Extraction of Lipids and Astaxanthin from Northern Atlantic Shrimp By-products: "Green"/Sustainable Extraction Process, Statistical Optimization Study and Mathematical Modeling of Kinetic Extraction

By

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Abstract

The shrimp by-products from shrimp processing are typically disposed of as "waste" into landfills or the oceans, representing an environmental and economic cost. Shrimp processing by-products from harvesting and processing are a source of valuable biomaterials/bioactive materials (such as lipids and astaxanthin) for use in the pharmaceutical, cosmetic, food industries, and biomaterials. Extraction of these compounds would reduce the environmental burden and enhance the industry's finances. However, in order to determine the feasibility, the quality and quantity of lipids/astaxanthin in the by-product as a function of extraction conditions and pre-treatment is required. This information on the quantity/quality of the extract in terms of lipid/FA and astaxanthin (ASX) compositions is required for product application (nutritional, medical, etc.). Traditionally, lipids and ASX are extracted using energy/waste intensive processes, which can degrade the product and/or by-products, are difficult to scale up, and/or operationally complex. However, the development of "green" valorization processes has made recovery of bioactive compounds from shrimp processing by-products feasible.

In this study, we outlined (1) literature review on the advances in the field of value-added lipid/ASX recovery from shrimp and other crustacean processing by-products with a particular focus on SC-CO_2, (2) a comprehensive analysis of the quality and yield of lipid/ASX extract as a function of the extractant solvent used (Soxhlet) and pre-treatment (freeze-drying) of the by-products, (3) the viability of ASX extraction using waste fish/sunflower oils and optimization of waste fish oil extraction of ASX from wet and freeze-dried (FD) shrimp by-products as a function of water content and operating

conditions (time, temperature and oil:waste ratio), (4) optimization of SC-CO_2 extraction of lipid/ASX from FD shrimp by-products as a function of temperature/pressure; study of the impact of static co-solvents adding to SC-CO_2 on lipid/ASX yields, quality and lipid/fatty acids distributions, and (5) mathematical model to predict lipid/ASX extraction rates at certain operating conditions of temperature, pressure and flow rate.

According to Soxhlet extraction results, in general, a mixture of polar/non-polar solvents maximized lipid/ASX yields, and the extract quality can be tuned with a proper solvent mixture to favour lipid yield, ASX yield, or a balance of both depending on the final application. ASX yields varied from 57-88 $\mu g/g_{waste}$ depending on Soxhlet solvent(s) for wet shrimp by-product to 118-218 $\mu g/g_{waste}$ for the freeze-dried. Lipid extracts are rich in omega-3 FAs and the composition of lipid classes varied with solvent(s) used and pre-treatment. Overall, pre-treatment to remove water decreased lipid yield but increased ASX yield/quality.

Edible waste oils are a potentially "green" solvent that could replace organic solvents and act to prevent degradation of the extracted ASX. This study investigates waste fish oil as a solvent for ASX extraction from Atlantic shrimp by-products (*Pandalus borealis*). This study observed that the higher the water content in the residues, the lower the ASX amount in the extract. As extended extraction times or high temperatures the ASX yield decreased; this is due to degradation of ASX with time/temperature. The optimal conditions to maximize the yield of ASX from both wet and freeze-dried shrimp by-products were 65 ˚C, 9:1 v/w and 1.5 h. ASX extractions were 40-60 % lower compared to Soxhlet, however the waste fish oil extracts were higher in triacylglycerols and omega-6 FAs.

SC-CO_2 extracted the highest lipid yield at 50 ˚C and 30 MPa, and the highest ASX yield and total carotenoid content (TCC) at 60 ˚C and 32 MPa. Lipid/ASX recovery increased with an increase in pressure; however, temperature had a complex impact on lipid/ASX yield at a constant pressure. SC-CO_2 extract fractions had high percentages of neutral lipids but low phospholipids. Static co-solvent in SC-CO_2 provided the same lipid yield as and the higher ASX yield than the published papers using continuous co-solvent/SC-CO_2. Polar co-solvent increased lipid/ASX recovery using SC-CO_2. Sunflower oil recovered higher ASX compared to waste fish oil. Polar co-solvents increased phospholipids and saturated fatty acids but decreased monounsaturated FAs and polyunsaturated FAs in the SC-CO_2 extract. Lipid compositions of sunflower oil extract was the same as sunflower oil, but the extract using waste fish oil in SC-CO_2 had higher sterols and free FAs compared to the lipid profile of waste fish oil. The highest lipid yield extracted using SC-CO_2 was 0.5 times of lipids extracted using ethanol in Soxhlet. The highest ASX yield with SC-CO_2 was 0.3 of ASX yield using a mixture of 40:60 vol% hexane/acetone in Soxhlet, and the highest TCC was 0.4 of the highest TCC obtained using Soxhlet (60:40 vol% hexane/isopropanol). The SC-CO_2 kinetic data were in good agreement with the mathematical model (Goto et al. 1993) used for both lipid/ASX extraction rates. The AARD values were 6 % for lipid extraction and 7.8 % for ASX extraction. Overall extraction rates of lipid/ASX were controlled by the strong solid–solute interaction.

