Development of Value-Added By-Products from Abundantly Available but Underutilized European Green Crab (*Carcinus Maenas*)

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August 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Masters of Science in Food Science and Agricultural Chemistry

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ABSTRACT

Global food waste is an issue that we are faced with each and every day. Poor management practices at each level of the food chain contributes to the overall loss of food. The fishery and aquacultural industry, for instance, generates a large percentage of unavoidable waste and losses that are rich in nutrients, such as proteins, lipids, and pigments. Fish and crustacean by-products today, are used to produce value-added products, including fishmeal, fish oil, and fertilizer for agriculture. Therefore, the use of fishery waste to generate value-added products is a part of sustainable practices that have become a growing trend to reduce global food waste altogether. However, another form of waste that lacks exposure is the destruction of invasive aquatic species in nature. Invasive species are foreign organisms in a particular area that are known to disrupt the environment and native species causing great harm to ecosystems and the economy. European green crab (Carcinus maenas) is an underutilized invasive species distributed on the shallow waters in the northern-western Atlantic coastline. Green crabs are known to be a very small aggressive specie with a large appetite that poses a threat to Canadian ecosystems and the fishery industry. To control their population in Canadian waters, licensed fishermen are permitted to destroy green crabs when captured to allow native species to re-emerge in the area. Currently, the green crab has little use as a food source, and are often discarded into the environment as waste. Thus, there is an interest to explore green crab to generate high value-added products. In this study, the biochemical composition and enzyme-assisted extraction of carotenoprotein and chitin from green crab were investigated. Initially, green crab harvested from Nova Scotia coastline was analyzed for lipid and protein composition. The crab samples contained comparable crude protein (20.98% dry wt. basis) and lipid (3.00 % dry wt. basis) content as those previously published in the literature. The lipid profile of green crab characterized using GC-MS showed a higher content of unsaturated fatty acids, including MUFAs mainly, oleic acid and n-3 PUFAs, such as EPA (20:5n-3) and DHA (22:6n-3). Therefore, green crab lipid extract showed a rich source of essential omega-3 fatty acids. Furthermore, carotenoprotein is a natural pigment comprised of a carotenoid and protein complex found in crustacean shells. The use of proteases, such as trypsin, is a mild and successful method to extract carotenoprotein from crab shells. The narrow specificity of trypsin to cleave at the carboxyl group of non-terminal amino acid arginine and lysine makes it efficient at recovering carotenoproteins. The enzyme-assisted extraction with the aid of trypsin from porcine pancreas

yield green crab carotenoprotein extract consisting of 67.58 % total soluble protein and 139.26 μg/g carotenoids. The amino acid analysis showed carotenoprotein extract was rich in essential amino acids (49.87 %), hydrophobic amino acids (44.17 %), and dominant in aspartic acid, asparagine, glutamic acid, and glutamine amino acids. Also, thin layer chromatography indicated the main carotenoids in carotenoprotein were astaxanthin and astaxanthin diester. The combined effect of carotenoid, amino acids, and peptides from extracted carotenoprotein showed the highest antioxidant activities at 4 mg/mL against DPPH and hydroxyl radicals and iron-reducing power ($p < 0.05$). Therefore, green crab carotenoprotein can be used as natural antioxidants or nutritive ingredients for foods and feeds. Moreover, crab shells are an excellent source to generate chitin for biomedical or pharmaceutical applications. However, the high content of ash (38.05 %) in green crab indicated that an extensive demineralization step was required to generate quality chitin. Consequently, the optimization of citric acid demineralization using Box-Behnken design to investigate the effects of time, concentration, and temperature on the total residual ash was conducted. The optimal condition was determined as 1.0 M of citric acid, at a temperature of 4°C and time of 18h to obtain 1.38 % total residual ash in green crab. Statistical analysis confirmed the effects of temperature and concentration of citric acid were the main parameters to significantly ($p < 0.05$) decreased the total ash in crab shells. The mineral analysis of green crab using ICP-MPS showed the shells were rich in calcium (196 mg/g), sodium (16.4 mg/g), phosphorous (12.8 mg/g), magnesium (8.50 mg/g), potassium (5.20 mg/g) and strontium (2.74 mg/g). However, extracted chitin following optimal demineralization conditions showed relatively lower levels of sodium (4.20 mg/g), calcium (1.40 mg/g), and phosphorous (0.900 mg/g). Chemical characterization using XRD analysis further confirmed $CaCO_3$ was effectively chelated with citric acid. The extracted chitin showed characteristic bands similar to commercial crab chitin, following FTIR analysis, indicating a potential source for further application.

In summary, green crab is an excellent source of nutrients, such as lipids and amino acid, as well as a rich source of value-added products, including carotenoprotein and chitin. Thus, exploiting invasive green crab as a means to reduce fishery and crustacean waste creates a new avenue of income within the food industry and an innovative source of bioactive nutrients.

RÉSUMÉ

Le gaspillage alimentaire mondial est un problème auquel nous sommes confrontés au quotidien. Les faibles pratique de gestion à tous les niveaux de la chaine alimentaire contribuent au gaspillage alimentaire. A titre d'exemple, l'industrie de la pêche et l'aquaculture génère un pourcentage important de déchets et de pertes inévitables qui sont riches en nutriments, tels que les protéines, les lipides et les pigments. De nos jours, les déchets ou issues de poissons et de crustacés sont utilisés pour développer les produits à valeur ajoutée, cela comprend la farine de poisson, l'huile de poisson et de l'engrais pour l'agriculture. Donc, la génération des produits à valeur ajoutée est une pratique durable utilisée pour réduire le gaspillage alimentaire mondial. Toutefois, une autre source déchets moins connu et moins exploité est celle provenant de l'élimination des espèces envahissantes dans la nature. Une espèce envahissante est un organisme vivant dans un milieu où ils n'avaient jamais été décelé auparavant perturbant ainsi l'environnement des espèces indigènes et causant des dommages aux écosystèmes et à l'économie. Le crabe vert (*Carcinus maenas*) fait partie des espèces envahissantes qui habitent le long des eaux peu profondes du nord-ouest de l'océan Atlantique. C'est une espèce agressive, d'une petite taille, et un gros appétit qui perturbe l'écosystème canadien et l'industrie de la pêche. Pour contrôler leur population dans les eaux canadiennes, les pêcheurs titulaires d'un permis sont autorisés à détruire les crabes verts lorsqu'ils sont capturés pour permettre aux espèces indigènes de ré-émerger dans la zone. Actuellement, le crabe vert est peu utilisé au Canada comme source d'aliment et est souvent rejeté dans l'environnement en tant que déchet. De fait, il y a beaucoup d'intérêt à les utiliser pour développer des produits à valeur ajoutée. Dans cette étude, la composition biochimique et l'extraction enzymatique des caroténoprotéines et chitine provenant des crabes verts ont été analysées. Premièrement, les crabes verts pêchés en Nouvelle-Écosse ont été analysés pour la composition en lipides et protéines. Les échantillons de crabes contenaient un niveau de protéine-brute (20.98% de matière sèche) et un niveau de lipide (3.00 % de matière sèche) comparable aux études précédemment publiées dans la littérature. La méthode GC-MS a été utilisée pour obtenir le bilan lipidique du crabe vert. Les résultats obtenus ont démontré un niveau haut en acide gras insaturé, qui inclut les acides gras mono-insaturés, notamment les acides oléiques, et les acides gras poly-insaturés, tel que EPA (20:5n-3) et DHA (22:6n-3). Ces résultats confirment que le crabe vert est une source riche en acides gras oméga-3. Les caroténoprotéines sont un type de pigment naturel

composés d'une partie caroténoïde et d'un complexe protéique provenant des carapaces de crabes. L'utilisation des peptidases, tel que la trypsine, est une méthode non agressive et effective pour extraire des caroténoprotéines. La trypsine agit comme une paire de ciseaux chimiques capable de couper la chaîne de protéine du groupe carboxyle des acides aminés, tels que l'arginine et la lysine non terminales pour récupérer les caroténoprotéines. L'extraction enzymatique des caroténoprotéines, à l'aide de la trypsine provenant de pancréas de porc, a atteint un rendement total de 67.58 % protéine soluble et de 139.26 μg/g caroténoïde. L'analyse d'acides aminés a démontré que les caroténoprotéines du crabe vert sont riches en acides aminés essentiels (49.87 %), acides aminés hydrophobes (44.17 %). Cette analyse a également démontré une richesse en acide aspartique, asparagine, acide glutamique, et en glutamine. La chromatographie sur couche mince a indiqué que l'astaxanthine et l'astaxanthine diester sont les caroténoïdes principaux des caroténoprotéines. L'effet combiné des caroténoïdes, des acides aminés et des peptides extrait des caroténoprotéines de crabe vert a démontré une forte activité antioxydante de 4 mg/ml contre le radicale DPPH et le radicale hydroxyle, de plus ils ont démontré la capacité de réduire le fer ($p < 0.05$). De ce fait, les caroténoprotéines du crabe vert peuvent être utilisés comme une source d'antioxydant naturel ou une source nutritive pour les humains et les animaux. Par ailleurs, le crabe vert est une source excellente pour générer la chitine pour l'application en l'industrie biomédicale ou l'industrie pharmaceutique. Par contre, le taux de cendre (38.05 %) élevé du crabe vert a indiqué qu'une étape déminéralisation efficace est requise pour générer une bonne qualité de chitine. Box-Behnken design a été utilisé pour optimiser la déminéralisation avec l'acide citrique. Les effets des paramètres de la déminéralisation du crabe vert (ex. le temps, la concentration de l'acide citrique et la température) sur le taux de cendre résiduel ont été déterminés. La condition optimale obtenue pour la déminéralisation du crabe vert a été 1.0 M d'acide citrique, a une température de 4°C et un temps de 18 heures pour un taux de cendre résiduel de 1.38 %. L'analyse statistique a confirmé que les effets de température et de concentration d'acide critique sont les paramètres principaux qui réduisent le taux de cendre du crabe vert ($p < 0.05$). De plus, l'analyse des minéraux avec ICP-MS a démontré que le crabe vert était riche en calcium (196 mg/g), sodium (16.4 mg/g), phosphore (12.8 mg/g), magnésium (8.50 mg/g), potassium (5.20 mg/g), et strontium (2.74 mg/g). Cependant, l'extrait de chitines obtenu après la déminéralisation optimale a démontré un taux faible de sodium (4.20 mg/g), calcium (1.40 mg/g) et de phosphore (0.900 mg/g). La caractérisation chimique par l'analyse XRD a confirmé que $CaCO_3$ a été en effet chélaté par l'acide

citrique durant. Finalement, l'analyse FTIR a démontré que le spectre d'absorption de l'extrait de chitines a été similaire au spectre de la chitine commerciale. Ce qui indique que l'extrait de chitine peut être utilisé dans de nombreuses applications.

Pour conclure, le crabe vert est une source excellente de nutriment, tel que les lipides et les acides aminés, en plus d'être une source riche pour développer des produits à valeur ajoutée, comme des caroténoprotéines et chitine. Ainsi, l'exploitation de cette espèce envahissante nous permettrait de réduire considérablement les déchets poissonniers et crustacés pour développer une nouvelle avenue économique dans l'industrie alimentaire et une nouvelle source de nutriments bioactives.

ACKNOWLEDGEMENTS

I would like to sincerely thank my Supervisor Dr. Benjamin K. Simpson for his expertise in the field, guidance, support and most importantly his encouragement to always pursue for more. I would also like to deeply thank Yi Zhang, my amazing lab colleague, for her guidance and her advice throughout my thesis.

Thank you to Dr. Yixiang Wang my committee member for his advice and allowing me to use the equipment necessary from his lab.

Thank you to Dr. Michael Ngadi for allowing me to use the equipment from his lab, as well as Ogan Mba for the training.

A big thank you to my incredible colleagues Camelia Oliva and Shuting Huang for their friendship, support and encouragement.

I would like to end by thanking my family and my friends for all the love and support they continuously provide me with.

LIST OF ABBREVIATIONS

ABTS:	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA:	analysis of variance
Asp+Asn:	aspartic+asparagine
BBD:	Box Behnken design
BC-Cs:	bacterial cellulose-chitosan
BCA:	bicinchoninic acid
BHA:	butylated hydroxyanisole
BHT:	butylated hydroxytoluene
BsChi:	*Bacillus subtilis* novel chitinase
CA:	citric acid
CC:	commercial chitin
COS:	chitooligosaccharides
CP:	carotenoprotein
Cs-AKEO:	chitosan-apricot kernel essential oil
Cs:	chitosan
d.f.:	dilution factor
DDA:	degree of deacetylation
DES:	deep eutectic solvents
DHA:	docosahexaenoic acid
DP:	degree of polymerization
DPPH:	2,2-dipheyl-1-picrylhydrazyl
EAA:	essential amino acid
EC:	extracted chitin
EDTA:	ethylenediaminetetraacetic acid
EO:	essential oil
EPA:	eicosapentaenoic acid
FAME:	fatty acid methyl esters
FFA:	free fatty acid
FTIR:	Fourier transform infrared

G:	green crab
GC-MS:	gas chromatography-mass spectrometry
GlcN:	glucosamine
GlcNAc:	N-Acetylglucosamine
Glu+Gln:	glutamic acid + glutamine
GsCsn46A:	*Gynuelle sunshinyii* novel cold-adapted chitosanase
HAE:	hydro-alcoholic extracts
HPAA:	hydrophobic amino acid
ICP-MS:	inductively coupled plasma Mass Spectrometry
LA:	lactic acid
LC-PUFA:	long-chain polyunsaturated fatty acid
MUFA:	monounsaturated fatty acid
MW:	molecular weight
NEAA:	non-essential amino acid
$OH^{\cdot}$:	hydroxy radicals
PES:	Polyethersulfone
PFCP:	peptidic fraction of carotenoproteins
PITC:	phenylisothiocyanate
PTC:	phenylthiocarbamoyl
PUFA:	polyunsaturated fatty acid
PVA:	polyvinyl alcohol
R^2:	coefficient of determination
RPH-Cs:	rapeseed protein hydrolysate- chitosan
RSM:	response surface methodology
SFA:	saturated fatty acid
SPD:	plasticized with spermidine
TCA:	trichloroacetic acid
$T_{g:}$	glass transition temperature
TLC:	thin-layer chromatography
TMS:	turkey meat sausages
UPLC:	ultra-performance liquid chromatography

WVP:	water vapor permeability
WVTR:	water vapor transmission rate
XRD:	x-ray diffraction

CHAPTER I.
INTRODUCTION

One of the major challenges of Fisheries and Aquaculture today is the large percentage of post-harvest loss and waste contributing to the issues of world hunger, food security, and malnutrition of our growing population, specifically in those countries that cannot afford waste. In a recent article, The Food and Agricultural Organization (FAO) published a report focused on "The State of World Fisheries and Aquaculture — Meeting the Sustainable Development Goal" (F.A.O., 2018). A part of the report highlights on the fact that the apparent consumption of fish and crustacea have risen considerably since the 1950s; however, the level of waste generated still remains problematic. It is estimated that approximately 35 % of the global fishery catch is loss at all levels of the food chain, resulting in loss of high quality protein, fatty acids and other micronutrients (F.A.O., 2018). This fact alone, has initiated the quest for sustainable approaches for fisheries and aquaculture practices which is inevitable to reduce waste.

Seafood consumption is abundant as it serves as a primary alternative source to animal protein in some countries around the world. Crustacean in particular, is a growing market for human consumption in Asian countries, since it is of nutritional value with minimal saturated fat and rich source of omega-3s (F.A.O., 2018). It is considered a healthier option in comparison to other animal sources, such as beef, chicken, and pork. According to the Canadian food guide, shellfish, such as shrimp, crab and lobster are recommended as a good source of protein to protect against cardiovascular diseases (Government of Canada., 2020). However, in contrast to fish species, the edible part of crustacean accounts for about 20 to 25 % of total body mass only, which also contributes to an unavoidable amount of waste (Özogul, Hamed, Özogul, Regenstein., 2019). Consequently, remains that are rich in nutrients end up as discard in the environment, landfilled or incinerated, which rises safety concerns (Al Khawli et al., 2020).

The destruction of invasive aquatic species is another form of waste that lacks exposure in the fishery and aquaculture industry. The term "invasive species" is defined as a foreign organism in a particular area that can disrupt native species and the ecosystem causing great economic and environmental harm (Rutledge et al., 2011). European green crab (*Carcinus maenas*) is amongst one of the most aggressive invasive species in the world (Fisheries and Oceans Canada, 2019). In North America, green crab has limited use in the fishery and food industry as

they are small in size, and are of no economic value (Khiari, Kelloway, & Mason, 2020). Therefore, when green crabs are accidently captured in fishing nets and traps while harvesting commercial species, they are often destroyed and discarded into the environment (Fisheries and Oceans Canada, 2019). Nonetheless, invasive species have shown to be an excellent source of nutrients, that are currently underutilized (Chen, Zhang & Shrestha., 2007).

The rising global food waste in conjunction with the growing rate of food security and malnutrition, makes it crucial for food scientist to find innovative and effective ways to reduce food waste and explore new food sources to feed a growing population. Thus, generating value-added products from waste material has become a new trend in food science. The use of fishery waste to generate value-added products, including fish meal, fish oil, bioactive compounds and digestive enzymes is a significant area of research (Al Khawli et al., 2020). Crustacean waste have shown to be an excellent source of lipid, raw material for the production of chitinous materials, protein, as well as, production of natural pigments (Özogul et al., 2019). The compositional analysis of various species of crustaceans have shown a rich content of essential fatty acids (i.e. linolenic acid, linoleic, omega-3s, including DHA and EPA) and rich in essential amino acid content (Balzano, Pacetti, Lucci, Fiorini, & Frega, 2017). It has also been previously shown that pigments extracted from the shells of shrimp, lobster and crab have served as a source of natural pigmentation for aquaculture of fish and crustacean species (Wade, Gabaudan, & Glencross, 2017). In addition, chitinous material have serve as a precursor for its derivative forms chitosan and chitooligosaccharides that has been used in the fields of agriculture, medical, food, pharmaceutical and cosmetics as a natural polymer (El Knidri, Belaabed, Addaou, Laajeb, & Lahsini, 2018).

Enzymatic-assisted extraction method is preferably used in contrast to the conventional chemical method to generate value-added products, including carotenoproteins (a natural pigment comprised of carotenoid and protein complex) and chitin (a linear polysaccharide chain of *N*-acetyl-*D*-glucosamine residue bonded by β –(1-4) linkages) from crustacean waste. The use of enzymes is a milder treatment, as well as, it is a more efficient catalysts to accelerate chemical reactions (El Knidril et al., 2018). Proteases are predominately used in literature to extract carotenoprotein and deproteinize crustacean shells to extract chitin from various species of shrimp and crabs (Hamdi et al., 2017; Hamdi et al., 2018; Poonsin et al., 2018; Younes et al., 2014) . In previous study, proteases, such as trypsin, pepsin, papain, visceral protease enzymes have

successfully extracted peptidic portions of crustacean waste (Klomklao et al., 2009; Poonsin et al., 2018; Sánchez-Camargo, Almeida Meireles, Lopes, & Cabral, 2011).

The overall aim of the present study was to evaluate the nutritional composition and characterize the biochemical properties of European green crab for the generation of value-added products through a mild treatment and with the use of enzyme-assisted extraction. More specifically, the objectives of the present thesis were : i) to characterize the composition of fatty acid and amino acid profile of extracted lipid and protein from green crab; ii) to extract carotenoprotein using enzyme-assisted extraction method and assess the biochemical activities, which, to date, have not been previously reported in European green crab species; iii) to investigate the effects of combining demineralization parameters using response surface methodology to extract chitin from green crab using a mild treatment; iv) to characterize extracted chitin from green crab using instrumental analysis to validate the chemical properties with commercial chitin.

A literature review describing the most recent extraction methods of carotenoproteins, chitin, chitosan and chitooligosaccharides in crustacean waste are reported. Furthermore, their application in the food industry and challenges are also identified in Chapter II. The extraction and biochemical characterization of lipids, protein and carotenoprotein from green crab are presented in Chapter III. The optimized extraction and characterization of chitin from green crab using mild treatment are presented in Chapter IV. Finally, Chapter V provides a general summary and conclusion for this study and future prospects for further work are proposed.

CHAPTER II.
LITERATURE REVIEW

2.1. Introduction:

In 2016, the world's total production of crustacean was reported to be 14 572 704 tonnes with Asia accounting for 80.5 % (11 737 701 tonnes) of the total production followed by the Americas at 14.2 % (2 073 139 tonnes) (FAO, 2018). The increasing demand for crustaceans, as an alternative source of animal protein, has shown to have great economic importance across the globe. However, over the years, the number of waste and by-products generated by shellfish processing industries represents up to 75% of raw material (Hamed, Özogul, & Regenstein, 2016). During the processing of shellfish, a significant amount of waste is generated from the removal of heads, tails, shells, roe, claws and viscera.

Recent studies have shown that crustacean shells are excellent sources of bioactive compounds that can further be used as value-added products (El Knidri, Belaabed, Addaou, Laajeb, & Lahsini, 2018; Rakkhumkaew & Pengsuk, 2018; Su, Huang, & Liu, 2018). The exoskeleton of crustaceans is composed of mainly chitin (20-30%), proteins (30-40%), ash (30-50 %) and the balance being lipids and pigments (Hamed et al., 2016). In general, the composition of each component varies with species, seasons, habitat and dietary intake (Table 2.1.).

Many recent studies and reviews, have investigated the feasibility of extracting from waste by-products generated by shellfish processing industries to produce natural colorants, bioactive and nutraceuticals, chitosan, chitooligosaccharides, bioplastics, hydrogels (Arancibia, Alemán, López-Caballero, Gómez-Guillén, & Montero, 2015; El Knidri et al., 2018; Hamed et al., 2016; Saini, Moon, & Keum, 2018; Shariatinia & Jalali, 2018; Wade, Gabaudan, & Glencross, 2017). Saini et al. (2018) reviewed the use of tomato pomace and crustacean processing waste, rich in lycopene and astaxanthin, in the food, cosmetic and pharmaceutical industries as an antioxidant, provitamin supplement, natural pigment and aquaculture feed. Wade et al. (2017) reviewed dietary carotenoids effects on crustacean pigmentation and other benefits to improve survival, growth, reproductive capacity and disease resistance. El Knidri et al. (2018) reviewed the use of crustacean shells like crabs and shrimps, to extract chitosan through new methods, with emphasis on ecological techniques. Similarly, Hamed et al. (2016) overviewed the bioactivity of chitosan, such as antioxidant, antimicrobial and immunological activities and use in various fields, including

food, cosmetic, medical, biotechnology, agriculture and textiles. Arancibia et al. (2015) reported on using mild treatments to extract chitin and derivatives, such as chitosan and chitooligosaccharides from shrimp waste. Furthermore, they elaborated on developing chitosan based biofilms incorporated with protein hydrolysates extracted from shrimp waste (Arancibia et al., 2015) . Recently, Shariatinia and Jalali (2018) discussed the antimicrobial activities of chitosan hydrogels and applications in drug delivery, tissue engineering, as well as, in water treatment.

Table 2.1.

Proximate composition from different crustacean species based on dry weight basis.

Sources	Protein (%)	Ash (%)	Chitin (%)	Lipid (%)	References
White shrimp *(Penaeus vannamei)* heads	54.4	21.2	9.3	11.9	(Trung & Phuong, 2012)
Pink shrimp (*Parapenaeus longirostris*) shells	31.3	28.61	34.86	3.97	(Sila, Nasri, & Bougatef, 2012)
Blue Crab (*P. segnis*) shells	11.25	59.11	27.53	1.07	(Hamdi et al., 2017)
Green Crab (*Carcinus maenas*) shells	14.08	38.00	43.9	3.23	Kirubanandan, S. (2016)
Lobster shells	25.83	40.64	-	-	(Zhu, Gu, Hong, & Lian, 2017)

Currently, there are various research articles on general use of fishery and shellfish wastes, to produce bioactive compounds; however, in this literature review we will focus on recent methods of extracting carotenoproteins, chitin, chitosan and chitooligosaccharides in crustacean waste. Furthermore, we will discuss their applications in the food industry and various other fields.

