,492	1,7393	0,1901	122,5126	0,1141	3	0,0340	4,2569	23,3790	19,0829			74,8612	14,6011	0,2672	172,1870	13,5777		
5I a	I	10,026	5,1251	1,7393	0,1744	112,3736	0,1175	3	0,0320	3,8906	19,9396	17,7440			74,8612	13,3928	0,2451	157,9371	12,6250		
	II	10,026	5,1251	1,7393	0,1744	112,3736	0,1127	3	0,0310	3,9295	20,1392	17,9216			74,8612	13,3928	0,2451	157,9371	12,7514		
	III	10,026	5,1251	1,7393	0,1744	112,3736	0,1277	3	0,0360	4,0273	20,6403	18,3676			74,8612	13,3928	0,2451	157,9371	13,0687		
5II a	I	11,0437	5,5244	1,7393	0,1921	123,7803	0,1108	3	0,0170	2,1919	12,1087	9,7824			74,8612	14,7522	0,2700	173,9686	6,9603		
	II	11,0437	5,5244	1,7393	0,1921	123,7803	0,1156	3	0,0180	2,2244	12,2886	9,9277			74,8612	14,7522	0,2700	173,9686	7,0637		
	III	11,0437	5,5244	1,7393	0,1921	123,7803	0,1237	3	0,0200	2,3097	12,7599	10,3085			74,8612	14,7522	0,2700	173,9686	7,3346		
4I b	I	10,0799	5,056	1,7393	0,1753	112,9778	0,1302	3	0,0500	5,4861	27,7375	24,5513			74,8612	13,4648	0,2464	158,7861	17,4685		
	II	10,0799	5,056	1,7393	0,1753	112,9778	0,1103	3	0,0430	5,5692	28,1580	24,9235			74,8612	13,4648	0,2464	158,7861	17,7333		
	III	10,0799	5,056	1,7393	0,1753	112,9778	0,1046	3	0,0410	5,5996	28,3114	25,0593			74,8612	13,4648	0,2464	158,7861	17,8299		
4II b	I	10,1031	5,0573	1,7393	0,1757	113,2378	0,1233	3	0,0340	3,9393	19,9222	17,5932			74,8612	13,4958	0,2470	159,1516	12,5177		
	II	10,1031	5,0573	1,7393	0,1757	113,2378	0,1514	3	0,0410	3,8687	19,5649	17,2778			74,8612	13,4958	0,2470	159,1516	12,2933		
	III	10,1031	5,0573	1,7393	0,1757	113,2378	0,1065	3	0,0290	3,8900	19,6729	17,3731			74,8612	13,4958	0,2470	159,1516	12,3611		
5I b	I	10,0721	5,0793	1,7393	0,1752	112,8903	0,1136	3	0,0410	5,1559	26,1885	23,1982			74,8612	13,4544	0,2462	158,6633	16,5057		
	II	10,0721	5,0793	1,7393	0,1752	112,8903	0,1025	3	0,0370	5,1568	26,1929	23,2021			74,8612	13,4544	0,2462	158,6633	16,5085		
	III	10,0721	5,0793	1,7393	0,1752	112,8903	0,1139	3	0,0420	5,2678	26,7566	23,7014			74,8612	13,4544	0,2462	158,6633	16,8638		
5II b	I	9,9231	4,2678	1,7393	0,1726	111,2203	0,1081	3	0,0210	2,7752	11,8440	10,6492			74,8612	13,2553	0,2426	156,3161	7,5770		
	II	9,9231	4,2678	1,7393	0,1726	111,2203	0,1080	3	0,0220	2,9101	12,4195	11,1666			74,8612	13,2553	0,2426	156,3161	7,9451		
	III	9,9231	4,2678	1,7393	0,1726	111,2203	0,1177	3	0,0240	2,9130	12,4320	11,1778			74,8612	13,2553	0,2426	156,3161	7,9531		