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Nomenclature

Abbreviations

ASE	accelerated solvent extraction
AMPLs	acetone mobile polar lipids
ALCs	alcohols
ANOVA	analysis of variance
ASX	astaxanthin
AARD	average absolute relative deviation
BBD	Box–Behnken Design
CER	constant Extraction Rate
DDD	desorption-dissolution-diffusion
DC	diffusion-Controlled
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
EEs	ethyl esters
EKs	ethyl ketones
FER	falling Extraction Rate

FAs fatty acids

FFAs free fatty acids

FD freeze-dried

GEs glycerol ethers

HCs hydrocarbons

HPE high-pressure extraction

MEs methyl esters

ME-SF methyl ester of sunflower oil

MKs methyl ketones

MAE microwave-assisted extraction

MUFA monounsaturated FAs

PC phosphatidylcholine

PE phosphatidylethanolamine

PI phosphatidylinositol

PLs phospholipids

PLE pressurized liquid extraction

PUFA polyunsaturated FAs

SFA saturated FA

STs sterols

SEs steryl esters

SC-CO_2 supercritical CO_2

TLC thin layer chromatography

TCC total carotenoid content

TAGs triacylglycerols

UAE ultrasound-assisted extraction

Symbols

τ residence time

r_p radius of particles

d_p particle diameter

a_p specific surface

C_p solute concentration in pore of particles

C solute concentration in SC-CO_2

k_f external mass transfer coefficient

α void fraction in the bed

C_s solute concentration in the solid

β solid porosity

D_e effective intraparticle diffusion coefficient

K adsorption equilibrium constant

ka	first- order kinetic adsorption
kd	first-order desorption constant
$C_{s,0}$	initial concentration of solute in solid phase
t	time
k_p	overall mass transfer coefficient
m	solute mass at the bed outlet
m_{s0}	initial mass of the solutes in the solid using the Soxhlet
β	particle porosity
α	bed void fraction
T_r	reduced temperature
P_r	reduced pressure
D_{21}	binary coefficient obtained
ρ_1	solvent density
μ_1	solvent viscosity
ρ_r	reduced solvent density
M	solvent/solute molecular weight
V_{c_1}	critical molar volume of CO_2
V_1	moral volume of CO_2

P_{c_1}	critical pressure of CO_2
T	temperature (K)
k	correlation parameter
k_f	mass transfer coefficient
D_e	effective intra-particle diffusion coefficient
u	superficial velocity
N	the number of data point

Chapter 1 Introduction and Overview

1.1. Introduction and review

The Atlantic fishery accounts for about 84 % of the total commercial fish landings in Canada. Crustacean species in the Atlantic region includes mostly lobster, crab, shrimp and scallops [1]. Shrimp harvesting and processing play a key role in Canada's employment, food security, and economic growth [2]. Globally, the value of shrimp exports worldwide was US$19.3 billion in 2017 [3]. In 2016, world shrimp capture production was approximately 6 million metric tonnes [4]. In 2020, shrimp landing was 68,580 metric tonnes in Canada (20 % of total shellfish landings). The value of landed shrimp in Canada was $262,697 and or approximately 13 % of total value of shellfish [5]. While the processed shrimp is a valuable commodity, there is also value in the shrimp processing residues (shells and heads) which accounts for 45 to 60 % of the shrimp's body mass depending on the species and processing method [6–8]. Shrimp by-products are sources of natural astaxanthin and lipids [6,9–12]. Shrimp by-products are also a protein source, providing a range of essential amino acids which have been incorporated into fish and livestock feed [13].

Astaxanthin ($C_{40}H_{52}O_4$), a red-orange carotenoid pigment, is an extremely dominant antioxidant compared to many other carotenoids (10 times) and vitamin E (500 times) [14,15]. An extensive assortment of astaxanthin (ASX)-based bioactivities involves anti-

cancer, immunomodulating, anti-diabetic and anti-inflammatory activities, which lead to increasing its market value [15–25]

Natural and synthetic ASX are available with different value in market, approximately USD $2500–7000/kg for natural ASX and USD $1000/kg for synthetic ASX [26]. The study in 2017 has predicted that the global market for both ASX will increase continuously to USD $2.57 billion in 2025 [27]. Synthetic ASX with much lower antioxidant activity (compared to natural ASX) is not authorized for human consumption [28], but natural ASX has been used as an additive in a variety of market products such as food and beverages, nutritional supplements and biomedicals [29]. The aquaculture industry has been interested in using ASX as a feed additive for pigmentation and nutrient supplementation purposes [29,30]. Algae and yeast are primary natural sources of ASX [31], but aquatic organisms such as marine fishes (salmon, trout and red seabream) and mostly crustaceans (shrimp, lobster, crawfish and crab) have high ASX through the food chain [32].