2.2. Carotenoids in crustacean shells:

Carotenoids are natural lipid-soluble pigments produced mainly by photosynthetic plants and algae (Wade et al., 2017). In crustaceans, like other animals, carotenoids are obtained through their diet; however, they can synthesize derivatives, including astaxanthin, from dietary carotenoids (Hooshmand, Shabanpour, Moosavi-Nasab, & Golmakani, 2017; Wade et al., 2017). Carotenoids can be classified into two groups: (I) carotenes, composed of unsaturated hydrocarbons (e.g., α-carotene, β-carotene, lycopene); and (II) xanthophylls, which consist of oxygenated functional groups, including hydroxyl and keto groups (e.g., astaxanthin, canthaxanthin, lutein) (Hooshmand et al., 2017; Saini et al., 2018). Although crustacean species intake a variety of carotenoids in their diet, astaxanthin is predominant (Hooshmand et al., 2017). However, in the shells of crustacean species, astaxanthin can be found in the form of carotenoproteins (Anahi Martinez-Delgado, Khandual, & Josefina Villanueva-Rodriguez, 2017). In addition to being natural colorants, carotenoids and carotenoproteins function as potent antioxidants. The presence of long conjugated aliphatic chains of carotenoids function as an antioxidant to free radical species by resonance-stabilization (Saini et al., 2018). In astaxanthin, the hydroxyl and keto groups further enhances its antioxidant properties by free radical scavenging and singlet oxygen quenching without pro-oxidative effects (Hamdi et al., 2018; Saini et al., 2018). In previous studies, astaxanthin was shown to be a powerful antioxidant compared to other carotenoids: with an activity 100 times more effective than alpha tocopherol (Saini et al., 2018).

Astaxanthin in its native state is red in color. When complexed with proteins to form carotenoproteins, it produces a green, or blue to purple color in crustacean shells (Anahi Martinez-Delgado et al., 2017; Saini et al., 2018). Crustacyanin are the main lipoprotein complex involved in the carotenoprotein of crustacean exoskeleton and hypodermal tissues (Wade et al., 2017). They belong to the family protein “lipocalin” which are a diverse group of proteins responsible for the binding of small hydrophobic molecules, such as carotenoids (Wade et al., 2017). The interaction of crustacyanin with astaxanthin modifies the neutral astaxanthin α-hydroxyketone form (native

red color) (Begum et al., 2015); as a result, a diverse array of colors are produced in crustacean shells (Wade et al., 2017). The most commonly found carotenoproteins in the exoskeleton of crustaceans are β-crustacyanin (dimers of crustacyanin-astaxanthin) and α-crustacyanin (octamer of β-crustacyanin) with a maximum absorbance between 580-590 nm and 630 nm, respectively (Saini et al., 2018; Wade et al., 2017). Upon heating, the carotenoprotein complex denatures and astaxanthin is released into its α-hydroxyketone form to produce a red color (Anahi Martinez-Delgado et al., 2017). In addition, astaxanthin can be found either esterified or in free form (Anahi Martinez-Delgado et al., 2017). Previous studies on Green crab (*Carcinus maenas*) shells have shown "astaxanthin pigment" in crustacea to consist of predominantly astaxanthin diester and monoester followed by free form astaxanthin (Naczk, Williams, Brennan, Liyanapathirana, & Shahidi, 2004). Similar results were reported by Senphan, Benjakul, and Kishimura (2014) for the composition of carotenoproteins from shells of Pacific white shrimp: suggesting astaxanthin can be found present in carotenoprotein esterified to fatty acids as mono or diester and free form. Astaxanthin is commonly found esterified with saturated or unsaturated fatty acids on one or both hydroxyl groups (Saini et al., 2018). In astaxanthin monoesters and diesters of shrimp (*L.vannamei*) discards, saturated fatty acid palmitic acid (C16:0) and unsaturated fatty acids oleic acid (C18:1n9c), linoleic acid (C18:3n3), docosahexanoic acid (DHA,C22:6n3) and eicosapentaenoic acid (EPA, C20:5n3) were the most abundant fatty acids reported (Gómez-Estaca, Calvo, Álvarez-Acero, Montero, & Gómez-Guillén, 2017). Therefore, astaxanthin can be a source of essential fatty acids.

2.2.1. Extraction of carotenoproteins and carotenoids

In recent years, researchers have been improving the methods of extracting carotenoids and carotenoproteins from crustacean shells. The two type of methods used for extraction are chemical and biological.

2.2.1.1. Chemical extraction

Traditionally, acetone was the most common solvent used during chemical extraction of carotenoids from aquatic animal tissues (K. L. Simpson, Katayama, & Chichester, 1981). For example, Hooshmand et al. (2017) reported that acetone had the highest yield in carotenoids from blue crab (*Protunus pelagicus*) and shrimp (*Penaeus semisulcatus*) discards (6.632 μg/g and

61.321 μg/g) compared to vegetable oils (0.207 μg/g and 4.025 μg/g, respectively). However, acetone among other organic solvents, have shown to break carotenoproteins apart due to carotenoids' high affinity to hydrophobic mediums (K. L. Simpson et al., 1981). Carotenoproteins are a hydrophilic complex compared to the native carotenoid compound in nature. Therefore, the extraction of carotenoproteins requires aqueous solvents, including dilute salt solutions (K. L. Simpson et al., 1981). In the chemical extraction of caretonoproteins from lobster (*Jasus lalandii*) shells, Timme, Walwyn, and Bailey (2009) used 10% (w/v) EDTA solution to suspend grinded shells. However, in the study, Timme et al. (2009) failed to quantify the yield of carotenoproteins extracted, instead the focus was on the qualitative analysis, including the effects of temperature and pH on the bathochromic shifts of carotenoproteins (α-crustacyanin and β-crustacyanin). Although, traditional solvent extraction is effective at extracting total carotenoid from crustacean shells, it is limited in maximizing the yield of carotenoids and carotenoproteins compared to other methods, such as enzymatic hydrolysis (Babu, Chakrabarti, & Surya Sambasivarao, 2008).

2.2.1.2. Biological extraction

Proteolytic enzymes have shown to increase the total yield of carotenoids compared to chemical methods (solvent extraction, supercritical fluid extraction and oil based extraction) (Senphan et al., 2014). In a study, Babu et al. (2008) compared the carotenoid yield of papain, pepsin and trypsin during the extraction of carotenoprotein from head waste of different shrimp species (wild *P. monodon, P. indicus, M. monocerous* and cultured *P. monodon*). Trypsin yielded the highest amount of carotenoids among all species of shrimp head waste (987, 531, 596 and 430 μg carotenoids/g sample, respectively), however, pepsin and papain also showed competitive carotenoid yields (Babu et al., 2008). Similar results were reported in brown shrimp (*Metapenaeus monoceros*) shell waste (Chakrabarti, 2002). The high success rate of commercial trypsin has promoted researchers to further explore alternative sources to reduce cost for carotenoprotein extraction (Poonsin et al., 2018). As shown in Table 2.2, several studies have been focused on developing extraction protocols using enzymatic and microbial methods to investigate bioactive properties of carotenoproteins from crustacean waste.

Trypsin is a digestive enzyme in the intestine that is characterized for its specificity to hydrolyze at the carboxyl group of non-terminal amino acids arginine and lysine (Klomklao, Benjakul, Visessanguan, Kishimura, & Simpson, 2009; Assaâd Sila et al., 2012). Mammalian

pancreatic trypsin, such as bovine trypsin have been extensively investigated, while interest in isolated trypsin from fish viscera has been emerging in present work as value-added products from fishery waste (Assaâd Sila et al., 2012). Bluefish trypsin was shown to aid in the recovery of carotenoproteins from black tiger shrimp shells (Klomklao et al., 2009). The recovery of protein and carotenoids were reported to be stable after 60 min of proteolytic treatment with bluefish trypsin (Klomklao et al., 2009). In the extraction of carotenoproteins from deep-water pink shrimp (*Parapenaeus longtrostris*) A. Sila et al. (2012) reported similar results for barbel (*Barbus callensts*) trypsin suggesting that 1h was the most effective hydrolysis time. However, when comparing the two methods of extraction Klomklao et al. (2009) reported that bluefish trypsin at 1.2 unit/sample was the most effective, while Sila et al. (2012) concluded that 1.0 unit/sample was the most effective concentration for barbel trypsin. This suggest that the two trypsins slightly differ in their proteolytic activities. This demonstrates that the origin of species contributes to the overall activity of trypsin. In each study, trypsin facilitated the release of protein and fats from shrimp waste. The protein content was used as an indicator of peptide cleavage to release carotenoproteins while fat content reflected the recovery of pigments during hydrolysis (Klomklao et al., 2009). As a result, the carotenoprotein proximate composition showed high content of protein and fats with the aid of bluefish trypsin and barbel trypsin (70.20 and 19.76 % dry weight basis; 71.09 and 16.47 % dry weight, respectively) (Klomklao et al., 2009; A. Sila et al., 2012). Furthermore, the aid of bluefish trypsin and barbel trypsin reduced the ash content and chitin contents in carotenoproteins (6.57 and 1.50% dry weight basis; 7.78 and 1.79% dry weight basis), thus suggesting they were retained in the shrimp shells (Klomklao et al., 2009; A. Sila et al., 2012). According to Klomklao et al. (2009), bluefish trypsin was as effective as bovine trypsin in recovering carotenoproteins from shrimp waste. In this regard, bluefish trypsin differed from barbel trypsin, which was reported to be more effective than bovine trypsin for recovering carotenoproteins from shrimp waste (A. Sila et al., 2012). Similar results were reported for Atlantic trypsin, which was shown to be more effective than bovine trypsin at recovering carotenoproteins at 4°C (Cano-Lopez, Simpson, & Haard, 1987).

Table 2.2.

Carotenoprotein extraction from shrimp discards using biological methods

Extraction sample	Methodology	Major research output	Reference
Head waste of different shrimp species (wild *P. monodon, P. indicus, M. monocerous* and cultured *P. monodon*)	Extraction of carotenoproteins by proteolytic enzymes: papain (pH 6.2 at 55°C), pepsin (pH 4.0 at 45°C) and trypsin (pH 7.6 at 45°C).	Trypsin extracted the highest amount of carotenoids from all species of shrimp head waste (987, 531, 596 and 430 μg/g, respectively).	(Babu et al., 2008)
Shrimp (*Litopenaeus vannamei*) discards	Extraction of carotenoproteins by lactic acid fermentation followed by enzymatic treatments: (1) protein hydrolysis using Savinase™ and (2) protein-lipid hydrolysis Savinase™ and Lipolase™.	The combination of proteinase and lipase was the most effective treatment for carotenoprotein hydrolysis with 15 proteolytic and 10 lipolytic units (900 and 66 mg/g soluble protein and total carotenoids, respectively).	(Armenta & Guerrero-Legarreta, 2009)
Pink shrimp (*P. longirostris*) waste	Extraction of carotenoproteins by Alcalase® enzyme (pH 8.0 at 50°C).	The peptidic fraction of extracted carotenoproteins consisted of 80.8% protein, 2.74% lipid, 14.4% ash, 1.13 % chitin and 1.08 μg carotenoids/g sample.	(Sila et al., 2014)
Shells of Pacific white shrimp	Extraction of carotenoproteins using	Carotenoproteins extracted with the aid of hepatopancreas	(Senphan et al., 2014)

(*Litopenaeus vannamei*)	hepatopancreas proteases (pH 8.0 at 60℃).	proteases consisted of 73.58 % protein, 21.87 % lipid, 2.63 % ash and 1.42 % chitin. The carotenoprotein was composed of major carotenoids astaxanthin and diester astaxanthin.	
Shrimp shell waste	Extraction of carotenoproteins using *S. scriba* crude alkaline protease (pH 10 at 30℃).	The extracted carotenoproteins showed increasing antioxidant activity with increased concentrations through DPPH radical scavenging activity (IC_{50} 3 mg/ml) and inhibition of β-carotene bleaching assay.	(Nasri, Abed, Karra-châabouni, Nasri, & Bougatef, 2015)

Other sources of enzymes have been shown to be as effective as trypsin enzymes in the extraction of carotenoproteins. Senphan et al. (2014) used crude proteases from pacific white shrimp (*Litopenaeus vannamei*) hepatopancreas to extract carotenoproteins from the shells of the same species shrimp. It was reported that hepatopancreas proteases aid in the extraction of carotenoproteins consisting of 73.58% protein, 21.87% lipid, 2.63 % ash and 1.42 % chitin (Senphan et al., 2014). The proximate composition of carotenoproteins obtained from the hepatopancreas proteases followed a similar trend to that of bluefish trypsin (Klomklao et al., 2009) and barbel trypsin (A. Sila et al., 2012). Furthermore, Nasri et al. (2015) investigated crude alkaline proteases from the viscera of *S. scriba.* It was reported that crude alkaline proteases had stability over a wide pH range (pH 7.0-13) compared to bovine trypsin (pH 7.0 – 9.0) and was also effective in the extraction of carotenoproteins from shrimp shells. This suggests that crude enzymes from viscera or other digestive organs of aquatic animals have potential use for enzymatic extraction of carotenoproteins. In addition, enzymes from microbial sources have also been shown to aid in the extraction of carotenoproteins from crustacea. The highest yield of carotenoids and soluble proteins was obtained when lactic acid fermented carotenoproteins were subjected to a mixture of proteinase and lipase (Armenta & Guerrero-Legarreta, 2009). The combination of

commercial enzymes Savinase™ and Lipolase™ was the most effective treatment for carotenoprotein hydrolysis with 15 proteolytic units (one proteolytic unit refers to the amount of protease required for the release of 1μmol of tyrosine/min at 40°C) and 10 lipolytic units (one lipolytic unit refers to the amount of lipases required for the release of 1μmol of butyric acid/min at 40°C) at pH 8 for 24 h at 40°C (900 and 66 mg/g soluble protein and total carotenoids, respectively) (Armenta & Guerrero-Legarreta, 2009). Carotenoproteins subjected to only proteases obtained a high yield in soluble proteins and carotenoids following hydrolysis treatment with 15 proteolytic units at pH 8 for 24h at 40°C (852 and 48 mg/g soluble protein and total carotenoids, respectively (Armenta & Guerrero-Legarreta, 2009). When comparing the efficiency of the two treatments reported, the use of only protease is sufficient to yield soluble protein content and carotenoid content if budget is a concern. For instance, Sila et al. (2014) used commercial Alcalase® enzyme from microbial source to extract carotenoproteins from Pink shrimp (*P. longirostris*) waste and further used it as an additional step to extract the protein fraction of the carotenoproteins (peptidic fraction of extracted carotenoproteins consisted of 80.8% protein, 2.74 % lipid, 14.4% ash, 1.13 % chitin and 1.08 μg carotenoids/g sample). Therefore, enzymatic hydrolysis can be used to hydrolyze extracted carotenoproteins as a modification to B. K. Simpson and Haard (1985) method that has been frequently reported in literature: involving chemical solvents, including cold acetone and petroleum ether to dissociate carotenoids from carotenoproteins.

2.2.2. Application in the food industry

Carotenoproteins are used as a source of carotenoids and proteins which are known to have potent antioxidant properties, essential nutrient benefits and many other properties that can be used in the food industry. In modern application these bioactive compounds have been growing in demand to add value, as well as be a source of natural colorant to improve foods (Hamdi et al., 2018).

2.2.2.1. Antioxidant properties

Oxidative stress is an imbalance of free radicals (singlet oxygen, peroxides and hydroxy radicals) and antioxidants that play a pivotal role in the development of chronic diseases, including cardiovascular diseases and cancer (Saini et al., 2018). In the presence of free radicals unsaturated

fatty acids undergo autoxidation in which hydroperoxides (H_2O_2) and hydroxy radicals ($OH^{\cdot}$) are involved in an oxidative chain reaction. Thus, the role of antioxidants is to donate electrons or hydrogen atoms to radials without becoming a prooxidant agent. Carotenoids have received significant attention for their potential to preserve lipid rich foods to prevent lipid oxidation. Poonsin et al. (2018) reported that carotenoproteins extracted from Pacific white shrimp (*Litopenaeus vannamei*) shells showed strong antioxidant activity as indicated by DPPH and ABTS radical scavenging activities, ferric reducing antioxidant power, chelating activity, H_2O_2 and $OH^{\cdot}$ scavenging activity comparable to BHT and EDTA. These results were consistent with Senphan et al. (2014), who reported extracted carotenoproteins at levels up to 5 mg/mL elicited antioxidant activity. Although carotenoproteins showed increasing antioxidant activity with increasing concentrations Poonsin et al. (2018) reported that BHA and EDTA antioxidant properties and metal chelating properties respectively, are more superior. Similar results were reported by Nasri et al. (2015), where β-carotene bleaching inhibition activity in the presence of extracted carotenoproteins was compared to BHA. The discoloration of β-carotene was instantly hindered with the addition of BHA, while carotenoproteins gradually slowed down the reaction (Nasri et al., 2015). From the literature, carotenoproteins have been shown to have potential as a natural antioxidant; however, synthetic antioxidants BHT, BHA and chelating agents EDTA are far more potent in inhibiting lipid oxidation. Sila et al. (2014) reported that peptidic fraction of carotenoproteins (PFCP) extracted from shrimp (*Parapenaeus longirostris*) by-products showed significantly ($p < 0.05$) higher metal chelating capacity than EDTA at concentrations 1, 2 and 3 mg/ml. This suggest that the peptidic portion of carotenoproteins can slow the oxidation reaction more effectively than when complexed to carotenoids. The functional groups of amino acids are able to donate electrons to pro-oxidant species. However, similar to extracted carotenoproteins studied in literature, PFCP showed lower antioxidant activity than synthetic antioxidants (Sila et al., 2014). Therefore, the composition of extracted carotenoproteins can be studied further to improve their overall antioxidant activity.

2.2.2.2. Preservative

The incorporation of natural preservatives in foods prone to oxidation and microbial growth has become a growing trend. The influence of combined carotenoids and reduced-nitrite on the overall quality of cured meat has been of interest in the literature. In one study, Hamdi et

al. (2018) investigated the effect of carotenoprotein (CP) extract from blue crab (*Callinectes sapidus*) shells on the quality of reduced-nitrite Turkey meat sausages (TMSs). In the procedure, Hamdi et al. (2018) reported that six different samples of TMSs were prepared: (1) 72 ppm ammonium nitrite, 0.05 % vitamin C and 0.01 % synthetic colorant; (2, 3 and 4) 36 ppm ammonium nitrite, CP (0.1, 0.25 and 0.5 %, respectively) and no vitamin C or synthetic colorants; (5) contained only 36 ppm ammonium nitrite; and (6) contained only 0.5 % CPE. In the study, different quality parameters were investigated, including antioxidant potential, microbial assay, pH, color of TMSs within a shelf life of 10 days of storage at 4°C. Hamdi et al. (2018) reported that antioxidant and antimicrobial activities of CP in reduced nitrite TMSs was as efficient as the positive control containing 72 ppm ammonium nitrite, 0.05 % vitamin C and 0.01 % synthetic colorant. The antioxidant activity indicated by Free fatty acid (FFA) stability and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging test revealed that the reduced-nitrite TMS samples containing varying concentrations of CP had the lowest content of FFA and highest DPPH activity over the 10 days of storage (Hamdi et al., 2018). The results suggest that CP can be an alternative to vitamin C in cured meat, since it was shown to have higher antioxidant activity. This suggest CP showed an antioxidant activity that is concentration-dependent (Poonsin et al., 2018). However, the TMSs containing only 0.5 % CP had a lower antioxidant activity (Hamdi et al., 2018). This shows that the presence of nitrite contributes to the overall antioxidant activity. Further studies can be conducted to investigate the minimum concentration of nitrite required to enhance the antioxidant activity of CP. For the microbial analysis of TMSs, all four samples containing CP were able to delay microbial proliferation over the ten days storage period. In the absence of CP, microbial growth increased from 5.59 to 8.48 log cfu g^{-1} TMS during 10 storage days (Hamdi et al., 2018). Hamdi et al. (2018) suggested that the antimicrobial property of CP could be assigned to carotenoids, predominately astaxanthin. Nonetheless, there is not sufficient literature published to support this thesis. In recent literature essential oils are more frequently discussed as an alternative antimicrobial preservative option for various products, such as cheese, rainbºw trout fillets, and other food products (Calo, Crandall, O'Bryan, & Ricke, 2015; Hassoun & Emir Çoban, 2017; Khorshidian, Yousefi, Khanniri, & Mortazavian, 2018; Ozogul et al., 2017). The incorporation of CP in reduced-nitrite TMSs was shown to have potential as an alternative preservative for cured meats, while maintaining pH stable and color; however, further assessments including sensory evaluation is recommended for future works.

2.2.2.3.Essential nutrients

Carotenoproteins are also a source of essential amino acids. Upon hydrolysis of the complex, further separation techniques are applied to identify the components of proteins. Senphan et al. (2014) reported that carotenoproteins extracted from pacific white shrimp shells were rich in glutamic acid/glutamine and aspartic/asparagine (85.29 and 68.98 mg/g sample respectively). The presence of all nine essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) in adults were determined in the extracted carotenoproteins. The most prominent essential amino acids were leucine and lysine (47.77 and 40.33 mg/g sample, respectively) (Senphan et al., 2014). These results were in agreement with those of Armenta and Guerrero-Legarreta (2009), who reported that fermented carotenoproteins from shrimp (*Litopenaeus vannamei*) waste were rich in aspartic acid and glutamic acid, as well as the essential amino acids leucine and lysine (126.7 and 142.2 mg/g carotenoproteins, as well as 110.1 and 99.2 mg/g carotenoproteins, respectively). Therefore, based on these findings the protein fraction of carotenoproteins can be utilized to produce protein hydrolysates enriched in essential amino acids. Sila et al. (2014) reported that the protein hydrolysates extracted from deep-water pink shrimp (*P. longirostris*) carotenoproteins were rich in essential amino acids, including arginine, lysine, histidine and leucine, which shows it to be a potential dietary protein supplement.

2.3. Chitin, Chitosan and Chitooligosaccharides

2.3.1. Chitin and chitosan

Chitin is the second most abundant polysaccharide after cellulose. It is the major component of exoskeleton and cuticles of various arthropods, including crustaceans. Chitin is characterized by its β-(1-4)- N-acetylglucosamine chain. As shown in figure 2.1., chitin exist in three polymeric forms: α-chitin (anti-parallel chains), β-chitin (parallel chains) and γ-chitin (combination of anti-parallel and parallel chains) (El Knidri et al., 2018; Feng et al., 2012). The α-chitin found in crustacean shells is the most stable and abundant form (El Knidri et al., 2018). Chitin is insoluble in water and most organic solvents (Feng et al., 2012). The strong inter and intramolecular hydrogen bonding of chitin creates a compact network which provides the limited solubility properties (El Knidri et al., 2018). For industrial application, derivative forms of chitin, including chitosan obtained by partial deacetylation is preferred (El Knidri et al., 2018). When the

degree of deacetylation (DDA) if above 50%, the polymer is referred to as chitosan, and when the DDA is below 50%, the polymer is referred to as chitin (El Knidri et al., 2018). In most literature, chitosan is reported with a minimum of 70% DDA (Chang et al., 2019; Guerrero, Muxika, Zarandona, & de la Caba, 2019; Sabbah et al., 2019). As shown in figure 2.2., the exposure of amino groups in the chitosan structure gives rise to many properties. Chitosan is a cationic polymer. The positively charged groups enhances the solubility of chitosan in dilute acidic solvents, and further provides antimicrobial properties and can interact with anionic polymers, such as xanthan gum, sodium carboxymethyl cellulose, sodium alginate and carrageenan (Hamed et al., 2016;(Shao, Li, Gu, Wang, & Mao, 2015). In general, chitin and its derivative forms are biodegradable, non-toxic, biocompatible and therefore have a wide range of industrial applications, including foods, pharmaceuticals, biomedical and many other fields (Hamed et al., 2016).