4I Noa	I	10,4589	5,2389	1,7009	0,1779	114,6376	0,1259	3	0,0120	1,3616	7,1334	6,2226	6,4737	1,2566	81,2070	12,8793	0,2357	151,8818	4,6967	4,8862	0,9484
	II	10,4589	5,2389	1,7009	0,1779	114,6376	0,1146	3	0,0110	1,3712	7,1837	6,2665			81,2070	12,8793	0,2357	151,8818	4,7298		
	III	10,4589	5,2389	1,7009	0,1779	114,6376	0,1279	3	0,0150	1,6754	8,7773	7,6566			81,2070	12,8793	0,2357	151,8818	5,7791		
4II Noa	I	10,3851	5,2476	1,7009	0,1766	113,8287	0,1119	3	0,0090	1,1490	6,0294	5,2969			81,2070	12,7884	0,2340	150,8101	3,9980		
	II	10,3851	5,2476	1,7009	0,1766	113,8287	0,1007	3	0,0080	1,1349	5,9556	5,2320			81,2070	12,7884	0,2340	150,8101	3,9491		
	III	10,3851	5,2476	1,7009	0,1766	113,8287	0,1136	3	0,0100	1,2575	6,5991	5,7974			81,2070	12,7884	0,2340	150,8101	4,3758		
5I Noa	I	10,431	5,239	1,7009	0,1774	114,3318	0,1287	3	0,0160	1,7760	9,3045	8,1381			81,2070	12,8450	0,2351	151,4767	6,1425		
	II	10,431	5,239	1,7009	0,1774	114,3318	0,1245	3	0,0150	1,7212	9,0172	7,8869			81,2070	12,8450	0,2351	151,4767	5,9529		
	III	10,431	5,239	1,7009	0,1774	114,3318	0,1147	3	0,0130	1,6191	8,4826	7,4193			81,2070	12,8450	0,2351	151,4767	5,6000		
5II Noa	I	9,7708	4,887	1,7009	0,1662	107,0955	0,1098	3	0,0090	1,1710	5,7225	5,3433			81,2070	12,0320	0,2202	141,8894	4,0331		
	II	9,7708	4,887	1,7009	0,1662	107,0955	0,1079	3	0,0090	1,1916	5,8232	5,4374			81,2070	12,0320	0,2202	141,8894	4,1041		
	III	9,7708	4,887	1,7009	0,1662	107,0955	0,1250	3	0,0110	1,2571	6,1437	5,7366			81,2070	12,0320	0,2202	141,8894	4,3299		
4I Nob	I	10,2908	5,4261	1,7009	0,1750	112,7951	0,1162	3	0,0140	1,7212	9,3392	8,2798			81,2070	12,6723	0,2319	149,4407	6,2495		
	II	10,2908	5,4261	1,7009	0,1750	112,7951	0,1063	3	0,0080	1,0751	5,8337	5,1720			81,2070	12,6723	0,2319	149,4407	3,9037		
	III	10,2908	5,4261	1,7009	0,1750	112,7951	0,1123	3	0,0140	1,7809	9,6636	8,5674			81,2070	12,6723	0,2319	149,4407	6,4665		
4II Nob	I	10,354	5,2387	1,7009	0,1761	113,4878	0,1007	3	0,0090	1,2768	6,6887	5,8937			81,2070	12,7501	0,2333	150,3585	4,4485		
	II	10,354	5,2387	1,7009	0,1761	113,4878	0,1119	3	0,0100	1,2767	6,6880	5,8931			81,2070	12,7501	0,2333	150,3585	4,4480		
	III	10,354	5,2387	1,7009	0,1761	113,4878	0,1021	3	0,0080	1,1194	5,8639	5,1670			81,2070	12,7501	0,2333	150,3585	3,9000		
5I Nob	I	9,7283	4,8907	1,7009	0,1655	106,6297	0,1195	3	0,0150	1,7932	8,7699	8,2247			81,2070	11,9796	0,2192	141,2722	6,2078		
	II	9,7283	4,8907	1,7009	0,1655	106,6297	0,1164	3	0,0140	1,7182	8,4033	7,8808			81,2070	11,9796	0,2192	141,2722	5,9483		
	III	9,7283	4,8907	1,7009	0,1655	106,6297	0,1079	3	0,0130	1,7212	8,4177	7,8944			81,2070	11,9796	0,2192	141,2722	5,9585		
5II Nob	I	11,0384	5,6132	1,7009	0,1878	120,9894	0,1194	3	0,0100	1,1965	6,7160	5,5509			81,2070	13,5929	0,2488	160,2972	4,1897		
	II	11,0384	5,6132	1,7009	0,1878	120,9894	0,1251	3	0,0100	1,1419	6,4100	5,2980			81,2070	13,5929	0,2488	160,2972	3,9988		
	III	11,0384	5,6132	1,7009	0,1878	120,9894	0,1037	3	0,0080	1,1021	6,1862	5,1130			81,2070	13,5929	0,2488	160,2972	3,8592		

Table L.7: Raw data from astaxanthin analysis of rapeseed oil from combination experiment of snow crab rest raw material. Rest raw material was exposed to enzymatic hydrolysis using Corolase® 2TS as proteolytic agent, at 65°C for 1 hour. After centrifugation and separation of sludge from water fraction, the sludge fraction was heated together with rapeseed oil (oil:SF ratio 2:1) at 65°C for 1 hour. Concentration of astaxanthin is measured using spectrophotometry.