Lipids from shrimp by-products comprise fat-soluble vitamins, cholesterol, phospholipids (PLs) [33] and free fatty acids (free FAs). Among FA classes, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are found as major n-3 FAs in lipid fraction extracted from shrimp by-products [6,34,35]. DHA and EPA are well-known for their nutritional and therapeutic properties that help to reduce the incidence of depression, cancer, diabetes and issues linked to high blood cholesterol [36–40] Thus, lipids and ASX have health benefits [16,19,40,41].

Shrimp processing discards are made up of ASX (23-148 $\mu g/g_{dry\ waste}$), approximately 5-15 dry wt% lipids [42], 40 dry wt% proteins, 35 dry wt% minerals, and 14-30 dry wt%

chitin. Lipids and ASX contents in the Atlantic shrimp processing by-products (*Pandalus borealis)* vary from 2.3 wet wt% to 8.12 dry wt% lipids and ASX from 148- 284.48 $\mu g/g_{dry\ waste}$ [29,43]. Lipids and ASX levels can vary depending on extraction methods, treatment prior to analysis and shrimp species.

The value of the shrimp industry could potentially be increased by extracting the high-value compounds left in the processing by-products. Recovery of these compounds could also decrease environmental impacts associated with shrimp harvesting and disposal.

Conventional chemical extraction methods (alkali, acid and organic solvents), which have been most commonly used for lipid and ASX recovery from shrimp by-products, are expensive, time-consuming and hazardous although this method provides high lipid/ASX yields [8,18,29,42]. Such energy/waste intensive processes of lipid/ASX extraction are difficult to scale up, and/or operationally complex, and can degrade the product and/or by-products. There have been several studies on the solvent-based process of lipid or ASX extraction from shrimp by-products, but one study of the impact of solvents on the quality of extract (lipid compositions and lipid: ASX ratios) [44]. The lipid and ASX compositions in the extract determine the potential application, from nutritional supplement to fish feed additive, thus it is important to investigate the impact of solvents on lipid and ASX recovery from shrimp processing by-products.

However, the development of "green" valorization processes has made recovery of bioactive compounds from shrimp processing by-products feasible. "Green" solvent alternatives with enhanced selectivity and low environmental impact used for processing shrimp by-products are vegetable oils and supercritical CO_2 (SC-CO_2) [45–48].

Extraction using edible oils can protect thermolabile compounds such as ASX from oxidation/degradation and delay the oxidation time [49,50]. Furthermore, the edible oil extract can supplement aquaculture feed [49]. Extraction of ASX from animal, plant and marine sources have focused on the use of plant-based oils [7,44,49–52].

SC-CO_2 limits the degradation of temperature-sensitive compounds such as lipids and ASX due to the use of CO_2 at supercritical conditions, moderate temperature (above 30 ˚C) and high pressure (over 7.38 MPa) in the extraction process, and provides the high quality of extraction without chemical contamination by toxic solvents [46–48]. SC-CO_2 is a non-polar solvent and can extract non-polar and low/medium molecular weight compounds with slight polarity [53]. However, polar lipids and ASX have relatively low solubility in supercritical CO_2 and therefore must operate at the higher end of the pressure range (>40MPa), or a polar co-solvent must be added [10,42]. Adding polar solvents (e.g., ethanol) to SC-CO_2 increases the density and polarity of SC-CO_2 and consequently increases the target compound solubility. However, this can be costly and possibly contaminate the product(s). Most studies have added ethanol to SC-CO_2 to increase polar compound extraction [10,11,46,54–56]. There are a few studies using sunflower oil (as an alternative co-solvent) to SC-CO_2 in shrimp processing by-product recovery [55,57]and no study on marine oil as a co-solvent. Although there are several studies on SC-CO_2 extraction of lipids/ASX from shrimp by-products [6,10,11,54,55,57–59] there is no work on the impact of operating conditions (temperature, pressure and co-solvents) on lipid compositions (lipid profiles) and distribution of lipids/ASX in the extract. This data is important as the distribution (i.e., quality) has a vital role in decisions around the final application of the extract.

1.2. Motivation and Objectives

According to the literature review, several studies focused on the extraction of lipids and ASX from shrimp by-products using organic solvent-based methods, but there are only two studies mainly focusing on various organic solvent-based processes for the extraction of ASX [60] or lipids/ASX from shrimp by-products [44]. Any preprocessing (i.e., water removal) done on the shrimp by-products prior to extraction will impact both the quantity and quality of the extract. However, there is no comprehensive study on solvent and water removal impact on lipid and ASX yields, the quality of extract (lipid composition and lipid: ASX ratios), lipid/FA compositions. The lipid compositions and lipids/ASX ratio in the extract play a vital role in the determination of the potential application in food/medicine; thus, study on the solvent impact on lipid and ASX recovery from shrimp processing by-products. The Soxhlet process has advantages relative to the solvent process such as benefits of indirectly heating solid phase and no filtration/separation process of solid phase from solvent, but there are only a handful of studies using Soxhlet in lipid and ASX recovery. In this study, Soxhlet performance in lipid/ASX recovery was studied.