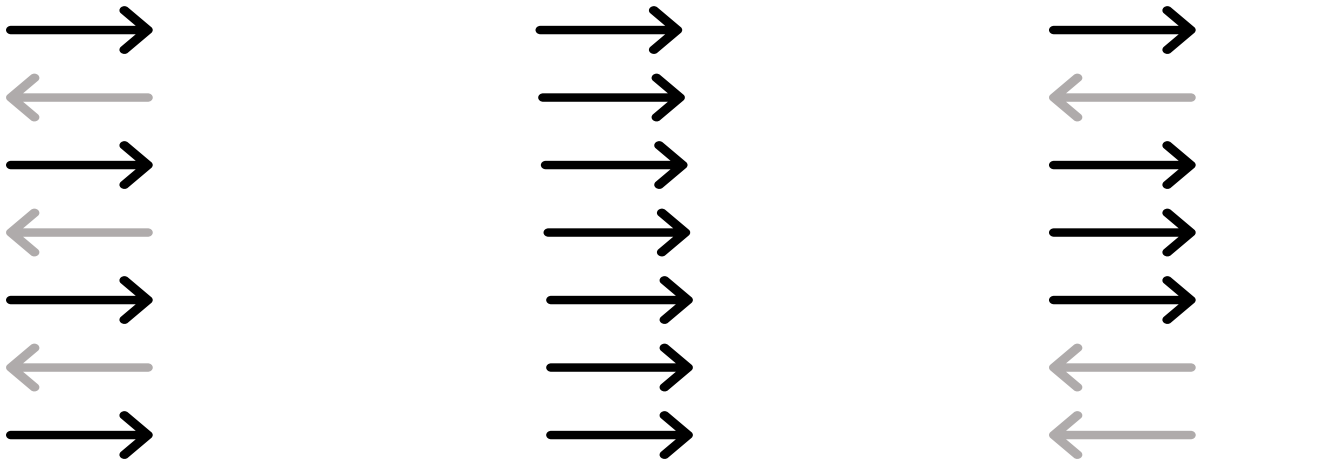


Figure 2.1.

The schematic representation of the three polymorphic forms of chitin (α-chitin, β-chitin and γ-chitin)

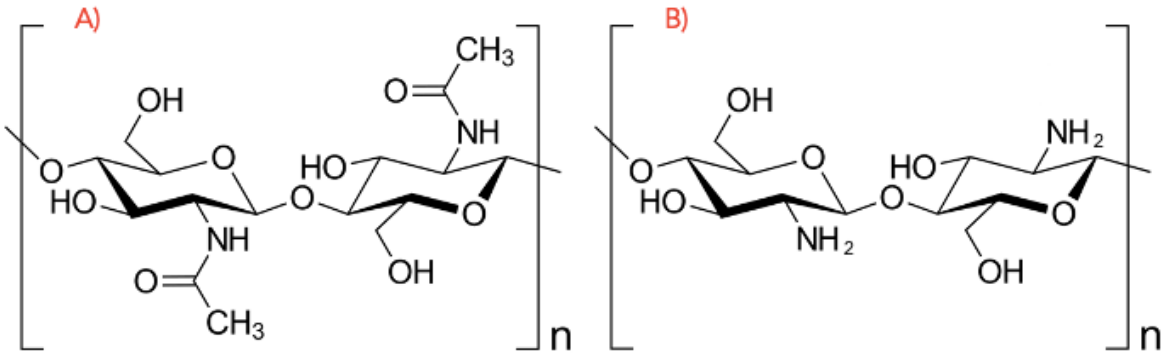


Figure 2.2.

Schematic representation of chitin (A) and chitosan (B) chemical structures.

2.3.2. Chitooligosaccharides

Chitooligosaccharides (COS) are produced by hydrolyzing chitin and chitosan polymers into oligomers of an average molecular weight (MW) less than 3.9 kDa and a degree of polymerization (DP) between 2-20 units (Lodhi et al., 2014). COS generally consist of β-(1-4)- N-acetylglucosamine or β-(1-4)- N-deacetylglucosamine units (Liaqat & Eltem, 2018; Lodhi et al., 2014). They can be classified based on their DDA as homochitooligosaccharides or heterochitooligosaccharides. Homochitooligosaccharides refer to oligomers that are exclusively composed of N-acetylglucosamine (A unit) or N-deacetylglucosamine (D unit), while heterochitooligosaccharides are comprised of both A and D units as shown in figure 2.3. The demand for COS is much greater than that of precursors chitin and chitosan as a result of being low MW (Liaqat & Eltem, 2018). COS possess water solubility properties, a wide range of bioactivity, including antioxidant properties, antimicrobial properties and various commercial applications, which is of interest in the food industry (Liaqat & Eltem, 2018).

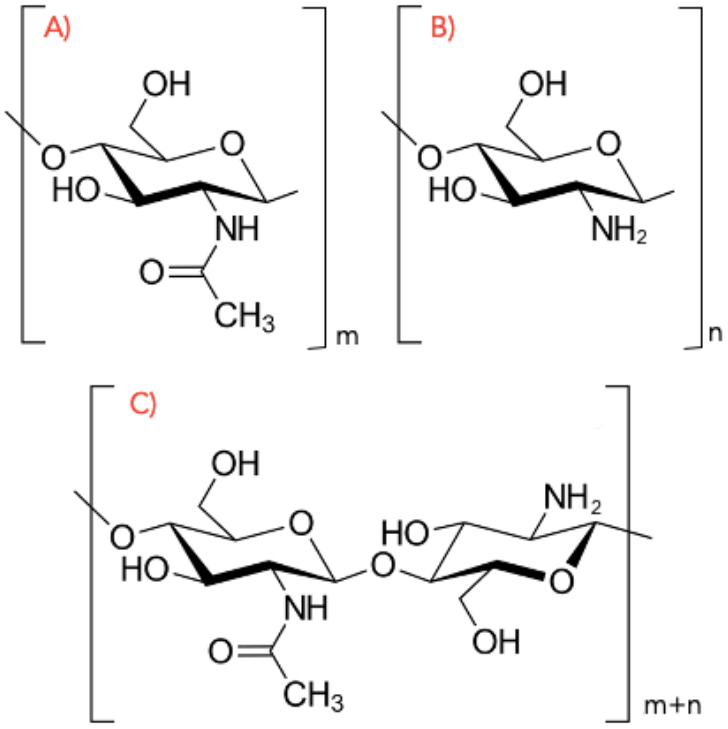


Figure 2.3.

Structure of *N*-acetylated COS (A), *N*-deacetylated COS (B) and partially *N*-deacetylated COS.

2.3.3. Extraction methods

Novel methods to extract chitinous material from crustacean waste and other sources, such as insect and fungus has been of interest in recent literature. Chitin is most frequently extracted by chemical methods. Although, chemical methods are simple and are a faster approach, there are some concerns regarding the quantity and type of solvents being used. Therefore, enzymatic and other new methods have been recommended to extract chitin and derivatives as an alternative to replace the noxious chemical agents. In general, there are three steps to extracting chitin: (1) Demineralization: to remove calcium carbonate ($CaCO_3$) the main inorganic salt of shells and various trace elements. (2) Deproteinization: to remove proteins and carotenoproteins. (3) Discoloration: to remove remaining pigments following deproteinization (El Knidri et al., 2018).

Additional steps are required for chitin derivatives which includes deacetylation to obtain chitosan and hydrolysis to obtain chitooligosaccharides.

2.3.3.1. Chemical and enzymatic extraction methods

Several scientific publications over the past several years have described detailed studies of extracting chitin and derivates from crustacean sources by chemical and enzymatic methods.

2.3.3.1.1. Chitin extraction

Saravana et al. (2018) describe extraction of chitin from shrimp shells (*Marsupenaeus japonicas*) using Deep eutectic solvents (DES). The use of DES involves a eutectic mixture of hydrogen bond acceptors and donors, consisting of various anionic and cationic functional groups (Saravana et al., 2018). The shrimp shells were treated with DES (25 mL/g sample) for 2h at 80℃ to reduce protein and ash content followed by 10% H_2O_2 (w/v) at 80℃ during discoloration. The chitin extracted using DES containing choline chloride and malonic acid (1:2 molar ratio) yield 23.86 % with low ash, protein and moisture content (0.74, 1.53 and 3.72 % respectively) comparable to commercial chitin. Zhu et al. (2017) reported a similar extraction method of chitin from lobster shells (Nanjing, Jiangsu, China) using choline chloride-malonic acid DES for 2h at 50^{o}C followed by depigmentation under the same conditions as the previous work mentioned. However, the extracted chitin yield 16.19% with lower ash content, a slight higher protein and moisture content (0.30, 2.02 and 4.21 % respectively) (Zhu et al., 2017). In both works, the use of choline chloride-malonic acid DES to extract chitin was as effective as the conventional chemical method which entails demineralization with 6% HCl (w/v) at room temperature for 2.5 h, deproteinization with 10% NaOH (w/v) at 90℃ for 3h and decolorization by 10% H_2O_2 (w/v) at 80℃ (Saravana et al., 2018; Zhu et al., 2017). However, the slight variation in ash, protein and moisture content may be a result of variation in temperature used during the experiment, the source and approximate composition of the shells. Hong, Yuan, Yang, Zhu, and Lian (2018) describe extraction of chitin from cooked lobster shell waste (Nanjing, Jiangsu, China) using four DES solvents at varying temperature treatments 50, 70 and 100℃ for 2h followed by discoloration with 10% H_2O_2 at 80℃. In Hong et al. (2018) findings, DES of choline chloride-malonic acid remained the most successful at isolating chitin, however, a slight variation in yield, ash, protein and moisture content was observed at different temperature treatments. This was further confirmed

when comparing the intrinsic viscosity and MW of extracted chitin using choline chloride-malonic acid at all three treatments (Hong et al., 2018). The following trend was observed: as the temperature increased from 50 to 100°C the intrinsic viscosity (mL g^{-1}) and MW (kDa) of chitin decreased (50°C: 1260 mL g^{-1} and 312 kDa; 70°C: 1128 mL g^{-1} and 278 kDa 100°C: 800 mL g^{-1} and 199 kDa , respectively) (Hong et al., 2018). When compared to the conventional method, the intrinsic viscosity and MW of chitin was greater than extracted chitin using DES at all three temperature treatments (2145 mL g^{-1} and 546 kDa respectively) (Hong et al., 2018). Saravana et al. (2018) reported a similar trend when comparing the viscosity (Cp) and MW (kDa) of extracted chitin using DES to the conventional method, as well as to standard commercial chitin (380 Cp and 79 kDa; 680 Cp and 132 kDa ; 1600 Cp and 286 kDa). Therefore, this suggest that DES is a more green approach to extracting chitin and is as effective as using conventional chemical method. However, it should be noted that the viscosity and MW of chitin was lowered with the use of DES when compared to conventional chemical extraction method.

Nguyen, Barber, Smith, Luo, and Zhang (2017) describes the extraction of chitin from deproteinized rock lobster (*Jasus edwarsii*) shells by microwave assisted demineralization with 7.5% lactic acid (18 mL/g sample) for 23 min. The mineral content of extracted chitin was 0.99% which showed to be significantly lower than 3.7% for chitin prepared by conventional demineralization with 7.5% lactic acid (50 mL/g sample) at 100°C for 60 min (Nguyen et al., 2017). Microwave induced lactic acid demineralization is a potential process that is less time consuming and requires less solvent to sample ratio, while reducing mineral content of chitin by 10 folds compared to the conventional method (Nguyen et al., 2017). More recently, Berton, Shamshina, Ostadjoo, King, and Rogers (2018) described ionic liquid-extracted chitin from raw black tiger shrimp shells using microwave pulsing with 1-ethyl-3-methylimidazolium acetate for 2.5 min. The DDA of chitin was determined by Cross Polarization Magic Angle Spinning (CP-MAS) nuclear magnetic resonance (NMR) indicating no deacetylation was observed from the ionic liquid-extract (Berton et al., 2018). Therefore, microwave pulsed ionic liquid-extraction is advantageous in terms of time, as well as maintaining the chitin fully acetylated. The use of a non-thermal technology may be a promising technique that is environmentally friendly and requires less resources for a pilot plant production scale. However, further studies are required to determine the quality of chitin, such as MW, crystallization, and solubility to compare with conventional extraction methods.

2.3.3.1.2. Chitosan extraction

Muley, Chaudhari, Mulchandani, and Singhal (2018) describe preparation of chitin and chitosan from prawn shells (Mumbai, India) by deproteination using 5 % NaOH at 90℃ for 24 h, demineralization using 4 % HCl for 36 h, and decolorization with chilled acetone treatment. The extracted chitin was further deacetylated with 50 % NaOH to obtain chitosan at 100℃ for 2 h (Muley et al., 2018). The chitosan yield extracted was 22.08 %, with a molecular weight of 173 kDa and a DDA of 78.4 % (Muley et al., 2018). Similarly, Al-Manhel, Al-Hilphy, and Niamah (2018) reported on extracting chitin and chitosan from shrimp (*Penaeus semisulcatus*) shells using 1N HCl (1:15 w/v) at 30℃ for 6 h to demineralize followed by deproteinization step using 3.5% NaOH solution (1:10 w/v) at 65℃ for 2 h and decolorization with 0.315% NaOCl. The deacetylation of chitin was carried out in 50 % NaOH (1:10 w/v) at 100℃ for 5 h to obtain chitosan. The chitosan yield extracted reached 12.93 % (Al-Manhel et al., 2018). Kheirandish et al. (2017) described extracting chitin from lobster shells (Persian Gulf, Iran) by demineralization using 10 % HCl for 24 h, followed by deproteinization using 10 % NaOH for 24 h. The extracted chitin was subjected in 45 % NaOH treatment to obtain a degree of deacetylation of 92 % (Kheirandish et al., 2017). Srinivasan, Kanayairam, and Ravichandran (2018) reported shrimp (*Panaeus monodon*) chitin extraction yield reached 30 % after demineralization using 1 M of HCl (1/30 (w/v)) for 74 min, deproteinization using 3 M of NaOH (1/30 (w/v)) for 74 min, and decolorization using acetone treatment (1/10 (w/v)) for 10 min at and 0.3155 NaOCl (1/10 (w/v)) for 15 min all at room temperature. After deacetylation of chitin using 50% NaOH at 90℃ for 50 min, the yield of chitosan reached 35% (Srinivasan et al., 2018).

As reported above, the deacetylation of chitin is carried out in extremely concentrated sodium hydroxide between 40-50% and high temperatures to obtain high degree of deacetylation. The yield of chitosan varies according to the method of extraction used as observed in published literature. From the literature reported, the method of Srinivasan et al. (2018) yield the highest content of chitosan. In contrast to the published articles, the methodology of Srinivasan et al. (2018) requires less time. For instance, the demineralization, deproteinization, decolorization and deacetylation steps require minimal time compared to the other methods described. As a result, the integrity of chitin and chitosan structure can be maintained during harsh chemical treatments, which factors into the overall extracted yield. Therefore, reducing the exposure time minimizes

partial deacetylation and chain degradation caused by demineralization and deproteinization steps to increase the quality of extracted chitin and chitosan (Srinivasan et al., 2018).

Arancibia et al. (2015) describes extracting chitosan from shrimp waste by mild treatment, which entailed a demineralization step using lactic acid (75.6 g/ L) for 36 h at 21℃. Deproteination was carried out through two enzymatic treatments with Viscozyme L (pH 4.5, 50℃) followed by hydrolysis with alcalase 2.4 L (pH 8.5, 50℃) to obtain chitin (Arancibia et al., 2015). Deacetylation of chitin was achieved by using 10 % NaOH solution for 72 h at 100℃ to obtain a DDA of 74 % and a MW of 320 kDa (Arancibia et al., 2015). In a recent article, Wang, Li, Han, Liu, and Yang (2018) described extracting chitin from crab shells with the aid of alcalase 2.4L FG (1g/100mL) in sodium phosphate buffer (pH 8.0) at 60℃ for 13 h, as opposed to using two enzymatic treatments. Younes et al. (2014) reported extracting chitin and chitosan from shrimp (*Metapenaeus monoceros*) shells with the aid of crude alkaline protease from microbial source *Bacillus mojavensis* A21 and viscera of Grey triggerfish (*Balistes capriscus).* The deproteination of shrimp shells was carried out at optimal conditions for each protease (20U/mg; 50℃ at pH 10 and 45℃ at pH 9, respectively) for 3 h to achieve an overall protein removal of 77 and 78 % respectively, followed by demineralization with 1.5 M HCl for 6 h at 25℃ (Younes et al., 2014). The extracted chitin was further treated with 12.5 M NaOH at 140℃ for 4 h to obtain chitosan (Younes et al., 2014). In Younes et al. (2014) work, chitosan extracted by enzymatic deproteinization with *B. mojavensis* A21 and *B. capriscus* showed the highest intrinsic viscosity (197 and 180 ml/g, respectively), which suggest a higher MW (17,650 and 19,780 g/mol, respectively), while chitosan obtained through chemical deproteination had a lower intrinsic viscosity and MW (75 ml/g and 5.820 g/mol, respectively). Similarly, Hamdi et al. (2017) describes extracting chitin from blue crab (*P. segnis*) and shrimp (*P. kerathurus*) shells with the aid of crude alkaline proteases from *P. segnis* viscera. The crab and shrimp shells were incubated with *P. segnis* protease (1-10 U/mg; pH 8.0) for 3 h at 50℃ to deproteinize followed by chemical demineralization with 0.55 M HCl for 48 h, at room temperature (Hamdi et al., 2017). The addition of *P. segnis* protease achieved a degree of 75 and 79 % deproteinization for crabs and shrimp shells, respectively (Hamdi et al., 2017).

As reported above, enzymatic hydrolysis is essential for the deproteinization of crustacean shells during chitin extraction. In the literature, various proteases, such as alcalase, viscozyme and crude alkaline protease showed to be successful at deproteinizing crustacean shells. However,

when comparing the different methods, deproteinization with the aid of crude alkaline protease seems to be the most efficient, since they achieve 75 to 80 % degrees of deproteinization at short incubation times of ~ 3h. This may be explained by the fact that crude alkaline proteases involve multiple mode of actions as opposed to purified enzymes that catalyze specific reactions. In the earlier sections, alkaline protease was also mentioned to be successful at extracting carotenoproteins from crustaceans, as well as trypsin. Therefore, utilizing enzymes is practical for deproteinization of shells to obtain chitin, carotenoproteins and protein hydrolysates. Moreover, when comparing chemical to enzymatic methods, a discoloration step is required for the chemical extraction of chitin from crustacea. However, during deprotonation of shells with the aid of enzymes it is assumed that pigments are successfully removed as carotenoproteins, thus a decolorization step is not mandatory. Consequently, less solvent is needed, and the use of harsh chemicals is also limited. In addition, Arancibia et al. (2015) and Younes et al. (2014) reported that deproteinization with the aid of proteases, as opposed to chemical deproteinization resulted in a higher MW chitosan. Therefore, these findings further supports the claim that chemical method results in low quality chitosan (Hamed et al., 2016).

2.3.3.1.3. Chitooligosaccharide extraction

Rakkhumkaew and Pengsuk (2018) prepared COS by chitosan hydrolysis using 6.25 N HCl at 56°C for 3 h. The chitosan carried out in acid hydrolysis yield 14.56 g/100g shrimp shells, with a low MW of 13 kDa and DDA of 54.83 % (Rakkhumkaew & Pengsuk, 2018). Varun et al. (2017) described hydrolysis of chitosan extracted from crab shells using two different acids: 0.5N and 1 N H_2SO_4 and 7 N HCl at 90°C for 1 to 6 h in a water bath. Acid hydrolysis carried out by 7N HCl produced a higher COS concentration followed by 1 N H_2SO_4 and 0.5 N H_2SO_4 (1.93, 0.84 and 0.55 mg/ml, respectively) (Varun et al., 2017). Varun et al. (2017) reported that acidic hydrolysis carried out in 0.5 N H_2SO_4 was preferred for further analysis, since it was simpler to neutralize to pH 6-7 by $CaCO_3$ although it had the lowest yield. Kazami et al. (2015) described hydrolyzing chitin from crab shells and squid pen (Tokyo, Japan) using concentrated HCl at 40°Cto prepare chitin oligomers of 4-6 GlcNAc units at varying times of hydrolysis 5, 15, 30 and 60 min. The MW of chitin reduced gradually as the time of hydrolysis increased (Control: 330, 256 to 60 min: 9645 Da, respectively) (Kazami et al., 2015). Kazami et al. (2015) reported that the recovery of chitin oligomers using acetone precipitation following acidic hydrolysis showed to be

the most efficient compared to using diluted NaOH neutralization method to reach pH 4-5. The acetone precipitation allowed for isolation of water-soluble oligomers from water-insoluble materials, which improved the yield of chitin oligomers (Kazami et al., 2015). In the study, Kazami et al. (2015) then tested if this procedure was also applicable to chitosan oligomers which was reported to be inefficient, possibly due to the presence of positively charged amino groups.

As reported above, neutralizing COS after acidic hydrolysis with concentrated acids is a struggle, especially for chitosan-based oligomers. The limitations of chemical acidic hydrolysis also involve the lack of control over hydrolysis mechanisms. As observed in the literature, temperature, concentration, time, starting material (chitin or chitosan) and acidic strength all play a role on the yield, MW, polarization and solubility of COS.

Fernández-de Castro et al. (2016) used chitosanase from *Streptomyces griseus* (EC 3.2.1.132) to hydrolyze chitosan (DDA 86%, MW 180KDa) from shrimp shells (*Pandalus borealis*) to produce COS (DDA 83%, MW 8.6 KDa). Polyethersulfone (PES) membranes were utilized to ultrafiltrate COS based on different MW cut off size (Fernández-de Castro et al., 2016). Berton et al. (2018) described using enzymatic hydrolysis of chitin hydrogel, raw black tiger shrimp shells, commercial chitin and extracted chitin from shrimp shells to generate COS.
The enzymatic hydrolysis of each sample was carried out in sodium phosphate buffer (50 mM, pH 6) with chitinase from *S. griseus* (0.1 mg/mL) for 48 h at varying temperatures 25, 37 and 50℃. The results revealed that enzymatic hydrolysis of shrimp shells generated purely monomeric N-acetylglucosamine, while a mixture of monomers and dimers were detected from commercial chitin and trimers were generated from extracted chitin (Berton et al., 2018). In addition, Berton et al. (2018) reported an increase in hydrolysis rate as the temperature increased from 25 to 50℃. These findings indicate that substrate and temperature highly influence the product and yield generated during enzymatic hydrolysis.

As reported above, chitosanase and chitinase from *S. griseus* have been shown to hydrolyze chitosan and chitin under mild conditions (optimum 37℃, pH 5-6) to produce low MW COS. Chitinase from *S. griseus* is a complex enzyme with the ability to degrade chitin in two enzymatic reactions: (1) chitin is cleaved into chitobiose units by chitodextrinase-chitinase, followed by (2) N-acetyl-glucosaminidase-chitobiase which cleaves the disaccharides into monomer acetyl-glucosamine units (Berton et al., 2018). Alternately, chitosanase catalyzes the endohydrolysis of β-1-4 linked N-acetyl-glucosamine and partially deacetylated glucosamine units

(Qin et al., 2018). Although, chitolytic enzymes from *S. griseus* have been shown to be effective at generating COS, other sources of chitinase and chitosanase haven been studied recently.