CO65 HT65 oil 2:1

Rep	Nr	SF (g)	Oil added/ (g)	Lipid in sample (%)	Lipid in SF (g)	SF (ug/g)	Lipid sample (g)	n-hexane (mL)	Abs.	Astaxanthin ug/g lipid	ug/OF	Extracted from available in SF (%)	Av. Ax extracted (%)	SD	SF % yield	Theoretical RRM sample for enz hydr	Lipid (g) in RRM sample	Astaxanthin (ug) in RRM sample	Astaxanthin recovery from RRM (%)	Av. Recovery from RRM	SI
4 a	I	10,7632	21,5189	1,7393	0,1872	120,6363	0,2176	5	0,0070	0,7659	16,4820	13,6625	17,2514	4,6451	74,8612	14,3775	0,2631	169,5500	9,7210	12,2745	3,305
	II	10,7632	21,5189	1,7393	0,1872	120,6363	0,2204	5	0,0070	0,7562	16,2726	13,4890			74,8612	14,3775	0,2631	169,5500	9,5975		
	III	10,7632	21,5189	1,7393	0,1872	120,6363	0,2071	5	0,0070	0,8048	17,3176	14,3552			74,8612	14,3775	0,2631	169,5500	10,2139		
4 b	I	10,0724	20,2908	1,7393	0,1752	112,8937	0,2118	5	0,0060	0,6745	13,6860	12,1229			74,8612	13,4548	0,2462	158,6680	8,6255		
	II	10,0724	20,2908	1,7393	0,1752	112,8937	0,2323	5	0,0060	0,6150	12,4782	11,0531			74,8612	13,4548	0,2462	158,6680	7,8643		
	III	10,0724	20,2908	1,7393	0,1752	112,8937	0,2491	5	0,0070	0,6691	13,5761	12,0255			74,8612	13,4548	0,2462	158,6680	8,5563		
5a	I	7,6976	15,0812	1,7393	0,1339	86,2764	0,2108	5	0,0090	1,0165	15,3306	17,7691			74,8612	10,2825	0,1882	121,2584	12,6429		
	II	7,6976	15,0812	1,7393	0,1339	86,2764	0,2277	5	0,0100	1,0457	15,7697	18,2781			74,8612	10,2825	0,1882	121,2584	13,0050		
	III	7,6976	15,0812	1,7393	0,1339	86,2764	0,2190	5	0,0100	1,0872	16,3962	19,0042			74,8612	10,2825	0,1882	121,2584	13,5217		
5b	I	3,8047	7,6094	1,7393	0,0662	42,6439	0,2390	5	0,0110	1,0958	8,3387	19,5541			74,8612	5,0823	0,0930	59,9345	13,9129		
	II	3,8047	7,6094	1,7393	0,0662	42,6439	0,2451	5	0,0110	1,0686	8,1311	19,0675			74,8612	5,0823	0,0930	59,9345	13,5667		
	III	3,8047	7,6094	1,7393	0,0662	42,6439	0,2074	5	0,0080	0,9184	6,9885	16,3880			74,8612	5,0823	0,0930	59,9345	11,6602		
6A	I	13,5725	27,0207	1,7393	0,2361	152,1236	0,2051	3	0,0160	1,1144	30,1129	19,7950			74,8612	18,1302	0,3318	213,8042	14,0843		
	II	13,5725	27,0207	1,7393	0,2361	152,1236	0,2021	3	0,0140	0,9896	26,7399	17,5778			74,8612	18,1302	0,3318	213,8042	12,5067		
	III	13,5725	27,0207	1,7393	0,2361	152,1236	0,2094	3	0,0130	0,8869	23,9643	15,7532			74,8612	18,1302	0,3318	213,8042	11,2085		
6B	I	14,1722	29,4181	1,7393	0,2465	158,8452	0,2115	3	0,0130	0,8781	25,8315	16,2621			74,8612	18,9313	0,3464	223,2511	11,5706		
	II	14,1722	29,4181	1,7393	0,2465	158,8452	0,2182	3	0,0130	0,8511	25,0383	15,7627			74,8612	18,9313	0,3464	223,2511	11,2153		
	III	14,1722	29,4181	1,7393	0,2465	158,8452	0,2066	3	0,0100	0,6915	20,3417	12,8060			74,8612	18,9313	0,3464	223,2511	9,1116		
6C	I	14,7691	29,4448	1,7393	0,2569	165,5354	0,2179	3	0,0250	1,6390	48,2607	29,1543			74,8612	19,7286	0,3610	232,6539	20,7435		
	II	14,7691	29,4448	1,7393	0,2569	165,5354	0,1935	3	0,0160	1,1812	34,7816	21,0116			74,8612	19,7286	0,3610	232,6539	14,9499		
	III	14,7691	29,4448	1,7393	0,2569	165,5354	0,2134	3	0,0230	1,5397	45,3361	27,3876			74,8612	19,7286	0,3610	232,6539	19,4865		
4NOa	I	8,9566	18,0342	1,7009	0,1523	98,1712	0,2103	5	0,0020	0,2264	4,0836	4,1596	5,4274	1,2499	81,2070	11,0293	0,2018	130,0658	3,1396	4,0965	0,943
	II	8,9566	18,0342	1,7009	0,1523	98,1712	0,2377	5	0,0030	0,3005	5,4193	5,5202			81,2070	11,0293	0,2018	130,0658	4,1666		
	III	8,9566	18,0342	1,7009	0,1523	98,1712	0,2117	5	0,0020	0,2249	4,0565	4,1321			81,2070	11,0293	0,2018	130,0658	3,1188		
4NOb	II	8,0528	16,1528	1,7009	0,1370	88,2649	0,2302	5	0,0030	0,3103	5,0120	5,6784			81,2070	9,9164	0,1815	116,9410	4,2860		