There have been studies using vegetable oils, however there is no work on marine oils as a green solvent. While plant-based oils show good performance, often shrimp processing occurs either in the same plant as finfish processing or are in close proximity. This provides an accessible source of "waste" fish oil from fish processing by-products and/or fishmeal production [61]. In this study, for the first time, waste fish oil was used as a solvent and also as a co-solvent in SC-CO_2 for ASX recovery from shrimp by-products.

While SC-CO_2 has advantages over solvent-based extraction processes [42], there are limited studies on lipid/ASX extraction using SC-CO_2 from shrimp by-products [6,10,11,54,55,57–59], and no comprehensive studies that focus on the impact of operating conditions (temperature, pressure and co-solvents) on lipid compositions (lipid profiles) and distribution of lipids/ASX in extract. This data is important as the distribution (i.e., quality) has a vital role in decisions around the final application of the extract.

The main objective of this work is to explore a much more sustainable approach to producing a high quantity and quality lipid/ASX product, that is free of toxic solvent residue. This residue is a concentrate of proteins, trace lipids and other compounds such as chitin and minerals. To address the objective (Fig. 1.1 and 1.2), the feasibility and impacts of using fish waste-derived oil on sustainable processing of shrimp by-products were evaluated in this study. Waste fish oil is much more sustainably extractable from fish processing waste [61] and was used as a solvent alternative to organic solvents/vegetable oils in this study. Waste fish oil was also added as a "green" co-solvent to modify SC-CO_2 extraction for ASX recovery.

In this study first, we focused on the Soxhlet method as a baseline to determine the maximum of extractable lipids and ASX in the Atlantic shrimp by-products (*Pandalus borealis*). In this method, various organic solvent/solvent mixtures with different polarities were applied to establish which methods favor lipids vs ASX vs lipids/ASX. The performance of the Soxhlet process was compared with published solvent processes [60,62]. According to the literature review (Chapter 2), among solvents studied in this area, we chose solvents proposed by other studies with respect to a maximum recovery of lipids and

ASX, and we also established a new solvent mixture (40:60 vol% of hexane/acetone) for high recovery of lipids/ASX from the shrimp by-products. In addition to solvent impact, water removal (using freeze-drying) impact was studied on lipid/ASX yields, quality and lipid/FA distributions from shrimp by-products. Two feedstocks, "wet" and freeze-dried (FD) shrimp by-products, were used in this study.

The second stage of this work was to improve the safety and environmental sustainability of the ASX extraction. Sunflower and waste fish oils were used to extract ASX from shrimp by-products. The edible oil ASX extraction process was first validated using sunflower oil and compared with the literature [7,44]. The protocol was then repeated using waste fish oil. In the end, the performance of waste fish oil extraction process was compared with the ASX yields obtained using the Soxhlet method. To achieve the maximum ASX yield using waste fish oil, further study on the impact of various process conditions (time, temperature and oil:waste ratio) was studied on two various feedstock, wet and FD shrimp by-products using response surface methodology, and lipid distributions were evaluated as a function of the process conditions and water content.

Another green alternative studied in this work was SC-CO_2 extraction**.** SC-CO_2 was optimized to maximize all lipid/ASX yields and extract quality. Extraction conditions (pressure and temperature) were studied to determine significant impacts on lipid/ASX yields using the central composite design (CCD). Furthermore, the impact of co-solvents on lipid/ASX recovery using SC-CO_2 at the "optimum" pressure and temperature from the CCD analysis. In this study, we chose to use a static co-solvent system as outlined in a published study [63] and then compared results with continuous approaches in the literature

to validate the approach. Co-solvents used in this study were ethanol, 40:60 vol% of hexane/acetone, sunflower oil, and waste fish oil. Waste fish oil is of particular interest as it is associated with finfish processing by-products and often represents a disposal cost. Lipid/FA compositions of SC-CO_2 extracts with or without co-solvents were studied, and the temperature/pressure impacts on SC-CO_2 extracts were compared. Furthermore, SC-CO_2 performance was compared to solvent-based extraction processes (Soxhlet, waste fish oil). In the final stage of the study**,** kinetic extraction at the optimal conditions and mathematical modeling of the SC-CO_2 process for lipids/ASX were studied.