Qin et al. (2018) investigated the catalytic mechanism of a novel cold-adapted chitosanase (GsCsn46A) from rhizobacterium *Gynuelle sunshinyii*. The enzymatic conversion of 2 % (w/v) chitosan (DDA of 95 %) to COS incubated with 1.5 U/mL GsCsn46A was carried out in 20 mM sodium acetate buffer (pH 5.5) at 30°C for 0.5, 2 and 6 h (Qin et al., 2018). The GsCsn46A showed high enzymatic activity immediately after 30 min incubation to form $(GlcN)_{2\text{-}7\ DP}$ (Qin et al., 2018). After 2 h incubation COS of mainly $(GlcN)_{2\text{-}4\ DP}$ and $(GlcN)_{5\ DP}$ were detected while after 6 h reaction time only $(GlcN)_{2\ DP}$ and $(GlcN)_{3\ DP}$ were detected (Qin et al., 2018). These findings indicate that GsCsn46A chitosanase is suitable for hydrolysis of chitosan into COS under mild reaction conditions. Santos-Moriano et al. (2018) described the biosynthesis of COS using chitosanase from *Bacillus thuringiensis* var. *aizawai*. The reaction was carried out in 50 mM sodium acetate buffer (pH 5.0), consisting of 2 % (w/v) chitosan (600-800 kDa and DDA of 90%) was incubated with 0.05 U/mL extracted chitosanase at 60°C for 72 h (Santos-Moriano et al., 2018). The catalytic activity of *B. thuringiensis* chitosanase during the conversion of chitosan to COS was maximized after 55 h (Santos-Moriano et al., 2018). The main chitooligomer products were $(GlcN)_{2\text{-}5\ DP}$, where $(GlcN)_{4\ DP}$ was predominant (Santos-Moriano et al., 2018). Wang et al. (2018) further evaluated the catalytic activity of novel chitinase (BsChi) from *Bacillus subtilis* for the bioconversion of chitin. The hydrolysis of extracted chitin from crab shell powder was carried out in 20 mM sodium phosphate buffer (pH 6.0) incubated with 0.25 mg/mL BsChi at 40°C for 12h (Wang et al., 2018). BsChi showed to have higher catalytic activity than commercial chitinase from S. *griseus* for the production of $(GlcNAc)_{2\ DP}$ and GlcNAc units (Wang et al., 2018).

As reported above, chitosanase and chitinase from bacterial sources have been shown to be as effective as commercial *S. griseus* chitolytic enzymes at hydrolyzing chitin and chitosan to produce COS. However, enzyme kinetics and characteristics differ from one source to another, as seen through the literature. According to Qin et al. (2018) findings, the cold adaptive chitosanase from *G. sunshinyii* is a promising alternative compared to chitosanase from *B. thuringiensis,* since it requires less thermal energy and incubation time. In terms of large-scale production of COS, cold adaptive chitosanase can be a better economical choice. Additionally, chitinase from *B. subtilis* was shown to be an effective alternative to hydrolyzing chitin from extracted crab shells. In the study, Wang et al. (2018) reported an incubation time of 12 h as opposed to 48 h, while

maintaining the temperature moderate at 40℃. It requires less incubation time which could possibly indicate that chitinase from *B. subtilis* follows a different mode of action compared to chitinase from *S. griseus* which performs two enzymatic reactions. Furthermore, in recent studies, novel acidic chitinase from marine bacterium *Paenicibacillus barengoltzii* (Fu, Yan, Wang, Yang, & Jiang, 2016), chitinase from *Escherichia fergusonii,* chitosanase from *Chryseobacterium indologenes* and *Comamonas koreensis* (Kim et al., 2018) were also shown to produce COS under moderate conditions ranging from 30℃ to 50℃. However, further studies are required to investigate how effective they are at hydrolyzing extracted chitin and chitosan from crustacean source.

Miscellaneous enzymes such as cellulase, papain and lysozymes have been shown to hydrolyze chitosan to produce low MW chitosan and chitooligosaccharides. G.-J. Wu, Wu, and Tsai (2015) described degrading shrimp chitosan with cellulase (10 U/mL) carried out in 0.425M acetic acid-bicarbonate buffer (pH 5.2) at 55℃ for 9 h. The chitosan hydrolysate obtained with the aid of cellulase was further fractionated into low MW of 20 kDa and chitooligosaccharides with DP between the range of 1-6 (G.-J. Wu et al., 2015). The effectiveness of cellulase may be due to its ability to cleave β-1-4-glycosidic bonds of chitosan (G.-J. Wu et al., 2015). Contrarily, Laokuldilok et al. (2017) reported that papain was the most effective non-substrate specific enzyme in hydrolyzing chitosan into COS. All three enzymes, cellulase, papain and lysozymes were each incubated with 1% (w/v) chitosan in sodium acetate buffer (pH 4.0) at 37℃ for 16 h (Laokuldilok et al., 2017). The results indicated that papain was able to randomly cleave between two glucosamines to produce COS at a faster rate (Laokuldilok et al., 2017).

2.4. Films

Chitosan based films have been of interest in recent studies as a potential alternative to petroleum-based synthetic packages. In literature, chitosan films have shown to be biocompatible, biodegradable, non-toxic and to have antimicrobial properties (El Knidri et al., 2018). In general, chitosan films tend to be brittle and have limited mechanical properties (Sabbah et al., 2019). Therefore, in recent works, researchers have been investigating the effects of using different plasticizers, crosslinkers, composite-films, solvents and neutralizers on chitosan films to further improve the mechanical, thermal, water-resistance, permeability, optical and biological properties

to compete with commercial bioplastics (Chang et al., 2019; Guerrero et al., 2019; Nair, Saxena, & Kaur, 2018; Sabbah et al., 2019; Sokolova et al., 2018; Uranga et al., 2019).

2.4.1. Plasticizers in chitosan films

Plasticizers are added to synthetic and bio-plastics to disrupt the intermolecular interactions of the polymer, impart flexibility and decrease the glass transition temperature (T_g) (Sokolova et al., 2018). In most studies, glycerol has been used frequently as a plasticizer to decrease the brittleness of chitosan films and improve the mechanical properties (Sokolova et al., 2018). Sokolova et al. (2018) reported on using DES composed of choline chloride and malonic acid ranging from 33 to 82 % (w/w) for the plasticizing of chitosan films. In the results, Sokolova et al. (2018) explained increasing the content of DES in the chitosan films led to a decrease in Young's modulus from 800 to 16 MPa, while elongation at break increased from 16 to 63 % at DES contents increasing to 67 % (w/w) . It was observed that beyond a DES content of 67 % (w/w) the elongation at break decreased (Sokolova et al., 2018). In terms of thermal analysis, Sokolova et al. (2018) reported that an increase in DES content led to a reduced T_g from 2.0 to - 2.3°C. These findings indicate that DES has a plasticizing effect on chitosan films as a result of decreasing the glass transition temperature, which indicates that the intermolecular chitosan-chitosan bonds were disrupted. Therefore, the intermolecular forces between chitosan and DES are weaker forces which may have led to an increase in elongation at break. Consequently, the weak intermolecular forces resulted in an easier deformation at less pressure, which indicates the chitosan films are less elastic. Sabbah et al. (2019) reported on the properties of chitosan films plasticized with spermidine (SPD) and glycerol. The aim of the study was to utilize SPD, which has shown to influence the properties of pectin and protein-based films, with glycerol and determine if the two plasticizers can improve the features of chitosan films. According to the results, 0.6% chitosan films containing SPD improved the thickness and elongation at break more than glycerol only (Sabbah et al., 2019). However, similar to the previous study, the use of plasticizers reduced the tensile strength and Young's modulus as a result of weak intermolecular forces between chitosan, positive aliphatic amine chain SPD and glycerol (Sabbah et al., 2019). Based on the results, chitosan films containing only 10 mM glycerol had a greater tensile strength (30.57 MPa) and Young's modulus (1194.38 MPa) than chitosan films containing 10 mM SPD alone (tensile strength 14.68 and Young's modulus 412.00 MPa) (Sabbah et al., 2019). Sabbah et al. (2019) explained that the plasticizing

effect of SPD is as a result of non-ionic interactions with chitosan, since both molecules are cationic. In the study Sabbah et al. (2019) concluded that an optimal concentration of 5 mM SPD and 25 mM glycerol in 0.6% chitosan films had an elongation break of 118.34%, which had the ability to heat-seal, as well as have improved permeability properties for CO_2 and O_2 but not water-vapor. In brief, through these studies a common trend that was observed was that plasticizers decrease tensile strength and Young's modulus, while increasing the elongation of break to produce less brittle chitosan films.

2.4.2. Crosslinked chitosan films

It has been reported that chitosan films, in which polymer chains are permanently crosslinked to citric acid, provide improved functional properties (Guerrero et al., 2019; Nataraj, Sakkara, Meghwal, & Reddy, 2018; Priyadarshi, Sauraj, Kumar, & Negi, 2018). In a recent study, Guerrero et al. (2019) reported on improved mechanical properties and homogeneous chitosan film structures when crosslinked with citric acid. The addition of 20 % citric acid in chitosan films resulted in smoother and a more homogenous structure when observed in scanning electron microscopy images (Guerrero et al., 2019). Guerrero et al. (2019) further suggested that homogeneity of the chitosan films and citric acid is a result of good compatibility between the molecules through covalent cross linkages. Therefore, the strong compatibility of chitosan and citric acid enhanced the tensile strength and elongation at break to reach 12.8MPa and 48.1%, respectively (Guerrero et al., 2019). This suggest that crosslinking chitosan films with citric acid is effective for improving mechanical properties. However, Guerrero et al. (2019) failed to emphasize on the fact that increasing citric acid concentration is a limiting factor, since the films become more hydrophilic. In the study, it was observed that the moisture content increased from 14.5 to 17.8 % when citric acid concentration increased from 0-20 % in the chitosan films (Guerrero et al., 2019). This was further confirmed with water contact angle test that decreased from 98.4 to 81.4°, which suggest the chitosan film surfaces were more hydrophilic (Guerrero et al., 2019). Furthermore, Priyadarshi, Sauraj, Kumar, and Negi (2018) reported chitosan films cross linked to citric acid on a 1:1 ratio showed a drastic decrease in tensile strength from 52.22 MPa to 9.48 MPa; however, elongation at break increased from 2.22 to 26.43 %, respectively. In addition, the moisture content decreased from 22.14 to 15.70 % and the water absorption decreased from 495.24 to 63.20 % (Priyadarshi, Sauraj, Kumar, & Negi, 2018). Therefore, it can be suggested

based on the studies by Priyadarshi, Sauraj, Kumar, and Negi (2018) and Guerrero et al. (2019) that 1:1 ratio of chitosan cross linked to citric acid leads to improvements in moisture content properties; however, in terms of tensile strength a higher ratio of chitosan to citric acid is suggested. Chitosan films cross linked to citric acid on a 1:1 ratio may promote weaker bonds and less exposed hydroxyl groups compared to when chitosan concentrations exceed citric acid. Therefore, this would explain the reason for improved moisture content, since hydroxyl groups of citric acid can interact with chitosan functional groups, however, the intermolecular forces maybe much weaker between citric acid—chitosan bonds as opposed to chitosan—chitosan bonds which would lead to decreased tensile strength and increased elongation at break. In a study, Nataraj et al. (2018) compared the effect of crosslinked chitosan films to alkaline treated chitosan containing citric acid. In the study three different films were investigated: (1) crosslinked chitosan films with citric acids, (2) non-crosslinked chitosan films containing citric acid, (3) non-crosslinked chitosan films containing citric acid further treated with 0.5 N NaOH solution, and the control films which was chitosan dissolved in 2 % acetic acid. In the results, Nataraj et al. (2018) reported crosslinked chitosan films exhibited the highest tensile strength (2.79 MPa), specifically at a concentration of 3 % citric acid. Alternatively, non-crosslinked chitosan films showed the lowest water vapor transmission rate (WVTR) compared to crosslinked and alkaline treated, (837.7, 1601.2, and 2627.8 $g/m^2/24h$) (Nataraj et al., 2018). However, these results differ from Priyadarshi, Sauraj, Kumar, and Negi (2018) who reported that crosslinked chitosan films showed a lower WVTR (795 $g\ m^{-2}d^{-1}$) than non-crosslinked chitosan (841 $m^{-2}d^{-1}$). Lastly, alkaline treated chitosan films showed the lowest water sorption activity as opposed to crosslinked and non-crosslinked chitosan films containing citric acid (Nataraj et al., 2018). Nataraj et al. (2018) explained these findings with the notion that NaOH treatment neutralizes the films therefore, hydrogen bonding with water is disrupted. Chang et al. (2019) further investigated 1 % chitosan films (with 0.5 % acetic acid and 10 % glycerol) treated with different NaOH concentrations (0, 1, 5 and 10 % w/w) at varying times 10s, 50s and 90s. From the results, Chang et al. (2019) reported that chitosan films treated with 10 % NaOH for 90s exhibited the highest tensile strengths (104.0 MPa), while chitosan films treated with 1 % NaOH for 10, 50 and 90s resulted in high elongation at break values (3.6, 2.8 and 3.3 %, respectively). This trend indicates that increasing NaOH concentrations leads to opposing effects for tensile strength and elongation at break. Therefore, chitosan films treated with 5 % NaOH seems to be the optimal condition to elicit a synergistic effect on both mechanical

properties. In addition, Chang et al. (2019) reported that the chitosan films treated with 5 % NaOH for 90 s exhibited a higher tensile strength (97.7 MPa) and elongation at break (3.7 %) than at 10 and 50 s treatments. In terms of water interaction with chitosan films, Chang et al. (2019) reported similar conclusions to Nataraj et al. (2018) : (1) chitosan films treated with NaOH resulted in a greater water contact angle of 90°, which suggest neutralization increased the hydrophobicity of films, (2) an increase in NaOH concentrations further decreases swelling properties of films, which might be caused by a decrease in charged amino groups, leading to more hydrogen bonding between chitosan chains, thus less swelling. In brief, both Chang et al. (2019) and Nataraj et al. (2018) showed the effect of NaOH concentration and time on the mechanical, water absorption and WVP properties of chitosan films.

2.4.3. Chitosan-based composite films

Composite chitosan films have been extensively studied in literature. Chitosan is biocompatible with many different polymers, such as other polysaccharides (e.g., cellulose, starch, alginate), proteins and lipid-based macromolecules. Therefore, in literature there has been a diverse study of chitosan based composite films (Table 2.3).

As shown in Table 2.3., composite chitosan-based films are of interest, since properties of the films can be improved. It can be suggested based on the literature that chitosan films incorporated with proteins show improved mechanicals properties, including tensile strength and elongation at break (Uranga et al., 2019; Zhang et al., 2019). The incorporation of essential oils has been shown to increase the hydrophobicity of chitosan by decreasing the water content, water solubility, as well as water vapor permeability of films (Priyadarshi, Sauraj, Kumar, Deeba, et al., 2018; Souza et al., 2017). Furthermore, incorporation of carotenoproteins was shown to increase the antioxidant antimicrobial properties of chitosan films (Hajji et al., 2018; Hamdi et al., 2019). Therefore, optimizing different properties of chitosan films is highly dependent on the type and amount of polymer being added to form a composite film.

Table 2.3.

Chitosan based composite biofilms

Composite films	Methodology	Major research output	References
Fish gelatin-chitosan films incorporated with citric acid	Fish gelatin-chitosan films were prepared with varying citric acid 10 and 20% (w/w) based on gelatin weight. 20% (w/w) glycerol was used as plasticizer.	Citric acid acted as a plasticizer for fish gelatin-chitosan films by increasing the elongation at break to 22.0%. In addition, bacterial growth of *E. coli* was significantly reduced at a concentration of 20% citric acid.	(Uranga et al., 2019)
Rapeseed protein hydrolysate-chitosan (RPH-Cs) films	2 % (w/v) RPH - Cs solution was prepared with 20 % (w/v) glycerol (plasticizer) then casted and dried at 25℃ under 55 % relative humidity to form films.	RPH-Cs films showed increased tensile strength (from 16.04 to 23.46 MPa) and elongation at break (5 to 42 %) with increased hydrolysate concentration. In addition, zone of inhibition of *E. coli* and *S. aureus* increased to reach 21.79 and 23.44 mm, respectively.	(Zhang et al., 2019)
Chitosan films incorporated with natural antioxidants	1.5 % (w/v) chitosan solution was prepared with 30 % (w/w) glycerol incorporated with essential oil (EO) or hydro-alcoholic extracts (HAE) at a level of 1% (v/v) of film solution.	Chitosan films incorporated with HAE had an increase in water content and water solubility compared to films with EOs. The mechanical properties of chitosan films increased significantly with the presence of EOs. Chitosan films incorporated with sage and thyme EOs are the most promising due to their improved mechanical properties.	(Souza et al., 2017)

Chitosan films incorporated with Apricot kernel essential oil	2% (w/v) chitosan solution was mixed with 0.2% v/v Tween 80 and apricot kernel oil (AKEO) at different ratios (1:1, 1:0.5, 1:0.25, 1:0.125)	The Cs-AKEO films showed improved moisture content, water absorption, solubility and WVP at 1:1 ratio. In addition, tensile strength was improved at a 1:1 ratio; however, elongation at break was the highest at Cs/AKEO 0.125 ratio. In terms of antimicrobial activity, Cs/AKEO at a 1:1 showed no growth of bacterial strains *E. coli* and *S. aureus* and inhibition of fungal growth on bread pieces over 10 days.	(Priyadarshi, Sauraj, Kumar, Deeba, et al., 2018)
Carotenoprotein-chitosan films incorporated with PVA	Shrimp carotenoprotein solution (0-3% w/w) was mixed with chitosan solution and PVA (10% w/w) to form films. Glycerol at different amounts (0, 5 and 10% w/w) was added as a plasticizer.	The optimal concentrations for the films were found to be 2.43% Cs, 9.93% glycerol and 0.005% CP, which elicit a elongation at break (149.11%) and tensile strength (24.07MPa). In addition, Cs-CP films showed excellent antimicrobial properties and antioxidant activities.	Hajji, Younes, Affes, Boufi, & Nasri, 2018)
Carotenoprotein-chitosan films	Carotenoproteins (CP) and Chitosan (Cs) extracted from blue crab were dissolved in 2% (v/v) acetic acid at different weight ratios (5, 10,15, 25 and 50%) for film forming solutions. 15% (15 g/100g polymer) Glycerol was added as a plasticizer for film forming.	The CP-Cs films showed an increase in redness and darkness as the concentration of CP increased as well as lower transparency. In addition, a decrease in moisture content and water solubility was observed as concentration increased to 50% CP-Cs. Antioxidant properties of the films were enhanced as a composite film compared to pure chitosan films. However, the addition of CPs decreased the mechanical properties significantly.	Hamdi, Nasri, Li, & Nasri, 2019)

2.5. Hydrogels

Hydrogels have gained considerable interest in literature due to diverse applications such as biomedical tissue engineering material, drug delivery devices, antibacterial, antifungal properties and water treatment (Shariatinia & Jalali, 2018). Natural hydrogels composed of biopolymers seems to be of more interest due to properties, such as natural, biodegradable, environmentally friendly, economical and diverse in raw material (Shariatinia & Jalali, 2018). Hydrogels can be defined as a three-dimensional crosslinked network made up of hydrophilic polymer chains able to swell and absorb water without solvation (Shariatinia & Jalali, 2018). In recent years, researchers have been improving the properties (e.g., swelling degree, mechanical and antimicrobial properties) of chitosan based hydrogels to introduce new applications for biomedical and pharmaceutical industries.

2.5.1. Mechanical properties

Kumar, Behl, and Chadha (2020) reported chitosan hydrogels crosslinked to polyvinyl alcohol (PVA) with addition of silver nanoparticles resulted in improved Young's modulus and elongation of break in comparison to chitosan-PVA hydrogel. The increase in mechanical strength of composite chitosan-PVA hydrogel might be due to the additional intermolecular hydrogen bonding interaction between the polymer and the silver nanoparticles (Kumar et al., 2020). Similarly, Xie, Liao, Zhang, Yang, and Fan (2018) showed silver nanoparticles integrated into chitosan hydrogels was able to reinforce the mechanical properties by increasing the compressive strength due to inter and intramolecular interactions (i.e. ionic interaction and hydrogen bonding). In addition, Ritonga, Nurfadillah, Rembon, Ramadhan, and Nurdin (2019) described composite chitosan-EDTA hydrogels showed improved mechanical properties and swelling power by varying the ratio of chitosan to EDTA. The crosslinking of chitosan hydrogel with EDTA at a ratio of 7:3 promoted ionic interactions between functional groups to form a tight structure, thus water absorption was reduced causing a low swelling power and the strength of hydrogels increased (Ritonga et al., 2019).

2.5.2. Antimicrobial properties

Li et al. (2017) described improving chitosan hydrogels by adding concentrations of 0-0.384 (w/w) $AgNO_3$ to chitosan solutions containing 2% (v/v) acetic acid followed by gelation under ammonia atmosphere for 24 h. In the study, Li et al. (2017) reported that an increase in $AgNO_3$ led to an improved tensile strength in chitosan hydrogels: at a concentration of 1.5 % (w/w) chitosan and 0.192 % (w/w) $AgNO_3$ the highest stress of 0.33MPa was obtained. In addition, Li et al. (2017) compared antibacterial activities of hydrogels composed of chitosan-$AgNO_3$ to controls (without $AgNO_3$). It was observed that chitosan-$AgNO_3$ hydrogels had excellent antimicrobial properties against a broad range of gram negative and gram positive bacteria, while the control showed no to poor inhibition zones against the bacteria (Li et al., 2017). Therefore, Li et al. (2017) suggest that chitosan-$AgNO_3$ hydrogels are a promising product for wound dressing materials in the biomedical field. Wu et al. (2018) described preparing chitosan-alginate hydrogels by mixing 2.50% (w/v) sodium alginate aqueous solution with 2.75% (w/v) chitosan solution followed by gelation at 4℃ for 24 h. In addition, loaded hydrogels were prepared by dissolving lysozyme (1, 5, and 10 mg) in chitosan solution prior to mixing chitosan-alginate hydrogel solutions (Wu et al., 2018) . In the study, Wu et al. (2018) reported that enzymatic activity of loaded lysozyme was decreased from 100 to 87.72%, which indicates that chitosan-alginate hydrogels are a promising drug delivery material for enzyme loading and adsorption. In addition, chitosan-alginate hydrogels loaded with lysozymes showed significant ($p < 0.05$) antimicrobial growth against *E. coli* and *S. aureus* in comparison to the chitosan-alginate control hydrogels (Wu et al., 2018). Therefore, chitosan-alginate hydrogel has potential usage in the food industry (such as a preservatives for the meat industry) and pharmaceutical industry. Wahid et al. (2019) described preparing bacterial cellulose / chitosan (BC-Cs) semi interpenetrating networks hydrogels, which involves using a crosslinker, such as glutaraldehyde. In the results, Wahid et al. (2019) reported that BC-CS hydrogels showed significant antibacterial activity against both strains of *E. coli* and *S. aureus* with increasing concentrations of chitosan. Pure chitosan hydrogels reduced the bacterial population by 98%, which supports literature that indicates chitosan has antimicrobial activity due to the positive charged amino groups (Wahid et al., 2019).

In brief, the previous studies (Kumar et al., 2020; Li et al., 2017; Ritonga et al., 2019; Wahid et al., 2019; Wu et al., 2018; Xie et al., 2018) showed that chitosan-based hydrogels have excellent antimicrobial properties and potential mechanical properties. Therefore, based on these

results, further studies can be done on chitosan hydrogels as a preservative for meat-based foods to see if there is a potential use in the food industry.

2.6. Conclusion

With the rising concern of waste management in the seafood industry, carotenoproteins, chitin and its derivates have gained more attention in recent works. These valuable bioproducts constitute a new source of natural products that meet the needs of consumers and impact positively on the health. Carotenoproteins, chitin and its derivatives provide highly valuable functionalities and health benefits, including biodegradable, biocompatible, antioxidant, antimicrobial and anti-inflammatory effects. These properties, can be used in a wide range of industrial applications, such as the food industry, pharmaceutical, biotechnology, medical and others. Various conventional methods have usually been utilized to extract these biopolymers; however, the importance and benefits of new ecological methods, including biological and non-thermal methods, has been reported. Moreover, the applications of carotenoproteins in the food industry, and chitosan-based biofilms and hydrogels have been discussed. The diversity in value added products provides a potential economic benefit for seafood processing waste. Specifically, the shellfish processing industries are a great source of by-products, especially chitin and carotenoproteins. Therefore, these polymers have a promising future that various industries can exploit for commercial applications.