	III	8,0528	16,1528	1,7009	0,1370	88,2649	0,2190	5	0,0030	0,3262	5,2684	5,9688	81,2070	9,9164	0,1815	116,9410	4,5051
5NO a	I	6,638	13,381	1,7009	0,1129	72,7576	0,2480	5	0,0030	0,2880	3,8540	5,2970	81,2070	8,1742	0,1496	96,3956	3,9981
	II	6,638	13,381	1,7009	0,1129	72,7576	0,2232	5	0,0030	0,3200	4,2822	5,8856	81,2070	8,1742	0,1496	96,3956	4,4423
	III	6,638	13,381	1,7009	0,1129	72,7576	0,2103	5	0,0030	0,3397	4,5449	6,2466	81,2070	8,1742	0,1496	96,3956	4,7148
5NO b	II	6,0977	12,2468	1,7009	0,1037	66,8355	0,2213	5	0,0030	0,3228	3,9529	5,9143	81,2070	7,5088	0,1374	88,5495	4,4640
	III	6,0977	12,2468	1,7009	0,1037	66,8355	0,2175	5	0,0030	0,3284	4,0219	6,0177	81,2070	7,5088	0,1374	88,5495	4,5420
6NO A	I	16,8942	31,6266	1,7009	0,2874	185,1734	0,1986	3	0,0060	0,4316	13,6498	7,3714	81,2070	20,8039	0,3807	245,3339	5,5638
	II	16,8942	31,6266	1,7009	0,2874	185,1734	0,2111	3	0,0060	0,4060	12,8416	6,9349	81,2070	20,8039	0,3807	245,3339	5,2343
	III	16,8942	31,6266	1,7009	0,2874	185,1734	0,2257	3	0,0060	0,3798	12,0109	6,4863	81,2070	20,8039	0,3807	245,3339	4,8957
6NO B	I	16,3045	34,6293	1,7009	0,2773	178,7099	0,2233	3	0,0040	0,2559	8,8617	4,9587	81,2070	20,0777	0,3674	236,7704	3,7427
	II	16,3045	34,6293	1,7009	0,2773	178,7099	0,2055	3	0,0020	0,1390	4,8146	2,6941	81,2070	20,0777	0,3674	236,7704	2,0335
	III	16,3045	34,6293	1,7009	0,2773	178,7099	0,2324	3	0,0030	0,1844	6,3860	3,5734	81,2070	20,0777	0,3674	236,7704	2,6971

M. Supercritical fluid extraction

Supercritical fluid extraction (SFE) was performed by Elena Shumilina at Dikiy Lab, Department of Biotechnology and Food Science, NTNU Trondheim, 19.-20.3.19. Method is described in chapter 2.7, results in **Table M.1**. The extraction yielded an orange coloured fraction dissolved in ethanol. After evaporation of the ethanol, the resulting pigment material was measured and recovery calculated based on original RRM samples.

Table M.1: Super fluid extraction of astaxanthin from snow crab rest raw material experimental details and resulting extracted amount of astaxanthin μg/g.

Batch 2	Nr	RRM (g, ww)	Pigment (g)	Pigment (μg)	Pigment μg/g RRM (w/ww)	Average μg/g	SD
	B	15,3557	0,0144	14400	937,7625	969,9099	45,4632
	D	15,069	0,0151	15100	1002,0572		

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