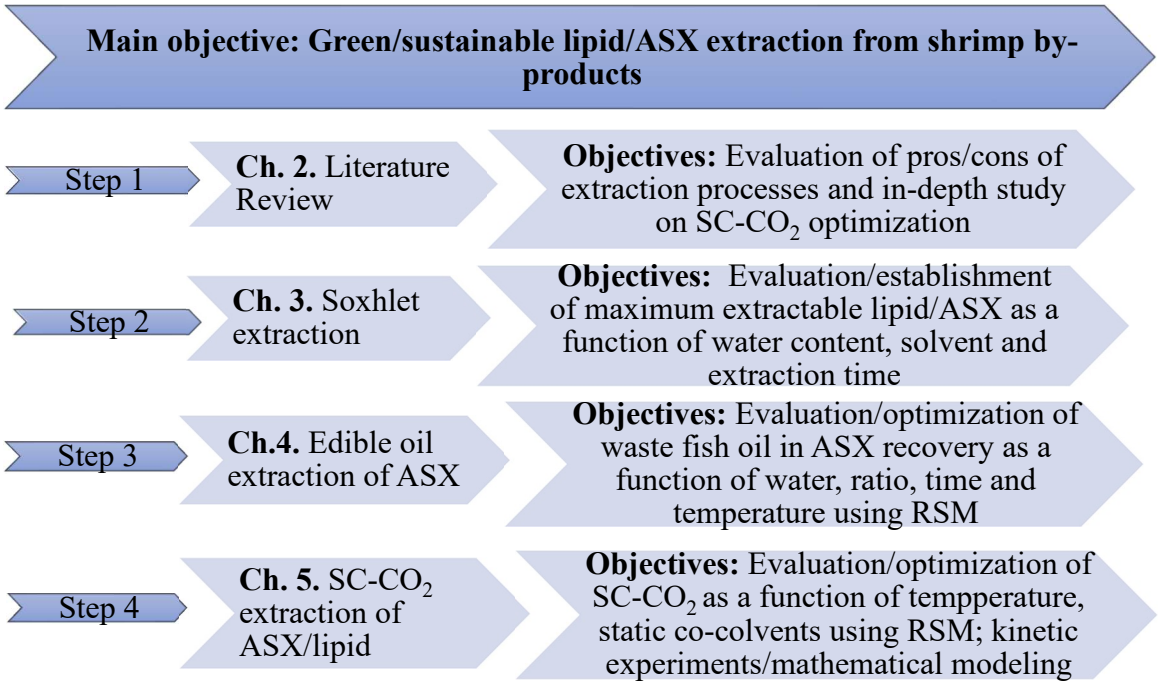

Figure 1.1: Flowchart of experimental design of the research

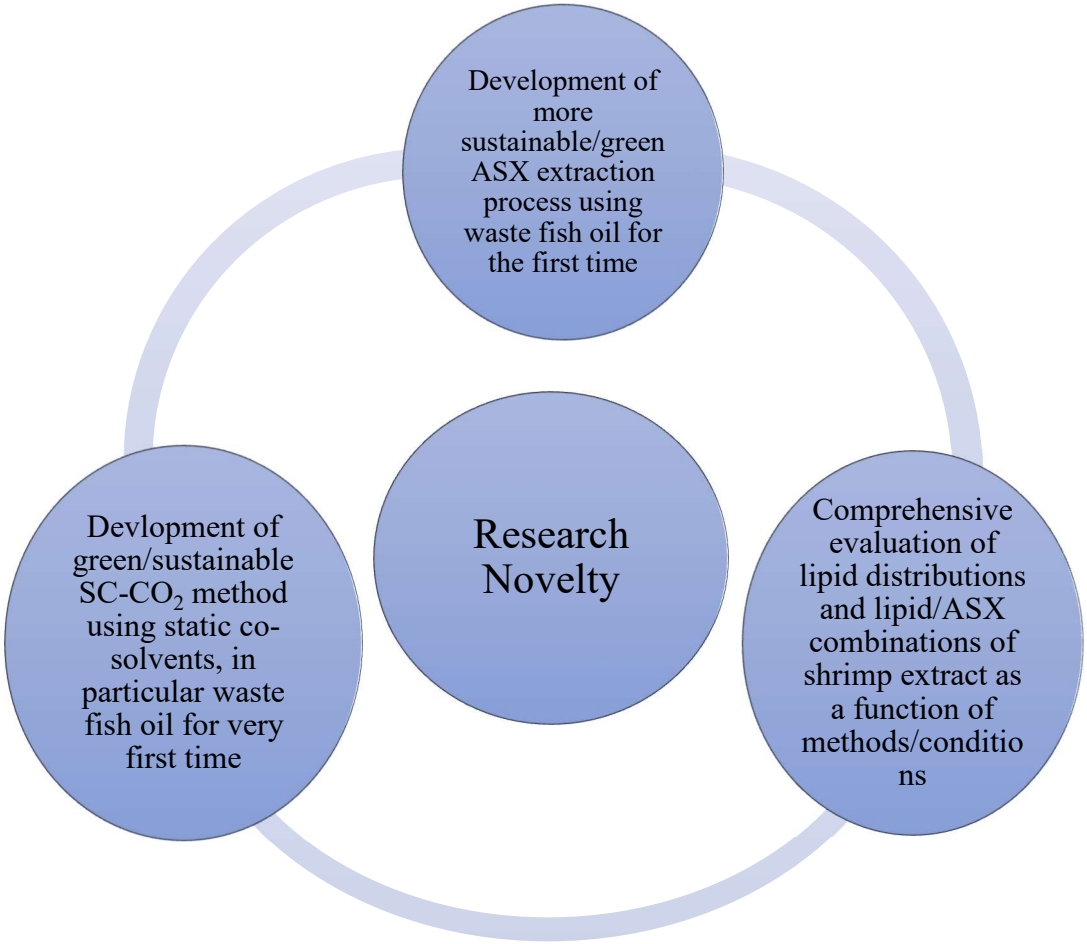

Figure 1.2: Flowchart of research novelty

The following approach is followed to address all objectives of this work:

- The review of literature on the advances in the field of value-added lipid/ASX recovery from shrimp and other crustacean processing by-products with a particular focus on SC-CO_2 is provided in Chapter 2. Data presented in Chapter 2 has been published in the Journal of *Trends in Food Science & Technology,* and the manuscript provides the pros and cons of the various processes are summarized and compared. Studies related to the optimization of supercritical extraction are outlined in greater detail.

- A comprehensive analysis of the quality and yield of lipid/ASX extract from the Atlantic shrimp by-products as a function of the extractant solvent used (Soxhlet), and pre-treatment (freeze-drying) of the by-products is provided in Chapter 3. The details of work in Chapter 3 have been accepted for the publication in *Chemical Engineering Communications.*

- Chapter 4 provides a preliminary study on the viability of ASX extraction using waste fish/sunflower oils and optimization of waste fish oil extraction of ASX from wet and FD shrimp by-products using Box-Behnken Design. Impacts of water and operating conditions such as time, temperature and oil:waste ratio on ASX yield and lipid/FA compositions are included in this Chapter. The details of work in Chapter 4 have been submitted to the *Journal of Cleaner Production*.

- CCD optimization of lipid/ASX extraction from FD shrimp by-products using SC-CO_2 is studied in Chapter 5. The effects of temperature/pressure and of static co-solvents adding to SC-CO_2 on lipid/ASX yields, quality and lipid/FA distributions are also outlined in this chapter. The co-solvents used in this work include ethanol, 40:60 vol% of hexane/acetone, sunflower oil and waste fish oil.

- Determination of kinetic experiment results and mathematical mass transfer model to predict oil extraction as a function of process conditions are also presented in Chapter 5. Details of the work in Chapter 5 has been published in the *Journal of CO_2 Utilization.*

- A summary, conclusions, and recommendations are presented in Chapter 6.