CHAPTER III.

CAROTENOPROTEIN FROM UNDERUTILIZED EUROPEAN GREEN CRAB (*CARCINUS MAENAS*): ENZYME-ASSISTED EXTRACTION AND BIOCHEMICAL CHARACTERIZATION

CONNECTING STATEMENT I

The utilization of crustacean by-products as a rich source to generate value-added products is mainly dependent on the source of crustacean specie and the method of extraction. The study reported in this chapter focuses on enzyme-assisted extraction of carotenoprotein with the aid of trypsin from porcine pancreas and biochemical characterization, including lipid and amino acid analysis of underutilized European green crab (*Carcinus Maenas*) harvested from the shallow waters of Nova Scotia coastline.

Note: This chapter constitutes the text of an article that is expected to be submitted to the Journal of *Aquaculture* for publication.

Contribution of author: Paola Sully designed and performed experiments, analyzed the data and drafted the manuscript.

Contribution of co-authors: Yi Zhang provided guidance and experimental assistance for developing experimental design. Simpson, B.K. supervised the research work, provided research funding, guided the lab performance and data analysis and revised the manuscript.

I Abstract

European green crab (*Carcinus maenas*) is an underutilized invasive species rich in bioactive compounds; however, limited studies have been conducted to generate value-added products. In this study, the nutritional composition and extraction of carotenoprotein from green crab were investigated. Whole crab contained high crude protein (20.98 % dry wt. basis) and low lipid (3.00 % dry wt. basis) content. The lipid profile characterized using GC-MS showed a higher content of unsaturated fatty acids, including MUFAs mainly, oleic acid and *n-3* PUFAs, such as EPA (20:5*n-3*) and DHA (22:6*n-3*). Trypsin assisted extraction of carotenoprotein consisted of 67.58 % total soluble protein and 139.26 μg/g carotenoids. It was rich in essential amino acids (49.87 %), hydrophobic amino acids (44.17 %) and dominate in Asp+Asn and Glu+Gln amino acids. The main carotenoids in carotenoprotein were astaxanthin and astaxanthin diester, and it showed the highest antioxidant activities at 4 mg/mL against DPPH and hydroxyl radicals and iron reducing power ($p < 0.05$). The green crab lipids and carotenoprotein can be used as natural antioxidants or nutritive ingredients for foods and feeds.

3.1 Introduction

Crustacean waste management is a major challenge faced by the shellfish processing industry each year. It is estimated that on the average, between 6 million to 8 million tons of waste is generated from crab, shrimp and lobster globally (Borić, Vicente, Jurković, Novak, & Likozar, 2020). The lack of waste management practices poses sanitation and environmental hazard concerns. Up to 75% of the total body weight, including the heads, carapace, tails and limbs of crustaceans are discarded into the ocean, landfills or incinerated (Hamed, Özogul, & Regenstein, 2016). Shrimp and crab processing by-product are amongst the most exploited raw materials for production of various value-added products, such as proteins, lipids, pigments and chitin (El Knidri, Belaabed, Addaou, Laajeb, & Lahsini, 2018; Saini, Moon, & Keum, 2018).

The invasive European green crab (*Carcinus maenas*) is classified amongst the "top 10 most unwanted species in the world" (Fisheries and Oceans Canada, 2019). This crab is small in size (approximately 10 cm) and low in meat for use as food. Furthermore, they reproduce and grow rapidly and have high tolerance for a wide range of environmental conditions (Leignel, Stillman, Baringou, Thabet, & Metais, 2014). In nature, green crabs are widely distributed along shallow waters in the northern-western Atlantic coastlines (Khiari, Kelloway, & Mason, 2020). In

Canada's Atlantic Ocean as example, green crabs are aggressive species that out-compete other crustacean species, including oysters, mussels, clams, lobsters and juvenile crabs, all of which serves as natural food source (Fisheries and Oceans Canada, 2019). The large appetite of green crabs poses a serious threat to Canadian ecosystem and fishery industry (Rayner & McGaw, 2019). Due to the large abundance over the years, Fisheries and Oceans Canada have permitted specific fishermen to destroy green crabs harvested to limit their population and allow native species to re-emerge in the area. However, these underutilized crabs have potential for profitable use. Based on previous composition studies reported, green crab are a potential rich source of proteins, lipids, pigments, minerals and chitin (Beth A & E. A. Fairchild, 2013; Naczk, Williams, Brennan, Liyanapathirana, & Shahidi, 2004).

The lipid profile of crustacean is an important nutritional quality index seeing as they are a main source of essential fatty acids namely, α-linolenic acid, linoleic acid and other omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs), including docosahexaenoic acid (DHA) and eicosatetraenoic acid (EPA), two vital fatty acids for fish dietary intake and human consumption (Xu, Cao, Wei, Zhang, & Liang, 2018). The use of crustacean to extract DHA and EPA was shown to be an alternative source of fish oil due to the relatively similar fatty acid composition (Balzano, Pacetti, Lucci, Fiorini, & Frega, 2017). Several studies have focused on beneficial roles of DHA and EPA on fish growth and health (Ma et al., 2014; Rombenso, Trushenski, Jirsa, & Drawbridge, 2016; Roy et al., 2020; Sprague et al., 2015; Toyes-Vargas et al., 2020; Zhang et al., 2019).

Carotenoproteins are natural pigments comprised of carotenoids and a protein complex (Wade, Gabaudan, & Glencross, 2017). In crustaceans, carotenoprotein complexes can be classified in two groups: lipovitellins and true carotenoprotein, a stable complex between carotenoid and protein complex called crustacyanin (Wade, Gabaudan, & Glencross, 2017). There is great interest in carotenoprotein due to its biological activities as an antioxidant, natural colorant and as nutritional feed source in aquaculture (Sila, Nasri, & Bougatef, 2012). The use of enzymes to aid in the recovery of carotenoprotein has been shown to increase the efficiency of the extraction process (Cano-Lopez, Simpson, & Haard, 1987). Proteases such as trypsin, has been reported in various studies to achieve higher yields of carotenoprotein extract as compared with other proteases because of its relatively narrower specificity (Chakrabarti, 2002; Klomklao et al., 2009; Poonsin et al., 2018). Crustacean shells from species, such as black tiger shrimp (*Penaeus monodon*), Pacific white shrimp (*Litopenaeus vannamei*), Brazilian redspotted shrimp

(*Farfantepenaeus paulensis*), blue crab (*Portunus segnis*) have been intensively used for carotenoprotein extraction (Hamdi, Nasri, Dridi, Li, and Nasri, 2020; Klomklao et al., 2009; Poonsin et al., 2018; Sánchez-Camargo, Almeida Meireles, Lopes, & Cabral, 2011).

Previous studies have been conducted on the nutritional content on green crab; however, to the best of our knowledge, little or no information regarding the extraction and characterization of lipids and carotenoprotein from green crab has been previously reported. Therefore, the present study aimed to investigate the extraction and compositional characteristics of green crab, with respect to the lipids and carotenoprotein profiles using a trypsin-assisted process, and characterizing them for various biochemical properties such as: total fatty acid content and fatty acid composition, as well as essential and non-essential and amino acid contents.

3.2 Material and Methods

3.2.1 Chemicals

Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce™ (Rockford, IL, USA). Ethylenediaminetetraacetic acid (EDTA), ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), sodium chloride (NaCl), and sodium phosphate were purchased from Fisher Scientific (Geel, Belgium). Acetone, acetonitrile, anhydrous sodium sulphate, 2,2-dipheyl-1-picrylhydrazyl (DPPH), ethanol, NHI-C FAME Mix, Folin's phenol reagent, hexane, hydrochloric acid (HCl), hydrogen peroxide (H_2O_2), methanol (MeOH), petroleum ether, phenylisothiocyanate (PITC), 1,10-phenanthroline, potassium ferricyanide, sodium hydroxide (NaOH), sodium hypochlorite (NaOCl), toluene, and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

3.2.2 Sample preparation

Green crabs (*C. maenas*) were harvested from Nova Scotia, Canada, and provided to the lab by Neptune Canada Inc. (Quebec, Canada). The Green crabs were washed, and oven dried at 65°C using Fisher Scientific gravity oven (Langenselbold, Germany). The dried samples were then crushed to obtain small particle size of 0.1 - 1.7 mm and stored at – 20°C for later analysis.

3.2.3 Analysis of crude fat and protein

The crude fat content of green crab (5 g) was measured using Soxhlet extraction with petroleum ether as solvent, according to AOAC method 991.36 (AOAC, 2006).

Crude protein was determined using the Dumas method. Two grams of dried green crab sample were deproteinized with 2.0% NaOH at 90℃for 2 h. The sample was filtered, thoroughly washed with deionized water and left to dry overnight in the oven at 60℃. Total nitrogen contents of green crab and deproteinated green crab (0.5-1.0 g) were measured using Dumas nitrogen analyzer (VELP SCIENTIFICA elemental analyzer, NDA 701; Usmate Velate, Italy). The crude protein content was estimated from the following equation given by Synowiecki and Al-Khateeb (2000):

$$\% \text{ Crude Protein} = ([N]_C - [N]_{Dp}) \times 6.25 \times 100\% \quad (1)$$

where $[N]_C$ and $[N]_{Dp}$ correspond to total nitrogen content in green crab and deproteinized green crab, respectively, and 6.25 represents the conversion factor. All analyses were completed in triplicate.

3.2.4 Total lipid extraction and fatty acid analysis

Total lipids in the dried crab samples were extracted with petroleum ether by Soxhlet extraction. The method of Ichihara and Fukubayashi (2010) was used for the preparation of fatty acid methyl esters (FAMEs) from the extracted lipids, with modifications. Briefly, 30 mg of total lipid were dissolved in 0.2 mL toluene, then 1.50 mL of MeOH and 0.30 mL of 8.0% HCl-MeOH were added to a final volume of 2 mL, then thoroughly mixed and incubated at 45℃for 16 h. After cooling down to room temperature (~ 22℃), 1 mL of hexane and 1 mL deionized water were added, mixed and left to stand for 10 min at room temperature (~ 22℃). The hexane layer was then collected using a pipette and sealed in an amber colored glass bottle. The collected hexane layer was referred to as “FAME”.

3.2.4.1 Fatty acid analysis using GC-MS

The FAMEs were analyzed according to Long et al., (2019), with slight modification using an Agilent 7890A-5975C GC-MS with an HP-5MS fused silica capillary column (30 m × 0.25

mm ID × 0.25 μm; Agilent technology, Santa Clara, CA, USA). The carrier gas was helium at a flow rate of 1.0 mL min^{-1}. One microliter of the sample was injected into the GC injector port with a 1:30 split ratio at an inlet temperature of 260°C. The column temperature was initially held at 40°C, rising to 170°C at a rate of 10°C min^{-1} and held for 1 min. It was then increased to 220°C at 2°C min^{-1} held for 1 min, followed by an increase at 3°C min^{-1} to 230°C and held for 5 min. It was then further increased at 20°C min^{-1} to a final temperature of 300°C until all FAMEs were eluted from the column. The temperature of the transfer line was maintained at 280°C. The ion-trap mass spectrometer was operated in electron impact (EI) mode and full scan monitoring mode (m/z 30 - 450). The ion source temperature of the MS was set at 230°C and the electron energy was set at 70 eV. The identification of FAMEs was carried out by comparing their retention times with known standards (NHI-C FAME Mix, Sigma-Aldrich, MO, USA) in combination with NIST (National Institute of Standard and Technology, US department of commerce) libraries. The fatty acid profile was expressed as the percentage of each fatty acid to total fatty acids (% Total fatty acid) based on integrated peak area using ChemStation software.

3.2.5 Extraction of carotenoprotein

Carotenoprotein from green crab was extracted with the aid of trypsin from porcine pancreas as per the method of Simpson and Haard (1985) with slight modification. The crushed crab shells (25 g) were demineralized with 250 mL of 5.3 % citric acid at 25°C for 4 h, filtered and washed with deionized water (1:100 w/v). The demineralized sample was subjected to deproteinization and further demineralization with 1:3 vol of 0.5 M EDTA (pH 8.0) , and trypsin (130 unit/g sample) and shaken continuously for 2 h at 25°C in a shaking water bath (Precision Scientific, Chicago, Il, US). After the protease treatment, the sample was filtered through four layers of cheesecloth and the filtrate was adjusted to pH 4.5 with 2 M HCl and left to sediment at 4°C overnight. The precipitate was collected by vacuum filtration using 0.22 μm microfilter (Millipore™, Darmstadt, Germany). The pellet obtained was suspended in 10 mL of 5 mM sodium phosphate buffer (pH 7.0) and lyophilized. The dried product was referred to as “carotenoprotein” and it was stored at – 20°C for subsequent analyses.

3.2.6 Characterization of carotenoproteins

3.2.6.1 Yield and color measurement

The yield percentage of extracted carotenoprotein from green crab was determined from the following equation:

$$\text{Yield (\%)} = \frac{\text{grams of carotenoprotein.}}{\text{grams of initial crab sample}} \quad (2)$$

Color in extracted carotenoprotein was measured using a colorimeter (Minolta, Model CM-3500d, NJ, USA). Approximately 0.1 g of carotenoprotein was inserted into a glass vial and was placed in the colorimeter for analysis. An empty glass vial was used as a blank. The colour was reported in CIELAB system. L*, a* and b* parameters indicate lightness, greenness to redness, and blueness to yellowness, respectively. The analysis was completed in triplicates.

3.2.6.2 Measurements of protein content in carotenoproteins

Total soluble protein and protein recovery in carotenoprotein and green crab was determined according to the method of Klomklao et al., (2009) and Senphan, Benjakul, and Kishimura (2014) with slight modification. Samples (100 mg) were mixed with 10 mL of 0.5 M NaOH and incubated at 85°Cfor 1 h. The mixture was cooled down to room temperature (~ 22°C) and filtered under vacuum using Whatman No. 1 filter paper. The total soluble protein content of supernatants was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA) following the manufacturer's instructions. Protein recovery was expressed as the percentage total soluble proteins in the extracted carotenoprotein relative to total soluble proteins of green crab.

3.2.6.3 Amino acid compostition of green crab shells and carotenoprotein

Amino acid composition analysis was determined according to the method of Zhang, Dutilleul, Li, and Simpson (2019). Crushed green crab shells and the extracted carotenoprotein samples were hydrolyzed separately with 6 M HCl and 1% phenol at 110°Cfor 24 h under nitrogen atmosphere. The hydrolyzed samples were derivatized with phenylisothiocyanate (PITC, Waters, MA, USA) solution at 25°C for 30 min to form phenylthiocarbamoyl (PTC) derivatives, and vacuum dried. The derivatized amino acids (PTC-amino acids) were dissolved in 0.5M sodium

phosphate buffer (pH 7.4) with 5 % acetonitrile, prior to ultra-performance liquid chromatography (UPLC) separation. To analyze the derivatized amino acids, AQUITY UPLC system (Waters, MA, USA) equipped with Pico-Tag system and Aquity BEH C18 column (2.1 mm x 10 cm, waters, MA, USA) was used. The column conditions were set at a column temperature of 48 ℃; with 0.14 M sodium acetate (pH 6.0), 0.05% triethylamine, 6% acetonitrile (Waters, MA, USA) as eluent A; 60% acetonitrile aqueous solution (Waters, MA, USA) as eluent B. Pierce amino acid standard H was firstly used as a calibration standard. Then PTC- amino acids were eluted through the UPLC system and detected at a wavelength of 254 nm with AQUITY TUV detector (Waters, MA, USA). The collected data were analyzed using Empower 2 software (Waters, MA, USA).

3.2.6.4 Measurement of carotenoid content in carotenoproteins

The total carotenoid content was determined according to method of Sila et al., (2012) and Senphan, Benjakul, and Kishimura (2014) with slight modification. One gram of extracted carotenoprotein was homogenized in 25 mL of cold acetone (-20 ℃) for 5 min and filtered under vacuum using Whatman No. 1 filter paper. The filtrate was transferred in a separatory funnel and was partitioned with 25 mL of petroleum ether. The separatory funnel containing both the sample and solvent solution was shaken gently and left to stand at 25℃ for 10 min. A solution of 10 mL 0.1 % NaCl was added to the mixture, then the lower layer was drawn off. The top layer was washed twice with 25 mL of deionized water. The petroleum ether layer collected was dried with 15 g of anhydrous sodium sulphate for 30 min, and the dried sample was filtered under vacuum. The sodium sulphate was continuously washed with petroleum ether to remove all remaining pigments. The petroleum ether fractions were pooled and evaporated under vacuum at 50℃ using a rotary evaporator. The residue collected was referred to as “carotenoid” pigment. The carotenoid pigment was redissolved in petroleum ether and made up to a final volume of 10 mL. The absorbance of appropriately diluted carotenoid was measured at 468 nm. The concentration of carotenoid was calculated using the equation given by Saito & Regier (1971):

$$\text{Carotenoid } (\mu g/g \text{ sample}) = \frac{A_{468} \times V \times d.f.}{0.2 \times W} \quad (3)$$

where A_{468} is the measured absorbance at 468 nm, V is the volume of extract, $d.f$ is the dilution factor, 0.2 is the A_{468} of 1 $\mu g/mL$ standard canthaxanthin and W is the weight of the sample in grams.

3.2.6.5 Separation of carotenoids using thin-layer chromatography

The carotenoid pigments from the extracted carotenoprotein were separated using thin-layer chromatography (TLC) with flexible 20 × 20 cm silica gel plates (Whatman, AL SIL G; W. Germany) following the method of Sánchez-Camargo et al. (2011). The concentrated extract was applied onto the plate and separated using a mobile phase (acetone/hexane (25:75% v/v)). The carotenoid pigments identification was carried out by comparison of the calculated R_f bands obtained with those reported in the literature (Sánchez-Camargo et al., 2011; Senphan et al., 2014).

3.2.6.6 Determination of antioxidant activity

Extracted carotenoprotein solutions at different concentrations 1, 2, 3, and 4 mg/mL, respectively, were prepared in deionized water at 25℃ and then filtered under vacuum using Whatman No. 1 filter paper. The antioxidant activities of those solutions were assayed via DPPH radical scavenging activity, hydroxyl radical scavenging activity, and Fe reducing activity measurements described below.

DPPH radical scavenging activity was determined as per the method of Wu et al., (2013), with slight modifications. An aliquot of 0.1 ml of sample (1 – 4 mg/mL) was added to 1 mL of 100 μM DPPH in 95% ethanol. The solution was mixed and incubated in the dark for 30 min at room temperature (~ 22℃). The absorbance of the incubated solution was measured with a spectrophotometer at 517 nm. The DPPH radical scavenging activity (%) was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = 100 \times \frac{A_{control} - A_{sample}}{A_{control}} \quad (3)$$

where $A_{control}$ and A_{sample}, corresponds to the measured absorbance of deionized water and carotenoprotein solution, respectively.

Hydroxyl radical scavenging activity was determined using the method of You, Zhao, Regenstein, and Ren (2011), and Zhang, Simpson, and Dumont (2018), with slight modifications. A solution of 0.6 mL of 5 mM 1,10-phenanthroline, 0.6 mL of 5 mM $FeSO_4$, 0.6 mL of 15 mM EDTA was mixed with 0.4 mL of 0.2 M sodium phosphate buffer (pH 7.5). Then 0.6 mL of sample (1 – 4 mg/mL) and 0.8 mL of 0.01% H_2O_2 were added. The mixture was incubated in a water bath at 37°C for 60 min and measured with a spectrophotometer at 536 nm. The hydroxyl radical scavenging activity (%) was calculated using the following equation:

$$\text{Hydroxyl radical scavenging activity (\%)} = 100 \times \frac{A_{sample} - A_{control}}{A_{blank} - A_{control}} \quad (4)$$

where A_{sample} was the measured absorbance of the sample, $A_{control}$ was the measured absorbance of deionized water instead of sample, and A_{blank} was the measured in absence of H_2O_2.

Iron reducing activity was determined using the method of Wu et al., (2013), with slight modifications. One mL of sample (1 – 4 mg/mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. After incubation of the mixture at °C for 30 min, 2.5 mL of 10% TCA were added and mixed for 10 min. Then 2.5 mL of deionized water and 0.5 mL of 0.1% $FeCl_3$ were added to the mixture and left to react for 10 min. The same steps were followed for the blank solution containing deionized water instead of sample. The absorbance was measured against blank solution at 700 nm. An increased absorbance indicated higher iron reducing activity.

3.2.7 Statistical analysis

All experiments were performed in triplicate and the data were expressed as means $\pm$ SD (standard deviations). Data were subjected to one-way analysis of variance (ANOVA). Comparison of means was carried out by Tukey's honestly significant difference (HSD) test. Statistical analyses were performed using the Statistical Package of Social Sciences (SPSS 11.0 for windows; SPSS Inc., Chicago, IL, USA).

3.3 Results and Discussion

3.3.1 Total fat and protein contents

The total fat and protein contents of whole green crab are shown in Table 3.1. Crude protein and fat contents of whole green crab (on a dry weight basis) were 21.0 %, and 3.0 %, respectively. Similarly, McNiven, Quijon, Mitchell, Ramsey, and St-Hilaire (2013) reported high values of crude protein (6.18 % -18.4 %) and low-fat (1. 86 % to 4.67 %) contents in whole green crab samples regardless of the month harvested and size of the carapace. In addition, Khiari et al., (2020) showed that whole green crabs were higher in protein and fat contents (38.60 % and 1.12 %, respectively) compared to the crab shells alone (15. 90% and 0.23 %, respectively); thus, making it a more nutrient rich source. According to the literature, our findings are consistent with the range of reported crude protein and fat values. Specifically, the fat content of our green crab was relatively high, which can serve as more nutrient-rich added-value product. The proximate composition of whole green crab in our study shows that an abundantly available underutilized species is high in useful nutrients and has good potential as an alternative agrifeed and aquafeed ingredient (Beth A & E. A. Fairchild, 2013). However, it has been reported in the literature that green crab is comprised of about 80 to 88 % shell, on a dry weight basis, which contributes to high levels of ash content (Khiari et al., 2020; Naczk et al., 2004), and renders unprocessed whole green crab as a limited primary animal feed ingredient for several species (Beth A & E. A. Fairchild, 2013). Therefore, extracting rich nutrients, such as proteins, carotenoproteins and fats from whole green crab could maximizes its application as a feed supplement.

Table 3.1.

Total crude fat and protein content of whole green crab.

Composition (% dry weight basis)	Whole Green Crab
Crude Protein	20.98 ± 0.39
Fat	3.00 ± 0.09
Means ± SD (n=3).	

3.3.2 Fatty acid profile

The total fatty acid composition of whole green crab is depicted in Table 3.2. Seventeen fatty acids were identified in the green crab fat extract. The fatty acid composition of whole green crab was high in monounsaturated fatty acids (MUFA), representing 36.86 % of total fatty acids. Oleic acid (18: 1*n*-9) was the predominant MUFAs comprising of about 17.16 % of total fatty acid, followed by palmitoleic acid (16:1*n*-7) and Eicosenoic acid (20:1*n-9*) representing 9.51 % and 5.82 %, respectively. Furthermore, saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) were similar in composition, representing 22.85 % and 24.14 % total fatty acid, respectively. The major SFA was palmitic acid (16:0), comprising of about 14.02%, followed by myristic acid (14:0) and steric acid (18: 0), each accounting for less than 4 % total fatty acid. The main PUFAs were eicosapentaenoic acid (EPA) (20:5*n-3*) which accounted for 16.86 %, followed by docosahexaenoic acid (DHA) (22:6*n-3)* representing 6.27 %, and the ratio of DHA/EPA was 0.37. These findings corresponded with previous studies where MUFAs and SFAs, such as oleic acid and palmitic acid levels were comparable in green crab (B. A. Beth A & E. A. Fairchild, 2013); however, our PUFA values differed from those reported by Naczk et al., (2004). In their study, Naczk et al., (2004) reported a total of 9 fatty acids as opposed to 3 fatty acids which were found in our study, representing almost half of the total fatty acid composition. These differences could be associated with the method of storage, since unsaturated fatty acids are prone to oxidation in the presence of heat, light and prooxidant. In addition, total fatty acid composition can vary based on crab physiology, which is highly dependent on size, feed, season and harvesting area (McNiven et al., 2013). In our study, *n-6* PUFAs were not detected in whole green crabs; therefore, the ratio of *n-3*/*n-6* was not determined. Nonetheless, the sum of *n-3* PUFAs was considerably higher than those reported in blue crab (*Callinectes sapidus),* swimming crab (*Portunus trituberculatus*) and Chinese mitten crab (*Eriocheir sinensis*) (Çelik et al., 2004; Chen et al., 2007; He et al., 2017).

EPA and DHA referred to as omega-3 long chain polyunsaturated fatty acids (*n-3* LC-PUFAs), are essential fatty acids included in farmed fish diets since fish species lack the ability to synthesize these essential fatty acids in sufficient amount for the animals' requirements (Xu, Cao, Wei, Zhang, & Liang, 2018). Moderate ratios of 1.02 to 1.46 DHA/EPA in feed formulations have been shown to improve the health and growth of fish species (Xu et al., 2018). The ratio of DHA/EPA in whole green crab was only 0.31 in our study, while in commercial fish oil from

Table 3.2.

Fatty acid composition of whole green crab (% of total fatty acid).

Fatty acid %	Mean ± SD
Saturated Fatty acids (SFA) composition %	
12:0	0.13 ± 0.01
14:0	3.64 ± 0.32
15:0	0.64 ± 0.07
16:0	14.02 ± 1.30
17:0	0.67 ± 0.10
17:0 *isomer*	0.52 ± 0.05
18:0	3.23 ± 0.22
Σ *SFA*	**22.85**
Monounsaturated Fatty acids (MUFA) composition %	
14:1*n*-3	0.36 ± 0.05
16:1*n*-7	9.51 ± 0.85
18:1*n*-9	17.16 ± 1.29
19:1*n-9*	0.58 ± 0.07
20:1*n-9*	5.82 ± 0.43
20:1 *isomer*	2.71 ± 0.23
22:1*n-9*	0.732 ± 0.01
Σ *MUFA*	**36.86**
Polyunsaturated Fatty acids (PUFA) composition %	
18:3*n-3*	1.01 ± 0.02
20:5*n-3 (EPA)*	16.86 ± 0.80
22:6*n-3 (DHA)*	6.27 ± 0.54
Σ *PUFA*	**24.14**
DHA/EPA	0.37

Means ± SD (*n* =2)

pelagic fish the value was approximately 1 (Toyes-Vargas et al., 2020) and in caramote prawn (*Penaeus kerathurus*) and mantis shrimp (*Squilla mantis)* at least 0.89 (Balzano, Pacetti, Lucci, Fiorini, & Frega, 2017). Although, whole green crab contains high levels of *n-3* LC-PUFAs, the ratio of DHA/EPA is not enough to replace fish oil as a primary ingredient in feed formulation. Therefore, whole green crab fat extract can be used in combination with fish oil or other crustacean lipid extract as an alternative.

3.3.3 Carotenoprotein characterization

3.3.3.1 Yield and color

The yield and color composition of extracted carotenoprotein from whole green crab are presented in Table 3.3. The extraction process with the aid of porcine pancreas trypsin yielded 5.73 mg/g of carotenoprotein. In comparison to the yield (22 mg/g) of carotenoprotein from blue crab (*Portunus segnis*), our process yield was low (Hamdi et al., 2020). This shows that various factors, such as the species and extract method can influence the process efficiency. Furthermore, the carotenoprotein was light orange in color with b^* -value of 15.29 and a^*-value of 9.00 corresponding to the colors yellow and red, respectively. The presence of yellow and red colors in carotenoproteins suggested that various carotenoids, including astaxanthin, the major carotenoid in crustaceans, were extracted with the protein complex from whole green crab. Similarly, Hamdi et al., (2020) reported extracted carotenoprotein from blue crab (*Portunus segnis*) was yellow and red in color, which corresponds to carotenoids.

Table 3.3.

The total yield and color composition of extracted carotenoproteins from whole green crab.

Composition	Carotenoproteins
Yield (mg/g)	5.73 ± 0.78
Color	
L^*	52.84 ± 1.61
a^*	9.00 ± 0.43
b^*	15.29 ± 0.40

Means ± SD (n = 3)

3.3.3.2 Protein and amino acid composition

The amino acid composition data of whole green crab and carotenoprotein extract are presented in Table 3.4. Whole green crab and extracted carotenoproteins were similar in amino acid profiles. The amino acid contents from whole green crab and its carotenoprotein were rich in glutamic acid/glutamine (14.32 and 14.41%) and aspartic acid/asparagine (10.52 and 11.00%) residues. These findings are consistent with those reported for carotenoproteins from various sources by Pattanaik et al., (2020), Senphan et al., (2014), Sila et al., (2012) and Klomklao et al., (2009), who all reported high glutamic acid/glutamine and aspartic/asparagine contents in carotenoprotein from various shrimp species. Similarly, Simpson and Haard (1985) reported comparable amino acid content in carotenoprotein recovered with bovine trypsin. The abundantly available glutamic acid/ glutamine and aspartic acid/asparagine are important energy substrates for fish and are essential for synthesis of purine and pyrimidine nucleotides in all cells (Li, Mai, Trushenski, & Wu, 2009).

The green crab carotenoprotein extracted with porcine pancreas trypsin yielded 95.7 % protein recovery, which showed similar essential amino acids profiles with that found in whole green crab. The total essential amino acid in whole green crab and carotenoprotein were 51.0 % and 49.9 %, respectively; and the corresponding non-essential amino acid contents were 49.0 % and 50.0 %, respectively. The ratio of EAA/NEAA in both were 1.1 and 1.0, respectively. These results were within the range of those reported by Pattanaik, et al., (2020) who reported that carotenoprotein extracted from four different shrimp species (*Penaeus monodon, Metapenaeus affinis, Parapeneopsis stylifera* and *Nematopalemon twnuipes*) showed a ratio of 0.69, 0.71, 2.00 and 0.71 of EAA/NEAA, respectively. This indicates that the whole green crab and green crab carotenoprotein are both good sources of essential amino acid. Furthermore, leucine, arginine, valine, isoleucine, phenylalanine and threonine were found in carotenoproteins in high amounts. In fish diets, these ten amino acids, including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine are considered indispensable (Kasozi, Iwe, Sadik, Asizua, & Namulawa, 2019). Alternative fish meals must be supplemented with those essential amino acids to satisfy the dietary requirements of salmonids. In our findings, carotenoproteins extracted from whole green crab comprised of most of the essential amino acids required for nutrition of farmed fish. However, low contents of histidine and methionine were found in the carotenoprotein. Therefore, carotenoprotein extracted from whole green crab would need to be supplemented with

Table 3.4.

Amino acid composition of whole green crab and extracted carotenoprotein.

Amino acid composition (%)	Green crab	Carotenoproteins
Asx (Asp+Asn)	10.52	11.00
Glx (Glu+Gln)	14.32	14.41
Ser	3.07	4.94
Gly[B]	5.02	4.26
His[A]	3.43	3.03
Arg[A]	5.16	6.31
Thr[A]	2.82	5.47
Ala[B]	4.99	5.00
Pro[B]	4.66	4.52
Tyr	6.43	6.01
Val[A,B]	6.56	6.24
Met[A,B]	4.26	3.62
Ile[A,B]	6.48	5.99
Leu[A,B]	9.79	8.55
Phe[A,B]	7.60.	5.99
Lys[A]	4.88	4.65
Total EAA	50.98	49.87
Total NEAA	49.01	50.14
EAA/NEAA	1.04	0.99
Total HPAA	49.36	44.17
Protein recovery (%)	—	95.27 ± 1.01*

[A] Essential amino acids (EAA) in fish

[B] Hydrophobic amino acids (HPAA)

Non-essential amino acids (NEAA)

*Means ± SD (n =3)

methionine and histidine to overcome these limitations. In addition, amino acid composition is widely recognized to play a role in the bioactivity of several peptides from proteins (Pownall, Udenigwe, & Aluko, 2010). Amino acids with hydrophobic side chains have shown antioxidant activities against various free radicles (Matsui et al., 2018). In our study, high content of hydrophobic amino acids (44.17 %) were found in carotenoproteins indicating potential for antioxidant properties.

3.3.3.3 Carotenoid pigment composition

Carotenoids are the basis of pigmentation in crustacean species. Like many other animals, crustaceans require carotenoid through their diets which is then distributed to various tissues of the body, such as carapace, hepatopancreas, and ovaries (Wade et al., 2017; Simpson, K. L., Katayama, T., & Chichester, C. O. 1981). Astaxanthin is the main carotenoid responsible for the bright red color in crustacean tissues, and provides numerous bioactivities, including photoprotection and antioxidant properties (Anahi Martinez-Delgado, Khandual, & Josefina Villanueva-Rodriguez, 2017).

The yield of carotenoid pigments of extracted carotenoprotein from whole green crab is presented in Table 3.5. The carotenoid pigment content recovered in carotenoprotein was 139.26 μg/g. According to the literature, the result obtained in our study is within the range of previous studies that reported levels ranging from 87.42 to 500 μg/g carotenoids for various crustacean species (Klomklao et al., 2009; Senphan et al., 2014; Sila et al., 2012).

Table 3.5

Total carotenoids in extracted carotenoprotein from green crab

	Carotenoprotein
Carotenoids (μg/g)	139.26 ± 0.49

Means ± SD (n = 3)

Carotenoid pigments from carotenoprotein extracted from whole green crab were separated and identified using TLC as depicted in Figure 3.1. Six distinct bands were observed with Rf values of 0.11 (green), 0.30 (orange), 0.32 (orange), 0.35 (orange), 0.73 (orange) and 0.97 (yellow).

According to Senphan et al. (2014) the yellow band with Rf of 0.97 corresponded to β-carotene and the orange bands having Rf of 0.32 and 0.73 corresponded to free astaxanthin and astaxanthin diester, respectively. Therefore, astaxanthins were present in extracted carotenoprotein in the form of free and diester. The presence of other pigments, including the band with Rf of 0.30 may correspond to astacene, an oxidative breakdown product of astaxanthin and the green band with Rf of 0.11 a non-carotenoid suggested their origin were probably diet-related (Liu, Hu, Sommerfeld, & Hu, 2003).

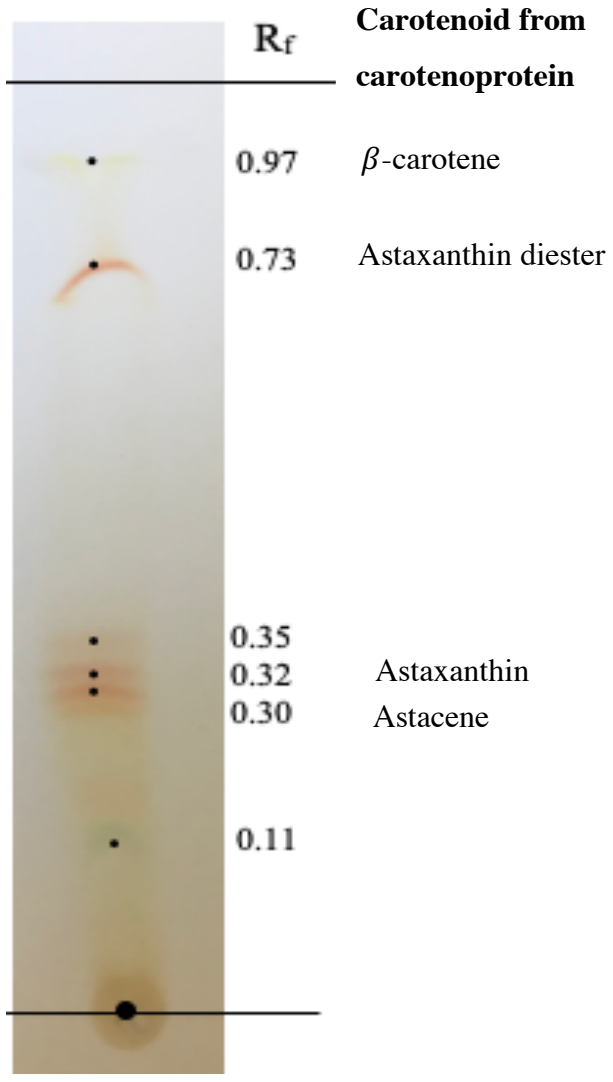


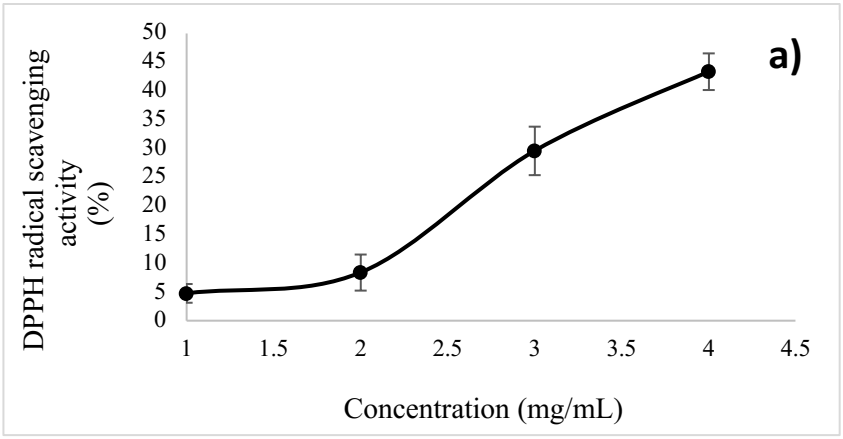
Figure 3.1.

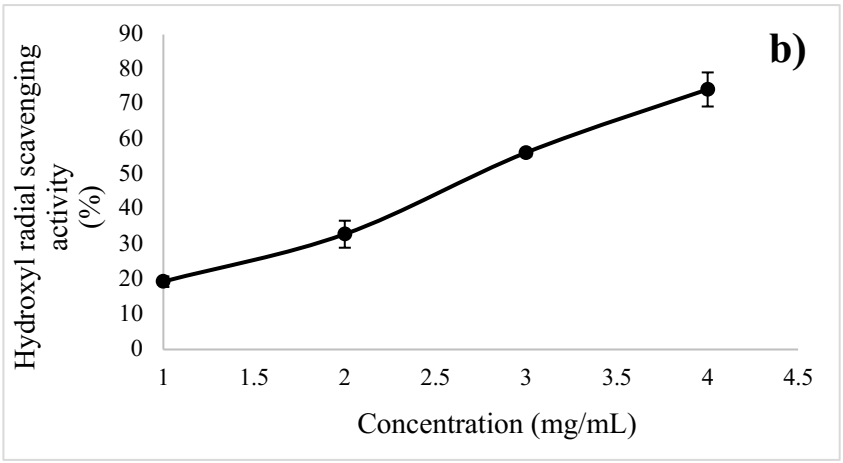
Thin layer chromatography of carotenoids extracted from carotenoproteins of green crabs.

Astaxanthin is a keto-carotenoid characterized by a conjugated double bond system, responsible for the color. Pigmentation of aquaculture is of economic importance, animals fed 100 &mg/kg astaxanthin for one month were shown to improve body color (Wade et al., 2017). Therefore, incorporating carotenoproteins from whole green crab in aquafeed provides a natural source of pigmentation.

3.3.3.4 Antioxidant activities

The antioxidant activity of carotenoprotein, extracted from whole green crab is shown in Figure 3.2. Carotenoprotein showed the highest DPPH activity at a concentration of 4 mg/ml ($p < 0.05$), however there were no significant difference at concentrations 1 mg/mL and 2 mg/ml ($p > 0.05$). The hydroxyl radical scavenging activity and iron reducing activity of carotenoprotein increased with increasing concentrations from 1 mg/ml to 4 mg/ml ($p < 0.05$). These results indicated that the antioxidant activity of carotenoprotein extract from whole green crab were concentration dependent. Therefore, carotenoprotein showed radical scavenging and reducing power capability as the concentrations were increased. These findings are in accordance with those of Poonsin et al., (2018), who reported carotenoprotein extracted from Pacific white shrimp shells showed antioxidant activity. The combination of amino acids, peptides and carotenoids comprised in carotenoprotein were able to donate electrons to free radicals and convert them into stable products to terminate free radical chain reactions (Poonsin et al., 2018). Sila et al., (2014) reported peptidic fraction of carotenoprotein from shrimp by-products showed strong radical scavenging activities and some degree of electron donation capacity at high concentrations. The antioxidant activities of peptides are most likely determined by the amino acid sequence and the properties of the amino acid side chains (Matsui et al., 2018). The presence of hydrophobic amino acid side chains, and functional groups such as the thiol in cysteine, thioether in methionine, indole group in tryptophan, phenolic hydroxyl group in tyrosine and imidazole group in histidine have hydrogen/electron donating activities able to interact with free radicles derived from lipid oxidation (Chalamaiah, Dinesh kumar, Hemalatha, & Jyothirmayi, 2012; Matsui et al., 2018). Therefore, in our study, the high content of hydrophobic amino acid shown in Table 3.2. corroborates with the antioxidant activities obtained in carotenoproteins. It has been reported that





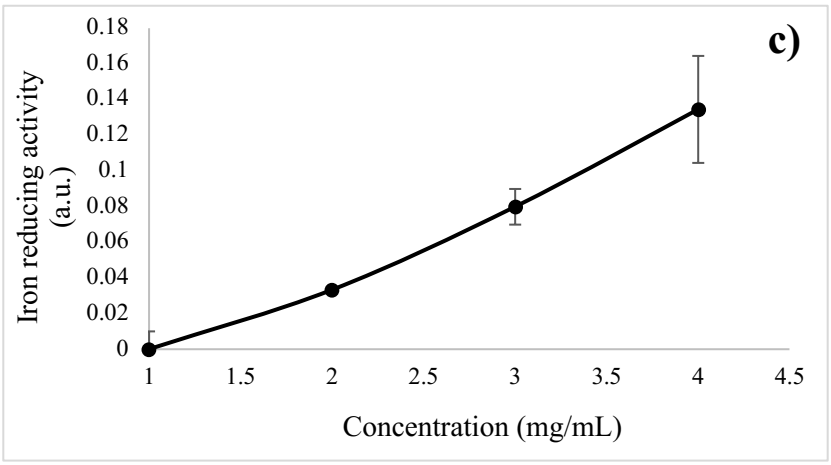


Figure 3.2.

Antioxidant activities of extracted carotenoproteins from green crabs at different concentrations as determined by DPPH radical scavenging activity (a) Hydroxyl radical scavenging activity (b) and Iron reducing activity (c). Bars represent SD (n = 3).

amino acids, such as valine, lysine, phenylalanine, leucine were among a few residues in isolated peptide from tuna backbone protein that showed quenching of free radicals DPPH, hydroxyl and superoxide in a dose-dependent manner (Je, Qian, Byun, & Kim, 2007). The amino acid composition plays an important role in the antioxidant activities of extracted carotenoprotein. In addition, carotenoids extracted from carotenoprotein have shown increased radical scavenging activities as well as metal chelating activities (Senphan et al., 2014). Sowmya and Sachindra (2012) reported that a carotenoid crude extract rich in astaxanthin showed antioxidant activities comparable to α-tocopherol. Free astaxanthin is prone to oxidation, therefore is it often found esterified with fatty acids or with proteins to form a carotenorprotein complex (Hamdi, Nasri, Dridi, Li, & Nasri, 2020). Nonetheless, astaxanthin in its free form is more susceptible to binding with free radicals, thus making it a potent antioxidant (Anahi Martinez-Delgado et al., 2017). The conjugated double bond chain and terminal ring containing hydroxyl and keto groups of astaxanthin stabilizes radicals through resonance. Therefore, carotenoprotein extracted from whole green crab demonstrated antioxidant properties through radical scavenging and reducing power which is dependent on concentration, amino acid composition and carotenoid profile.

3.4 Conclusion

Whole green crab showed high content of crude protein making it a rich nutrient source to extract carotenoproteins. Although fat content was low, the sum of *n-3* PUFAs, including EPA and DHA was considerably higher than those previously reported in other crab species. Carotenoprotein extracted with the aid of porcine pancreas trypsin showed a protein recovery of 95.72 %, which showed similar essential amino acids profiles with that found in whole green crab. Green crab carotenoprotein was high in essential amino acids and hydrophobic amino acids. In addition, astaxanthin and astaxanthin diester were the major carotenoids present. The interaction of carotenoids, amino acids and peptides comprised in carotenoproteins with free radical species through hydrogen/electron donation showed strong antioxidant activity. Thus, enzyme assisted extraction of carotenoprotein from underutilized green crab could be a potential alternative ingredient to enhance nutritive value and antioxidant activities in feed and food products.

CHAPTER IV.

CHITIN EXTRACTION FROM GREEN CRAB: OPTIMIZATION OF CITRIC ACID DEMINERALIZATION USING BOX-BEHNKEN DESIGN (BBD)

CONNECTING STATEMENT II

In the previous chapter, enzyme-assisted extraction of carotenoprotein and biochemical characterization of green crab was evaluated. The results indicated carotenoprotein recovered by trypsin-assisted extraction showed rich source of essential amino acids and carotenoids with antioxidant activity. This current study further assessed the extraction of chitin from green crab with mild treatment. The effects of parameters, temperature, concentration and time during the demineralization of green crab shells with citric acid treatment was optimized using Box-Behnken design. Moreover, the quality of extracted chitin following optimization was assessed by using ICP-MS, XRD and FT-IR for mineral analysis and chemical structure analysis.

Note: This chapter constitutes the text of an article that has not yet been published.

Contribution of author: Paola Sully designed and performed experiments, analyzed the data and drafted the manuscript.

Contribution of co-authors: Yi Zhang provided guidance and experimental assistance for developing experimental design. Simpson, B.K. supervised the research work, provided research funding, guided the lab performance and data analysis and revised the manuscript.

I Abstract

Crab shells are an excellent source to generate chitin of high quality for biomedical or pharmaceutical applications. In this study green crab showed high content of ash (38.05 %) and chitin (11.26 %). Organic acid was used to demineralize green crab (*Carcinus maenas*) to produce chitin under mild conditions. The chelation properties of citric acid was significantly ($p < 0.05$) more effective at demineralizing green crab in comparison to lactic acid and conventional HCl. The demineralization of green crab with citric acid was further optimized using the Box-Behnken design to investigate the effects of parameters (time, concentration and temperature) on the total residual ash. The optimal condition was determined as 1.0 M of citric acid, at a temperature of 4°C and time of 18h to obtain 1.38 % total residual ash in green crab. The effects of temperature and concentration were significant ($p < 0.05$) at decreasing the total residual ash content in green crab. The mineral analysis showed green crab was rich in calcium (196 mg/g), sodium (16.4 mg/g), phosphorous (12.8 mg/g), magnesium (8.50 mg/g), potassium (5.20 mg/g) and strontium (2.74 mg/g). However, following demineralization of green crab with optimal conditions extracted chitin showed relatively lower levels of sodium (4.20 mg/g), calcium (1.40 mg/g) and phosphorous (0.900 mg/g). XRD spectra confirmed $CaCO_3$ was effectively chelated with citric acid. The FTIR spectrum of extracted chitin showed characteristic bands similar to commercial crab chitin, indicating a potential source for further application.

4.1. Introduction

Chitin is the second most abundant biopolymer on earth, after cellulose. Chitin is known to be a linear polysaccharide chain composed of *N*-acetyl-D-glucosamine residues bonded by β-(1-4) linkages. The economic value of chitin and its derivates, chitosan and chitooligosaccharides are quite significant due to the wide range of application in various industries, including agricultural, biomedical, pharmaceutical, environmental, food and cosmetics (Nguyen, Barber, Smith, Luo, & Zhang, 2017). Crustacean waste have commonly been the primary source of commercial chitin production. In contrast to other sources, such as insects and fungi, the chitin content in crustacean waste varies from 20 to 30 % depending on the species (El Knidri, Belaabed, Addaou, Laajeb, & Lahsini, 2018). In crustacean shells, chitin is often associated with minerals, protein and pigments. Therefore, to extract chitin from crustacean shells three steps are required: demineralization, deproteination and depigmentation.