In general, this study addressed the research gaps such as lipid profiles of extracted shrimp oils and lipid/ASX distributions in the extracts as a function of solvent, water content and operating conditions. This work established a more sustainable process of lipid/ASX extraction from shrimp by-products using waste fish oil and developed the SC-CO_2 technique using static co-solvents, including waste fish oil for very first time.

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Chapter 2 Literature Review

Extraction of lipids and astaxanthin from crustacean by-products: A review on supercritical CO_2 extraction

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Abstract

Background

Crustacean by-products are potentially a source of high-value carotenoids and lipids for use in the pharmaceutical, cosmetic, food industries, and biomaterials. Traditionally, lipids and carotenoids are extracted using energy/waste intensive processes, which can degrade the product and/or by-products, are difficult to scale up, and/or operationally complex. The development of "green" valorization processes has made recovery of bioactive compounds from shrimp processing by-products feasible.

Scope and approach

In this review, we outline the advances in the field of value-added lipid and carotenoid recovery from shrimp and other crustacean processing by-products with a particular focus on supercritical CO_2. The pros and cons of the various processes are summarized and compared. Studies related to the optimization of supercritical extraction are outlined in more detail.

Key findings and conclusions

Overall, supercritical extraction using CO_2 and co-solvents has the potential to increase both polar and non-polar lipid/carotenoid recovery without the negative environmental and economic disadvantages associated with traditional extraction methods. There are particular advantages using edible oils as green co-solvent in supercritical CO_2 extraction as an alternative to organic co-solvents. However, there is still significant study required determining the range of ratios of solvents, developing batch extractive processes into continuous processes, balancing operating conditions (e.g., costs) with product purity, and scale-up for larger scale production.