Within the shell, chitin forms a complex network with protein onto which $CaCO_3$, the main salt in crustacea, deposit for reinforcement and rigidity (Gbenebor, Adeosun, Lawal, & Jun, 2016). The thickening of crustacean shell is based on the amount of $CaCO_3$. Therefore, the isolation of chitin depends on the type of shell. Crab shells have previously been identified as a source of chitin (Bernabé et al., 2020; Castro, Guerrero-Legarreta, & Bórquez, 2018; Gbenebor et al., 2016; Hajji, Ghorbel-Bellaaj, Younes, Jellouli, & Nasri, 2015; Hamdi et al., 2017; Khiari, Kelloway, & Mason, 2020; Taokaew, Zhang, Chuenkaek, & Kobayashi, 2020). The level of mineral content in the shells of crab species varies, which impacts the efficiency of isolating chitin (Boßelmann, Romano, Fabritius, Raabe, & Epple, 2007). In crab shells, generally the $CaCO_3$ content is higher than shrimp shells, therefore requiring extensive demineralization (Gbenebor et al., 2016). The quality of chitin relies heavily on the level of impurities that remains following extraction.

Acid treatment for the removal of minerals is required for the demineralization of crustacean shells. Strong inorganic acids, including hydrochloric acid (HCl) have conventionally been used to remove $CaCO_3$ in crustaceans shells (Al-Manhel, Al-Hilphy, & Niamah, 2018). However, the use of strong acids may initiate partial deacetylation and hydrolysis of chitin resulting in decreased physiological properties of final product (Younes et al., 2014). Moreover, the disposal of corrosive reagents pose an environmental hazard and creates a source of pollution (Saravana et al., 2018). In recent studies, organic acids have been used as an alternative approach for the demineralization of crustacean shells (Baron et al., 2015). Lactic acid has previously been reported in the demineralization process of lobster (*Jasus edwardsii*) shells (Nguyen et al., 2017), shrimp (*Pandalus borealis*) shells (Mahmoud, Ghaly, & Arab, 2007) and crab (*Chionoecetes japonicus*) shells (Jung, Jo, Kuk, Kim, & Park, 2005). However, being that lactic acid is a monoprotic acid, it is more reluctant to denote its only proton as a weak acid. As a result, to increase its efficiency of demineralization a combination of prolonged extraction times, addition of heat with thermal treatment or microwave assisted treatment have been employed (Nguyen et al., 2017). Citric acid is inexpensive and is a chelating agent that can favor the demineralization of crab shells. It is worth exploring its usage to generate high quality chitin in addition to its various use as an acidulant, a buffer, chelator and non-toxic crosslinking agent (Soccol, Vandenberghe, Rodrigues, & Pandey, 2006).

European green crab (*Carcinus maenas*) is an invasive species with little to no industrial application (Rayner & McGaw, 2019). Although being small in size, the shells are a rich source

of chitin (Khiari, Kelloway, & Mason, 2020). However, the high levels of calcium salts renders it a poor source for extraction of value-added products (Beth A & Fairchild, 2013). The demineralization for green crab is an essential step to generate quality chitin. Optimization of crustacean shell demineralization have been reported (Arbia, Adour, Amrane, & Lounici, 2013; Gamal, El-Tayeb, Raffat, Ibrahim, & Bashandy, 2016; Nguyen et al., 2017; Younes et al., 2016). Nonetheless, the efficiency of organic acid to remove minerals from green crab have not yet been investigated. The objectives in the present study were to evaluate the effectiveness of organic acids, citric acid and lactic acid to demineralize green crab as a preliminary study. The demineralization process was then optimized using the response surface methodology following the Box-Behnken design to investigate the effects of independent variables time, concentration and temperature on the total residual ash in green crab. The optimum conditions were determined to extract chitin and characterize the mineral composition and chemical properties by FTIR and XRD analysis.

4.2. Methods and Material

4.2.1. Chemicals

Citric acid, papain enzyme, sodium hypochlorite (NaOCl, and phosphate were purchased from Fisher Scientific (Geel, Belgium). Acetone, hydrochloride (HCl), lactic acid, and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

4.2.2. Sample preparation

Detailed information on sample preparation of green crab is presented in chapter 3.

4.2.3. Analysis of ash and chitin content

Ash content was determined according to the standard Association of Official Analytical chemists method (AOAC, 2000). The green crab ash were quantified by incineration at 550°C using a muffle furnace (AOAC method 942.05).

Chitin was determined by the method of Senphan, Benjakul, and Kishimura (2014) with slight modifications. Dried crab samples (2.0 g) were mixed with 30 mL of 2.0% NaOH at 25°C for 6 h. The mixture was filtered under vacuum using Whatman No. 1 filter paper. The residue was mixed with 15 mL 1M HCl for 30 min at 25°C, filtered and washed with deionized water. The

washed residue was then homogenized with cold acetone (-20 ℃) using a Fisher Brand homogenizer (Canada), filtered and washed with deionized water. The sample was mixed with 30 mL of 0.3 % NaOCl for 6 h at 25℃ to bleach the remining pigments. The mixture was filtered and washed with deionized water. The residue was oven dried at 65℃ for 24 h and the dried material was referred to as "chitin".

4.2.4. Organic acid demineralization

Green crab were demineralized by using different organic acids according to the modified method of Mahmoud et al. (2007). Dried green crab samples (5 g) were subjected to organic acids, citric acid and lactic acid at various concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 M) with a sample to acid ratio of 1:10 (w/v) for 6 h at 22 ℃. The demineralized sample was filtered and washed thoroughly several times with distilled water until neutral. The solid matter was then oven dried at 65 ℃ for 24 h. The dried demineralized sample were analyzed for their total residual ash content. Samples were placed in the muffle furnace (Thermolyne Corporation; Dubuque, Iowa, U.S.A) at 600℃ for 3 h. The samples were then transferred in a desiccator to cool down to room temperature and weighed. The total residual ash content was determined according to the following equation:

$$\text{Total residual ash } (\%) = \frac{W_a}{W_{ds}} \times 100\%, \quad (1)$$

where W_a is the weight of ash remaining and W_{ds} is the weight of dried demineralized sample in grams.

In addition, the demineralization of green crab samples with organic acid was compared to HCl at the same level (1.0 M) under the same conditions as described above.

4.2.5. Optimization of demineralization conditions

Box Behnken design (BBD) is amongst one of the commonly used design in Response surface methodology (RSM) for optimization of several chemical processes (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). BBD is considered an economical and efficient second-order design based only at three-factorial levels. In the present study, BBD was used to optimize demineralization of green crab with organic acid by investigating the effects of the three factors that were time (X_1), concentration (X_2) and temperature (X_3). Table 4.1 demonstrates the different factor levels coded as (-1, 0, + 1) of each independent variable.

Table 4.1.

Design of three-factorial levels

Factors	Symbols	Level		
		-1	0	+ 1
Time (h)	X_1	12	18	24
Concentration (M)	X_2	0.4	0.7	1.0
Temperature (°C)	X_3	4	22	40

The coded value (Z) of each variable were determined according to the following equation (Bezerra et al., 2008):

$$Z=\left(\frac{z_i-z_i^0}{\Delta z_i}\right), \quad (2)$$

The complete BBD consisted of 15 combinations from the three independent variables and three center points as shown in table 4.2. The % total residual ash (Y) were recorded as the response (dependent variable). The experimental response data generated by BBD were fitted by a second-order polynomial regression model to describe the interaction between the different independent variables and obtain a response surface (Nguyen et al., 2017) :

$$Y=\beta_0+\beta_1x_1+\beta_2x_2+\beta_3x_3+\beta_{12}x_1x_2+\beta_{13}x_1x_3+\beta_{23}x_2x_3+\beta_{11}x_1^2+\beta_{22}x_2^2+\beta_{33}x_3^2, \quad (3)$$

here, Y is the predicted response, x_1 , x_2 and x_3 are independent variables, β_0 is the constant coefficient, β_1, β_2 and β_3 are the linear coefficients, β_{12}, β_{13} and β_{23} are the cross-product coefficients and β_{11}, β_{22} and β_{33} are the quadradic coefficients.

Table 4.2.

Box-Behnken Design (BBD) with response of dependent variable to demineralization conditions.

Run	Independent Variables						Dependent Variable	
	Time (X_1)		Concentration (X_2)		Temperature (X_3)		Total Residual ash (Y_1) (%)	
	Coded Value	Actual Value (h)	Coded Value	Actual Value (M)	Coded Value	Actual Value (°C)	Exp.[a]	Pre.[b]
1	0	18	0	0.7	0	22	5.10	20.15
2	-1	12	0	0.7	-1	4	1.33	4.44
3	0	18	+1	1.0	+1	40	35.14	24.55
4	0	18	-1	0.4	-1	4	22.96	15.75
5	+1	24	0	0.7	-1	4	2.40	2.84
6	+1	24	-1	0.4	0	22	39.59	31.46
7	-1	12	+1	1.0	0	22	8.80	8.84
8	0	18	+1	1.0	-1	4	2.74	-8.47
9	-1	12	-1	0.4	0	22	39.57	33.06
10	0	18	0	0.7	0	22	10.98	20.15
11	+1	24	0	0.7	+1	40	39.89	35.86
12	0	18	-1	0.4	+1	40	44.70	48.77
13	+1	24	+1	1.0	0	22	3.23	7.24
14	0	18	0	0.7	0	22	4.04	20.15
15	-1	12	0	0.7	+1	40	41.78	37.46

[a] Experimental data

[b] Predicted data

4.2.6. Extraction of chitin

Green crab demineralized at optimum conditions was use to extract chitin according to the modified method of Marzieh, Zahra, Tahereh, and Sara (2019). The demineralized green crab was

enzymatically deproteinized using papain. Demineralized green crab (25 g) were added to sodium phosphate buffer (pH 6.0) adjusted with 2 M HCl at a sample to buffer ratio of 1:3. Then 0.1% (w/w) enzyme was added on the basis of protein content in crab sample. The mixture was placed in a water bath at 65°C with continuous shaking for 2 h. To inactivate the enzyme, the temperature was increased to 90°C for 15 min then filtered through with cheesecloth and washed with deionized water until neutral pH . The solid matter was further depigmented with 0.3 % (v/v) NaOCl for 1h, filtered and washed thoroughly with deionized water. The residue was oven dried at 65°C for 24h and the dried material was referred to as "chitin".

4.2.7. Characterizations

Green crab and extracted chitin from optimized demineralization conditions were characterized by a series of analytical techniques.

4.2.7.1. Mineral analysis

The mineral analysis of green crab and extracted chitin were conducted by elemental analysis using Inductively coupled plasma Mass Spectrometry (ICP-MS) (Agilent 8000-G3663A Triple Quadrupole; Santa Clara, CA, USA). The samples were thoroughly freeze-dried and ground to fine powder, prior to the digestion. The fine powder was then digested with hydrochloric, nitric, hydrofluoric and perchloric acids using microwave digestion for ICP-MS analysis.

4.2.7.2. Fourier transform infrared (FT-IR) spectroscopic analysis

The FT-IR spectrum of green crab, extracted chitin and commercial chitin were recorded on a Nicolet 6700 spectrophotometer (Thermo Fisher Scientific Inc.,, Waltham, MA, USA) using an attenuated total reflectance (ATR) accessory equipped with a Ge crystal. The spectra in the range of 400 – 4000 cm^{-1} were collected in 64 scans at a resolution of 4 cm^{-1} and 25°C, using the empty cell as a blank.

4.2.7.3. X-ray diffraction (XRD) analysis

The XRD patterns of green crab and chitin were collected on a Bruker D8 Discovery X-Ray diffractometer (40 kV and 44 mA) using a Cu $K\alpha$ radiation ($\lambda = 0.154\, nm$). The rotation mode (2θ was from 4° to 45°) with a scan rate of 1 °/min was used.

4.2.8. Statistical analysis

All studies on organic acid demineralization were performed in triplicates and the data was expressed as means ± SD (standard deviations). Data were subjected to one-way analysis of variance (ANOVA). Comparison of means was carried out by Tukey's honestly significant difference (HSD) test. Statistical analyses were performed using the Statistical Package of Social Sciences (SPSS 11.0 for windows; SPSS Inc., Chicago, IL, USA). For optimization analysis, Design Expert version 12.0 (State-Ease Inc. Minneapolis, MN, USA) was used for the experimental design, mathematical model fitting and used to analyze the interaction between the response and independent variables. It was also used to predict and verify the model equation. The ANOVA analysis was performed to statistically assess the repeatability of the three center points replicates (Hooshmand, Shabanpour, Moosavi-Nasab, & Golmakani, 2017).

4.3. Results and Discussion

4.3.1. Ash and chitin composition

The total ash and chitin composition of green crab is depicted in table 4.3. Green crab was high in ash content accounting for 38.05% of total dry mass and had a chitin content of 11.26% of total dry mass. McNiven, Quijon, Mitchell, Ramsey, and St-Hilaire (2013) reported ash levels of green crab, during the harvesting months of May to October, were 32.9 % to 40.9 % of dry mass, which was shown to be related to the size of carapace. The ash content of crabs with large carapace dimensions showed the highest ash levels (McNiven et al., 2013). Khiari et al. (2020) reported recovered shells from green crab showed a significantly ($p < 0.05$) higher ash (22.55 %) and chitin (11.25 %) content compared to the whole green crab (15.92 % and 5.76 %, respectively). Similarly, Naczk, Williams, Brennan, Liyanapathirana, and Shahidi (2004) obtained chitin content of 12.6 % to 14.5 % from green crab shells harvested from different sites. In the present study, our findings are in accordance with previous literature. Therefore, the level of ash and chitin in crustacean is directly related to the exoskeleton dimensions, season of harvest and species.

Table 4.3.

Total ash and chitin content of green crab.

Composition (% dry weight basis)	Green Crab
Ash	38.05 ± 2.14
Chitin	11.26 ± 3.83

Means ± SD (n=3).

4.3.2. Organic acid demineralization

The demineralization of green crab using organic acids, including citric acid and lactic acid were compared in figure 4.1. Green crab demineralized with organic acids showed a decline in total residue ash. The total residual ash differed significantly ($p < 0.05$) between the organic acids. Citric acid resulted in a significantly ($p < 0.05$) lower total residual ash in crab samples than lactic acid at concentrations 0.2 M to 1.0 M. The increase in citric acid concentration significantly ($p < 0.05$) reduced the total residual ash in green crab. Meanwhile, the reduction of total residual ash in crab samples with lactic acid was only significant ($p < 0.05$) at concentrations 0.4 M to 1.0 M.

These results indicated that the demineralization of green crab with organic acid is concentration dependent. Therefore, an increase in concentration rendered total residual ash. In addition, the demineralization of green crab using organic acids were compared to the conventional HCl demineralization at 1 M. Figure 4.2. showed citric acid (7.80 %) was significantly ($p < 0.05$) more effective at reducing total residual ash in green crab than lactic acid (42.89 %) and HCl (20.78 %). Similar to our study, Baron et al. (2015) showed that demineralization by weak acids of shrimp (*Litopenaeus vannamei*) cuticles can be done. They showed that weak acids, such as citric acid (1.0 %) led to lower residual mineral compared to HCl (1.4 %), acetic acid (16.4 %), formic acid (4.1 %) and phosphate acid (7.0 %). The mechanism of organic acids, weather mono or polyacids during demineralization of crustacean is quite different from that of mineral acids (Baron et al., 2015). Setoguchi, Kato, Yamamoto, and Kadokawa (2012) demineralized red queen crab shells with citric acid to extract chitin. A 1.5 % (w/v) citric acid aqueous solution was able to completely remove $CaCO_3$ from the crab shells. The citric acid was efficient at demineralizing the crab shells due to its chelating properties (Setoguchi et al., 2012). Previous studies have shown that small

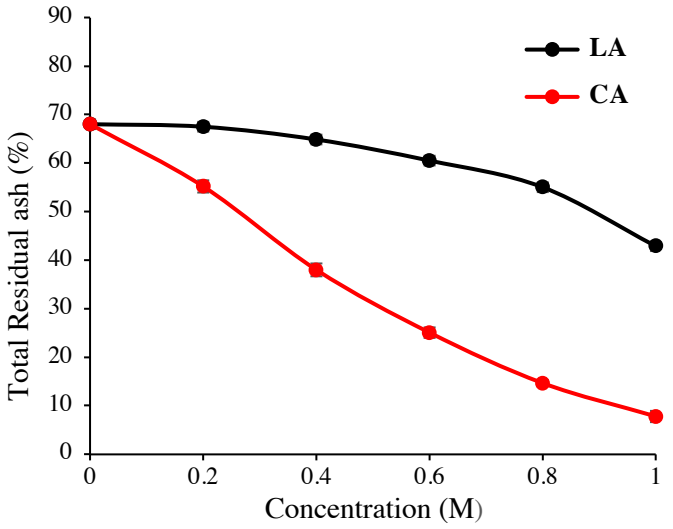


Figure 4.1

Total residual ash of green crab demineralized using different organic acids (CA: citric acid and LA: lactic acid) at various concentrations. The bars represent the standard deviation from triplicate determinations.

molecular organic acids, including citric acid can effectively remove heavy metals by chelation reactions (Ke et al., 2020; Li, Yang, Liang, & Guo, 2017). Citric acid can form stable complexes with metals through isoelectric interactions formed by functional groups, such as carboxyl groups (Abdullah & Ang, 2018). Jung et al. (2005) reported demineralization of red crab (*Chionoecetes japonicus*) by a chelating agent, such as Ethylenediaminetetraacetic acid (EDTA) was more effective at decreasing the ash content than lactic acid and showed comparable residual ash content to treatment with HCl. These findings show that chelating agents are as efficient as mineral acids at demineralizing crustacean waste. Citric acid is a chelating agent that is able to effectively bind to a metal at either three carboxylic groups (Abdullah & Ang, 2018). Therefore, the chelation properties of citric acid in this present research shows that it is a suitable organic acid for the purpose of this study.

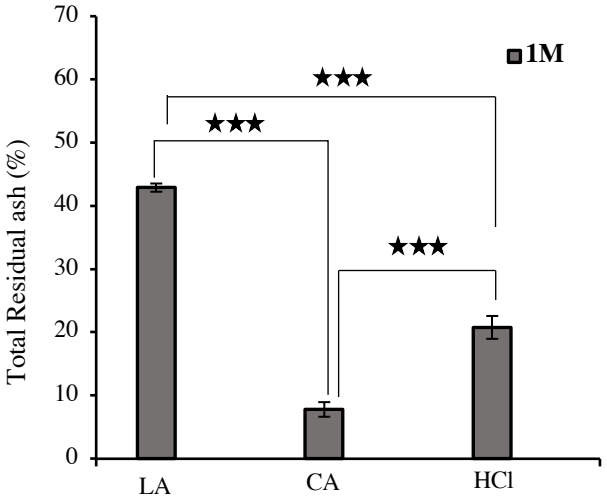


Figure 4.2

The total residual ash of green crab demineralized using organic acids, citric acid (CA) and lactic acid (LA) in comparison to HCl. The bars represent the standard deviation from triplicate determinations.

4.3.3. Statistical analysis and Model fitting

The combined effect of independent variables time, concentration and temperature on the demineralization of green crabs with citric acid was adopted to optimize total residual ash (table

4.2). The response for total residual ash was expressed as experimental and predicted data. The total residual ash in green crab ranged from 1.33 % to 44.70% ash as shown in table 4.2. The obtained response data from BBD design were expressed as the following regression equation:

$$Y = 20.15-0.7963X_1-12.11X_2+16.51X_3 \quad (4)$$

where, Y represents the % total residual ash, X_1, X_2, and X_3 are the coded levels of time, concentration and temperature, respectively.

The validation of the model was statistically evaluated based on the analysis of variance (ANOVA) and the coefficient of determination (R^2) as depicted in table 4.4. (Nam, Cho, Han, Her, & Yoon, 2018). The ANOVA results obtained from the second order polynomial regression showed that the model was significant (F-value = 11.78, $p = 0.0009$) for total residual ash in green crab. In addition, the lack of fit of the model relative to the pure error was insignificant ($p = 0.1148$) suggesting that the obtained linear model fitted the experimental data well. The coefficient of determination (R^2) is often referred to evaluate the degree in which the experimental data fits the regression equation (4). Thus, a high R^2 coefficient close to 1 assures that the model fits the experimental data (Gamal et al., 2016). In this study, the value of determination coefficient R^2 was 0.7626, which indicated 23.74 % of total variations in data response were not explained by the model. However, the difference between Adjusted R^2 and Predicted R^2 was less than 0.2 which implies reasonable agreement. Therefore, the response model can be used for predictions and optimization of total residual ash in green crab.

The p-value is commonly used as a tool to define the significance of model terms (Arbia et al., 2013). Thus, p-values < 0.05 indicates that the model terms are significant. In table 4.4, it can be seen that linear model terms X_2 and X_3 are statistically significant, suggesting that concentration (X_2) and temperature (X_3) are the main factors influencing the total residual ash. However, the model term X_1 had a p-value > 0.05, which indicated the independent variable time was insignificant. The insignificant term was removed from the model to refine the regression equation to describe total residual ash:

$$Y= 20.15-12.11X_2+16.51X_3 \quad (5)$$

The linear terms concentration and temperature showed opposite effects on the total residual ash. The regression equation showed that the temperature had a positive influence on the total residual ash, while the concentration showed a negative effect. Temperature showed the highest value of regression coefficient compared to concentration which denotes that this parameter had the most significant effect ($p = 0.0006$) on the process (table 4.4.).

Table 4.4.

ANOVA for the fitted model

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	*p*-value
Model	3359.66	3	1119.89	11.78	0.0009
X_1	5.07	1	5.07	0.0533	0.8216
X_2	1173.94	1	1173.94	12.35	0.0048
X_3	2180.64	1	2180.64	22.94	0.0006
Residual	1045.81	11	95.07		
Lack of Fit	1017.86	9	113.10	8.09	0.1148
Pure Error	27.95	2	13.98		
Cor Total	4405.47	14			

Note: X_1 is time, X_2 is concentration and X_3 is temperature. For response, $R^2 = 0.7626$ Adjusted $R^2 = 0.6979$ Predicted $R^2 = 0.6202$ Adeq. Precision = 11. 37.