Keywords:
Astaxanthin, Lipids, Shrimp by-products, Extraction, Supercritical CO_2, Green co-solvents/solvents

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2.1. Introduction

Aquaculture and the ocean fishery contribute to Canada's employment, food security, and economic growth [1]. In order to ensure the economic and environmental sustainability of the industry as a whole, innovative solutions are required to extract more value from the resource. These solutions should be "green", focused on minimal energy input and wastes generated [2–4]. The process selection and design and subsequent target product(s) require knowledge of chemical, physical, and thermal properties of the fishery residues.

The Atlantic fishery accounts for about 84 % of the total commercial fish landings in Canada. Crustacean species in the Atlantic region includes mostly lobster, crab, shrimp and scallops [5]. Northern Shrimp (*Pandalus borealis*) have been harvested by the Canadian fishery since the early 1960s [1]. While the processed shrimp is a valuable commodity, there is also value in the shrimp processing residues (shells and heads) which accounts for 45 to 60 % of the shrimp's body mass depending on the species and processing method [6–8]. Fishery by-products (krill, crab, shrimp, fish by-products, etc.) are excellent sources of bioactive proteins, peptides, amino acids, enzymes, oils, fatty acids (FAs) and minerals [2–4]. Shrimp and crab shells are sources of naturally occurring astaxanthin (ASX), a member of the carotenoids' family [9]. The shrimp residue is also a protein source, providing a range of essential amino acids which have been incorporated into fish and livestock feed [10]. Many studies have also reported that high-value carotenoid pigments, primarily ASX, and n-3 FAs such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) can be extracted from shrimp by-products [6, 11-13].

Crustacean shell waste contains between 30-40 wt% proteins, 30-50 wt% minerals (mainly calcium carbonate), and 20-30 wt% chitins [14]. Shrimp by-products are composed of 40 wt% proteins, 35 wt% minerals, and 14-30 wt% chitins and is rich in carotenoids [15]. In Sachindra et al.'s work [7,16,17], carotenoids ranged from 35-153 $\mu g/g_{wet\ waste}$ depending on Indian shrimp by-products [7,16,17]. Other sources include snow crab (71.7 $\mu g/g_{waste}$-119 $\mu g/g_{dry\ waste}$), lobster (98 $\mu g/g_{waste}$), and crawfish (153 $\mu g/g_{wet\ waste}$) by-products [18–21]. EPA is on average 8 wt% of total FAs and 10.7 wt% of total FAs is DHA in Northern Shrimp by-products while snow crab by-products contain 17.1 dry wt% lipids [6,18] and Antarctic Krill contains 20.6-22.1 wt% EPA and 14.9 -16.9 wt % DHA of total FAs [22]. Lipid and ASX levels of various crustacean residues are outlined in Tables 2.1 and 2.2

Table 2.1: Lipid contents in the residues of different species of crustacean.

Shrimp species	Common name	Total lipid yield (%)	Ref.
Pundulus borealis	Pink shrimp, Alaska	1-4[a]	[23]
Pandalus borealis	Pink shrimp, Canada	2.3[a]	[21]
Litopenaeus vannamei	Pacific white shrimp, Mexico	9.96[b]	[24]
Chionoecetes opilio	Snow crab, Canada	17.1[b]	[18]
Farfantepenaeus paulensis	Redspotted shrimp, Brazil	4.9[b]	[11]
Euphausia superba	Krill, Korea	16.12[b]	[25]
Paracoccidioides brasiliensis & *Penaeus paulensis*	Pink shrimp, Brazil	68[b]	[26]
Litopenaeus vannamei	Pacific white shrimp, China	14.65[b]	[27]
Pandalus borealis	Northern shrimp, Denmark	14.4[b]	[28]

[a] *Values on a wet weight basis*
[b] *Values on a dry weight basis*

Table 2.2: Astaxanthin (ASX) contents in the residues of different species of crustacean.

Shrimp species	Common name	ASX Yield ($\mu g/g_{waste}$)	Ref.
Procambarus clarkia	Crawfish	153[a]	[19]
Pandalus borealis	Pink shrimp, Canada	148[b]	[21]
Chinoecetes opilio	Back snow crab, Canada	119.6[b]	[21]
Penaeus monodon	Shrimp, India	145[a]	[16]
Penaeus indicus	Shrimp, India	95.6[a]	[16]
Metapenaeus dobsonii	Shrimp, India	134.6[a]	[16]
Parapenaeopsis stylifera	Shrimp, India	257.8[a]	[16]
Litopenaeus vannamei	Shrimp, Mexico	4.53 mg/mL	[24]
Farfantepenaeus paulensis	Redspotted shrimp, Brazil	53[b], 1.08 $mg/g_{extract}$	[11]
brasiliensis & *Penaeus paulensis*	Pink shrimp, Brazil	1223 $\mu g/g_{extract}$	[11]
Penaeus monodon	Tiger shrimp, Malaysia	86.52[b]	[29]
Pandalus borealis	Northern shrimp, Denmark	23.2[b]	[28]