4.3.4. Surface plot analysis for optimization conditions

The response surface plot was used to understand the interaction of the three independent variables and to optimize the level of each variable to minimize the response. As shown in figure 4.3., the response surface plot were generated by keeping one variable constant while varying the two other within the experimental domain. The interaction of temperature and concentration on the effect of total residual ash was depicted in figure 4.3. A decrease in total residual ash was observed when temperature was lowered, and concentration was increased. However, the parameter temperature was more influential than concentration. The total residual ash only varied from 44.70 to 35.14 % at 40°C when raising concentration to 1M. Nonetheless, when temperature decreased to a minimum of 4°C the total residual ash reduced significantly while increasing concentration. This suggest the effects of decreasing temperature plays a primary role in mineral removal followed by the effects of increasing concentration. It was observed during the experimental runs that the addition of high temperatures favored depigmentation of green crab instead of mineral removal, resulting in high total residual ash. This could be because high temperatures elicits protein hydrolysis, while demineralization kinetics remains unaffected (Baron et al., 2015). Similarly, Younes et al. (2016) reported increasing the temperature had no effect on mineral removal from shrimp shells. In contrast, the optimized conditions proposed for demineralization of shrimp shells was using 0.5 M HCl at 4°Cto obtain complete removal of minerals (Younes et al., 2016). Similarly, Nguyen et al. (2017) reported the effects of high temperature and time was insignificant at demineralizing lobster shells. In our study, a temperature of 4°Cand a concentration of 0.7 M citric acid resulted in the lowest total residual ash. These findings confirmed that the parameters temperature and concentration of citric acid need to be combined to optimize the total residual ash during the demineralization of green crabs. The total residual ash only varied from 44.70 to 35.14 % at 40°C when raising concentration to 1M. Nonetheless, when temperature decreased to a minimum of 4°C the total residual ash reduced significantly while increasing concentration. This suggest the effects of decreasing temperature plays a primary role in mineral removal followed by the effects of increasing concentration. It was observed during the experimental runs that the addition of high temperatures favored depigmentation of green crab instead of mineral removal, resulting in high total residual ash. This could be because high temperatures elicits protein hydrolysis, while demineralization kinetics remains unaffected (Baron et al., 2015). Similarly, Younes et al. (2016) reported increasing the

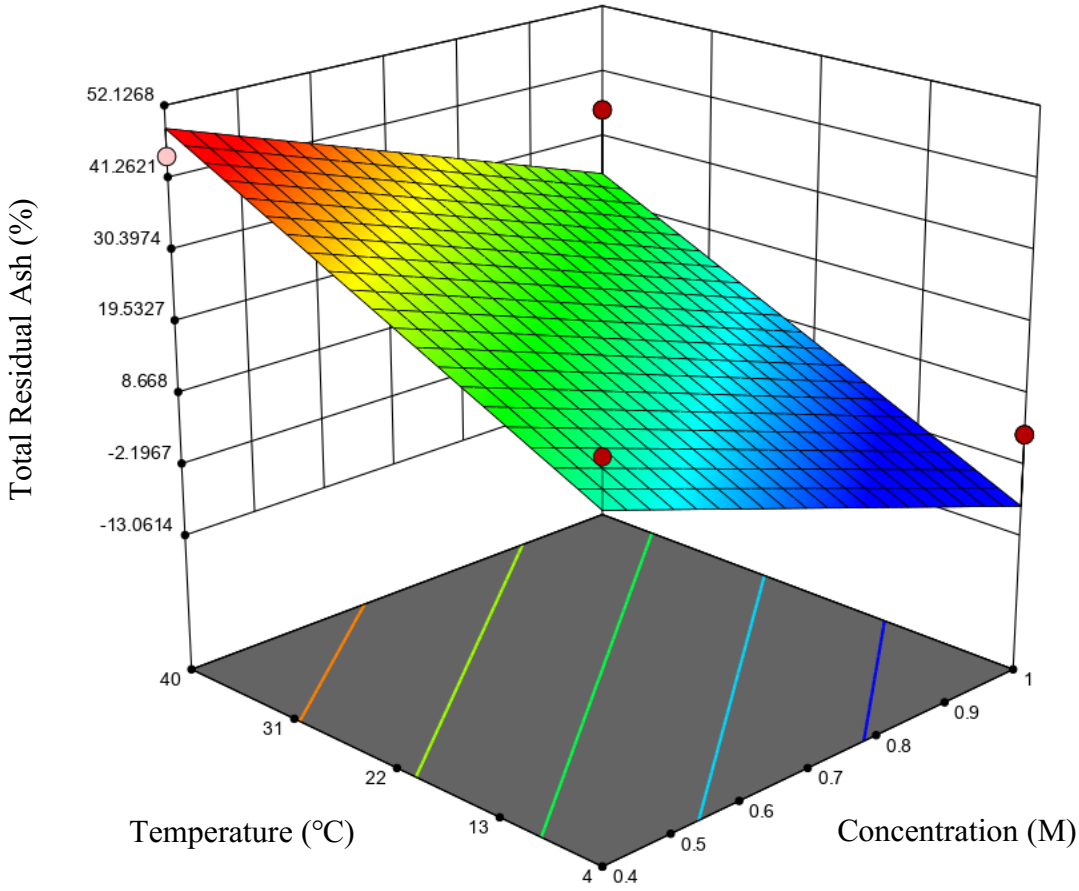


Figure 4.3.

Three dimensional response surface graph for total residual ash in whole green crab.

temperature had no effect on mineral removal from shrimp shells. In contrast, the optimized conditions proposed for demineralization of shrimp shells was using 0.5 M HCl at 4°C to obtain complete removal of minerals (Younes et al., 2016). Similarly, Nguyen et al. (2017) reported the effects of high temperature and time was insignificant at demineralizing lobster shells. In our study, a temperature of 4°C and a concentration of 0.7 M citric acid resulted in the lowest total residual ash. These findings confirmed that the parameters temperature and concentration of citric acid need to be combined to optimize the total residual ash during the demineralization of green crabs.

The desirability function was used for optimization of response. The objective of the method was to obtain an overall response desirability value close to 1 (Nam et al., 2018). In the present study, the desired target goal for response Y was set to minimize the total residual ash in green crab. The optimization condition was selected based on the level of variables giving the highest desirability (1.0) as shown in figure 4.4. The optimized condition for total residual ash was

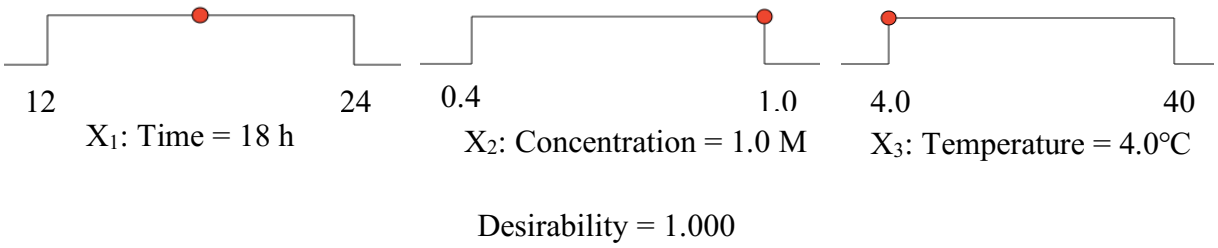


Figure 4.4.
Desirability ramp for numerical optimization

identified at time (X_1) of 18 h, concentration (X_2) of 1.0 M and temperature (X_3) of 4°C, with a predicted value of - 8.47 % (assumed as 0 %) total residual ash. The total residual ash obtained from the three replicates was 1.38 ± 0.09%, which was comparable to the optimum goal (0 %). Therefore, the demineralized green crab was further used to extract chitin for further analysis.

4.3.5. Mineral profile of green crab and extracted chitin

Table 4.5 shows the mineral composition (ug/g) of green crab and extracted chitin following optimized demineralization conditions. Of the 29 minerals analyzed, calcium (196 mg/g) was dominant in green crab followed by sodium (16.4 mg/g), phosphorous (12.8 mg/g), magnesium (8.50 mg/g), potassium (5.20 mg/g) and strontium (2.74 mg/g). However, the majority of the minerals found in green crab were removed during the optimized demineralization step to extract chitin. The remaining minerals in chitin were found at relatively lower levels and consisted of mostly sodium (4.20 mg/g), calcium (1.40 mg/g) and phosphorous (0.900 mg/g). The abundantly high content of calcium removed in green crab following demineralization suggests

Table 4.5.
Total mineral composition of whole green crab and extracted chitin following optimized demineralization.

Mineral composition (mg/g)	Green Crab	Chitin
Aluminum	0.130	0.056
Antimony	1.00 E-05	1.00 E-05
Arsenic	0.0065	7.30 E-04
Barium	0.0082	4.50 E-04
Beryllium	1.00 E-05	1.00 E-05
Boron	0.009	0.001
Cadmium	0.0012	4.30 E-04
Calcium	196	1.40
Chromium	6.50 E-04	5.50 E-04
Cobalt	7.40 E-04	1.40 E-04
Copper	0.025	0.027
Iron	0.190	0.120
Lead	2.60 E-04	9.50 E-04
Magnesium	8.50	0.050
Manganese	0.023	5.00 E-04
Mercury	3.90 E-05	7.80 E-05
Molybdenum	1.10E-04	5.00 E-05
Nickel	4.40E-04	3.50 E-04
Phosphorus	12.8	0.900
Potassium	5.20	< 0.050
Selenium	0.0012	7.60 E-04
Silver	6.80E-04	0.0013
Sodium	16.40	4.20
Strontium	2.74	0.016
Thallium	5.00E-06	5.00 E-06
Tin	5.00E-05	5.00 E-04
Titanium	0.004	0.0025
Uranium	2.60E-05	4.40 E-05
Vanadium	4.00E-04	2.00 E-04
Zinc	0.077	0.0028

that citric acid treatment under optimized conditions was efficient at chelating calcium salts, such as $CaCO_3$. These findings confirm that temperature strongly influenced the removal of calcium in green crab in addition to concentration of citric acid. This can be explained by the solubility properties of $CaCO_3$. In general, in the presence of low temperatures $CaCO_3$ is able to dissolve in water in large quantities, yet in contact with heat it precipitates (Geyssant, 2001). Therefore, low temperatures may have favored large quantities of $CaCO_3$ salts from green crab to dissolve under acidic conditions during the demineralization with citric acid. This suggest that the dissolution of calcium carbonate can be controlled by a combination of pH, concentration and temperature (Ehrlich, Koutsoukos, Demadis, & Pokrovsky, 2009).

4.3.6. Characterization of chitin

The XRD analysis was used to determine the crystalline structure of green crab and extracted chitin, as depicted in figure 4.5. The XRD patterns of green crab showed diffracted peaks at 2θ = 19.12º, 23.41 º, 29.63 º, 31.94 º, 36.64 º and 40.12 º. Apart from the peaks at 19.12 º and 23.41 º which are typical features for chitin, a peak at 29.63 º indicates the presence of $CaCO_3$ in green crab (Yang et al., 2019). The XRD patterns of extracted chitin was reduced to a few peaks at 19.45 º, 23.84 º and 26. 89 º suggesting the possible crystalline structure of α-chitin, which usually has distinct peaks at ~ 9 º, 19 º, 23 º and 26 º (Huang, Zhao, Guo, Xue, & Mao, 2018; Yang et al., 2019). The absence of the peak at 29.63 º which correspond to $CaCO_3$, indicated citric acid successfully reduced the mineral composition in extracted chitin, thus confirming the mineral analysis.

The FTIR spectra of green crab, extracted chitin and commercial chitin are depicted in figure 4.6. The FTIR spectrum of extracted chitin shows very similar major absorption peaks as the spectrum of commercial crab chitin, indicating a potential source for further application. A series of characteristic absorbance bands were observed on the spectra located at 3439 cm^{-1}(O—H stretching), 3248 cm^{-1} (N—H stretching), 2846 cm^{-1} (C—H stretching), 1631 cm^{-1} and 1651cm^{-1} (Amide I), 1560 cm^{-1} (Amide II) , 1300 cm^{-1} (Amide III) and 1018 cm^{-1} (C—O stretching) (Borić, Vicente, Jurković, Novak, & Likozar, 2020; Gbenebor et al., 2016). The absorption peak at 2916 cm^{-1} corresponds to the vibrational symmetric CH_3 stretching and asymmetric CH_2

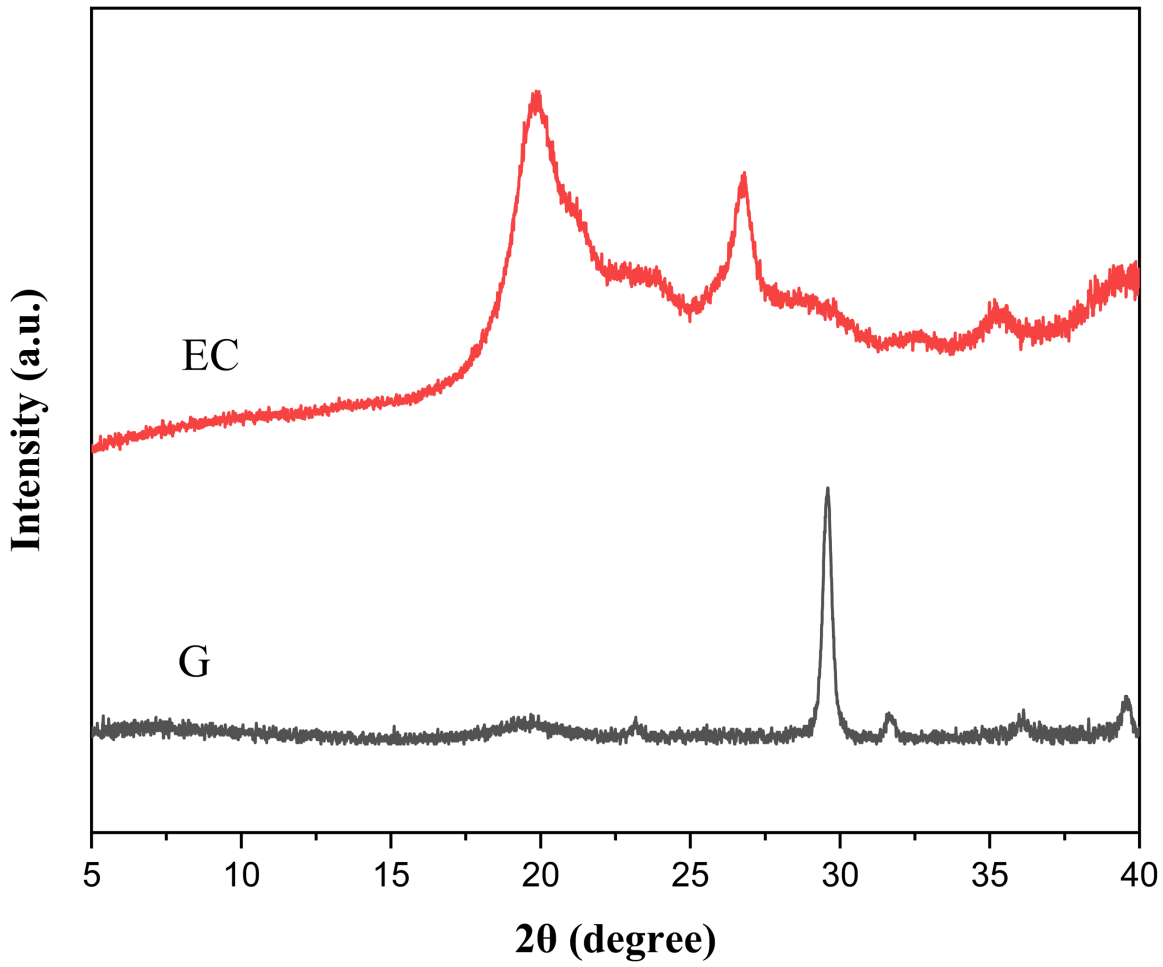


Figure 4.5.

XRD profile of (a) green crab and (b) extracted chitin.

stretching (Gbenebor et al., 2016). The splitting Amide I band which is a typical characteristic of α-chitin, corresponds to the inter and intramolecular hydrogen bonding of CO—NH and CO—$HOCH_2$, respectively (Hamdi et al., 2017). Specifically, amide II band is related to N—H bend and C—N stretch and amide III band denotes the stretching of C—N function groups and CH_2 wagging (Gbenebor et al., 2016). Other characteristic absorption bands for chitin are at 948 cm^{-1} representing CH_3 wagging along the polymer chain and 848 cm^{-1} indicating C—H stretching of the beta-1,4 glycosidic bonds (Kumari, Rath, Sri Hari Kumar, & Tiwari, 2015). These findings reported were in agreement with Borić et al. (2020), Yang et al. (2019), Hamdi et al. (2017), Gbenebor et al. (2016) and Kumari et al. (2015). The reduced absorption band at 1546 cm^{-1} indicates effective enzymatic protein removal (Hamdi et al., 2017; Yang et al., 2019). Furthermore,

the absence of the $CaCO_3$ characteristic bands at 829 cm^{-1}, 1402 cm^{-1}, 1747 cm^{-1} in extracted chitin as compared to green crab indicates desirable demineralization process (Gbenebor et al., 2016; Yang et al., 2019). The reduced absorption band at 1546 cm^{-1} indicates effective enzymatic

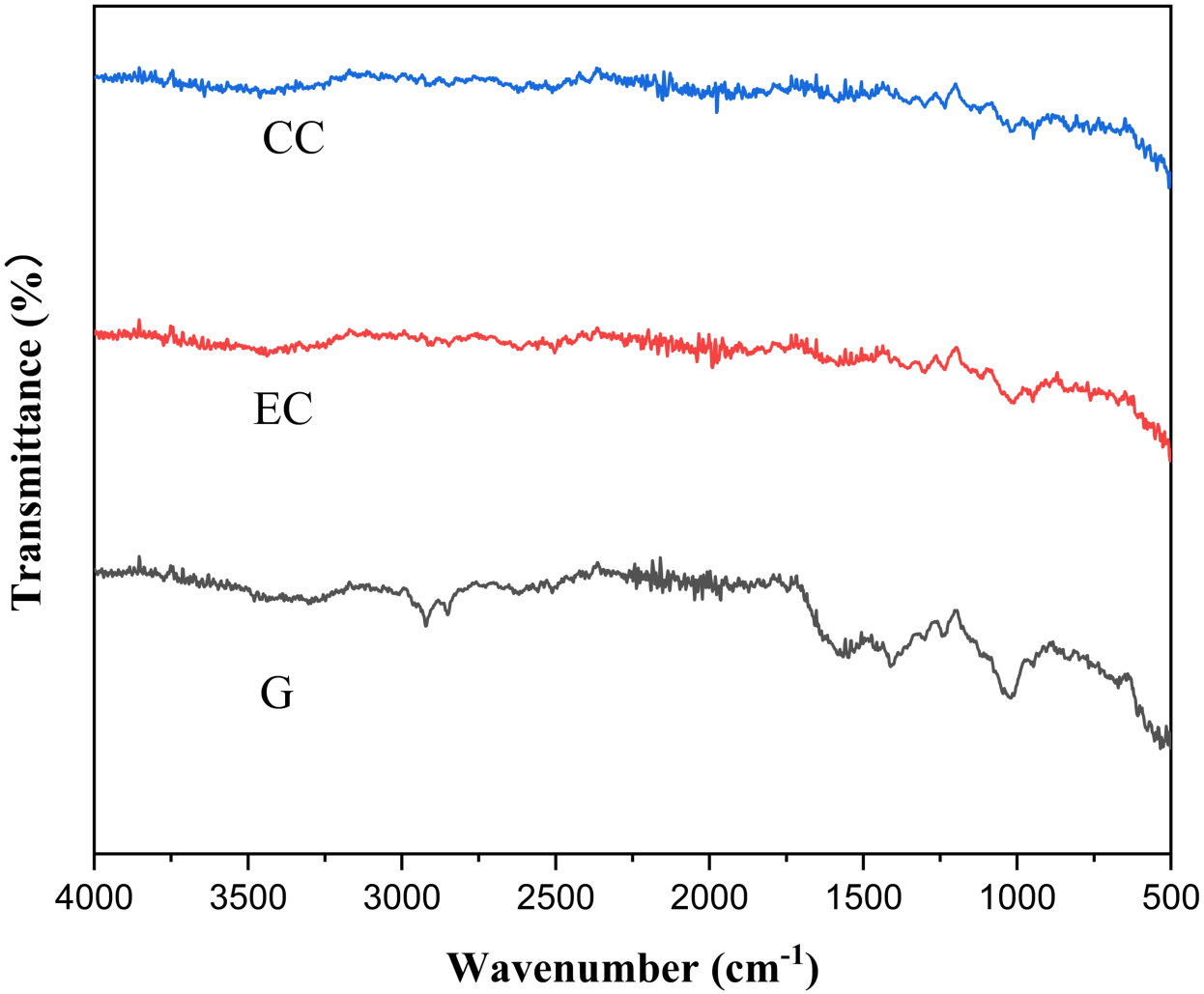


Figure 4.6.

ATR-FTIR spectra of (G) green crab shells, (EC) extracted chitin and (CC) commercial chitin from crab shells.

protein removal (Hamdi et al., 2017; Yang et al., 2019). Furthermore, the absence of the $CaCO_3$ characteristic bands at 829 cm^{-1}, 1402 cm^{-1}, 1747 cm^{-1} in extracted chitin as compared to green crab indicates desirable demineralization process (Gbenebor et al., 2016; Yang et al., 2019).

4.4. Conclusion

In this study, the removal of mineral in green crab under mild conditions was explored to improve the quality of extracted chitin. The demineralization with organic acid was successfully achieved for the reduction of ash content in green crab. Citric acid showed to be the most efficient upon optimization of total residual ash. A concentration 1.0 M of citric acid, at a temperature of 4°C and time of 18h led to 1.38 % total residual ash in green crab. The demineralized green crab was further enzymatically deproteinated to extract chitin. The remaining minerals in chitin were found at relatively lower levels and consisted of mostly sodium, calcium and phosphorous. Furthermore, the characterization studies confirmed $CaCO_3$ was successfully chelated by citric acid, as well as, extracted chitin was chemically similar to commercial crab chitin, indicating a potential source for further application.

CHAPTER V.
GENERAL SUMMARY AND CONCLUSION

The generation of value-added products from underutilized European green crab showed to be an excellent source of lipids rich in MUFAs and LC-PUFAs (i.e. EPA and DHA) and carotenoprotein using enzyme-assisted extraction. Several extraction methods from a variation of crustacean species have been studied; however, the extraction method and biochemical characterization presented, in this study, on green crab is limited in literature. The extracted carotenoprotein from green crab showed comparable amino acid profile to those reported in published studies (Klomklao et al., 2009; Pattanaik et al., 2020; Senphan et al., 2014; Sila et al., 2012). In addition, TLC showed that free and diester astaxanthin were the main carotenoid in extracted carotenoprotein. The antioxidant assay via DPPH radical scavenging activity, hydroxyl radical scavenging activity, and iron reducing activity showed the antioxidant activity of carotenoprotein were concentration dependent (1 – 4 mg/mL). The combination of amino acid, peptides and carotenoids from extracted carotenoprotein were able to donate free electrons to radicles and convert them to stable products. Therefore, extracted carotenoprotein from green crab is a potential alternative ingredient to enhance the nutritive value and provide antioxidant activity in feed and food products. However, further studied need to be conducted to evaluate the nutritional profile and health benefits of carotenoprotein, as an active ingredient in farm fish feed. For instance, the use of extracted carotenoprotein as a replacement ingredient in aquafeed to evaluate its effects on growth performance of farmed fish, pigmentation and composition analysis of fish meat can be further studies. In addition, incorporating carotenoprotein as a functional food ingredient to enrich protein deficient food for human consumption can further be evaluated. Thus, the use of extracted carotenopritein from green crab has an economic value and potential that can be exploited within the aquaculture and food industry.

The optimization of citric acid demineralization to extract chitin from green crab shells was validated for the first time. Green crab shells showed high content in chitin; however, the excessive amount of ash content present suggested that an effective demineralization treatment was required. In literature, the effects of various parameters on the demineralization of crustacean shells have been studied; however, in this study the parameters temperature and concentration of citric acid were significant at reducing the total residual ash in green crab shells. The BBD showed citric acid

demineralization at low temperature and high concentration resulted in low residual ash content in green crab shells. Therefore, under optimized conditions, citric acid was effectively able to chelate minerals in green crab shells to extract chitin. The mineral analysis using ICP-MS showed calcium, sodium and phosphorus salts were the main minerals removed from crab shells. Furthermore, FTIR and XRD analysis of extracted chitin confirmed similar characteristic peaks as commercial crab, indicating a potential source for further application. Additional characterization of extracted chitin is suggested for future studies, such as molecular weight analysis, degree of acetylation and structural analysis using scanning electron microscopy. This can provide information on the quality of chitin following a mild treatment compared to the conventional extraction methods used for commercial chitin. Moreover, generating derivatized form of chitin, such as chitosan and chitooligosaccharides can further be studied for application in the food, biomedical, pharmaceutical and agricultural industry.

Overall, European green crab is an underutilized invasive species that has showed to be an excellent source of nutrients, such as lipids and amino acid, as well as, a rich source of value-added products, including carotenoprotein and chitin. Therefore, exploiting green crab by-products as a means to reduce fishery and crustacean waste creates a new avenue of income within the food industry and innovative source of bioactive nutrients.

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