[a] *Values on a wet weight basis*
[b] *Values on a dry weight basis*

The extraction efficiency of lipids and carotenoids is controlled by; nature of the solid matrix, extraction method, extraction time, operational conditions (e.g., temperature and pressure), and solvent used and ratios of solvent: solid [30]. Solvent-based methods include atmospheric solvent extraction (e.g., Soxhlet extraction), accelerated solvent extraction, and supercritical fluid extraction. Ultrasonic, pulsed electric field assisted extraction and enzymatic processes are also used [8]. Many traditional solvent methods use potentially hazardous organic solvents such as dichloromethane and methanol. Complete extraction

can take several hours; the process poses safety and environmental hazards, and products require further processing to recover the solvent [8,30]. Therefore, the need for green alternatives with enhanced extraction efficiency and low environmental impact is desirable, such as supercritical CO_2 extraction [2].

Supercritical CO_2 extraction can improve yields of carotenoids and lipids from crustacean by-products [31, 32], is non-toxic, inflammable, inexpensive, and operates at relatively moderate temperatures (above 30 °C) and pressure over 7.38 MPa [33]. The low temperatures limit the degradation of temperature-sensitive compounds such as ASX and lipids and limit damage to the function of the extracts from hydrothermal stress. Thus, losses of ASX during extraction can be reduced compared to conventional extraction applied at high temperatures [25, 31, 32]. Once extracted ASX may need to be further purified depending on the application (e.g., to remove lipids). A purification method includes, among others, saponification [34–36].

While traditional solvent methods have been investigated extensively [26,37,38]there is little work comparing and contrasting supercritical CO_2 and these methods and looking at supercritical CO_2 application in the area as a whole. In this review, we will provide an overview of the key concepts and procedures in the extraction of lipids and ASX from crustaceans with a particular focus on shrimp by-products. This review will then focus on supercritical CO_2 extraction. The intent of this paper is to present the first thorough and in-depth review of the work to sustainably extract lipids and ASX from crustacean by-products.

2.2. Lipids

Lipids (oils extracted from biomass) are soluble in organic non-polar solvents [33] and include FAs, derivatives of their related compounds [25,33]. Lipid classes include triacylglycerols and associated compounds (diacylglycerols, monoacylglycerols), hydrocarbons, ketones, alcohols, sterols, wax esters, free FAs, glycerophospholipids, glycerol-glycolipids, cholesterol, ether lipids and sphingolipids [33,39]. Lipids are generally made up of a one subset consisting of a major functional group (ester) including waxes, triglycerides, and phospholipids (PL) and a second subset, such as steroids, FAs, soaps, sphingolipids, and prostaglandins [33].

FAs (carboxylic acids) are composed of long hydrocarbon chains with various degrees of saturation [33]. There are three types of FA in lipids: saturated FA (SFA) with single bonds, monounsaturated FAs (MUFA) with one unsaturated bond, and polyunsaturated FAs (PUFA) with multiple double bonds [33]. FAs are considered nonpolar and can be separated into two types, essential FAs and non-essential FAs based on the ability of the human body to synthesize them. Non-synthesized FAs (or essential FAs) must be supplied externally from the diet and non-essential FAs can be synthesized in the body. The prime lipid-derived compounds, extracted from aquatic sources are n-3 FAs, EPA and DHA [13,40]. Another classification of lipids, based on polarity, includes neutral lipids and polar lipids. Neutral lipids consist of fatty acids, alcohols, glycerides, sterols, triacylglycerols, diacylglycerols, and monoacylglycerols [41].

2.3. Astaxanthin

Astaxanthin (ASX) is a major member of the carotenoids' family (65-98 %) known as xanthophyll. ASX (3,3′-dihydroxy-β, β′-carotene-4,4′-dione) consists of a hydroxy (OH) functional group and one keto (CO) at each end of the polyene chain, which is a fat-soluble pigment ($C_{40}H_{52}O_4$) [8,42].

ASX has both lipophilic and hydrophilic properties [8,43] allowing up to 10x antioxidant activity compared to other carotenoids and up to 500x compared to vitamin E [43]. ASX is not synthesized in animals but transferred to them through the food chain and therefore, is used in animal feed supplementation and as a human nutraceutical [40]. ASX can be found in natural sources in two forms: unesterified (free) and esterified. The free form is unstable and particularly susceptible to degradation and oxidation if exposed to light, heat, oxygen and pH [43]. Hydroxyl groups (OH) of ASX esterify with different FAs such as palmitic, oleic, estearic, or linoleic. Esterified ASX has two forms, monoester (one hydroxyl group (OH)) and di-ester (two OH groups). Carotenoids may also interact to form a chemical complex with proteins (carotenoproteins) or lipoproteins (carotenolipoproteins) [40, 42, 43]. ASX exists mostly within the esterified form in algae (70 wt% in monoester form, 10-15 wt% in diester form and 4-5 wt% in free form). In Antarctic Krill, esterified forms of ASX dominate and Atlantic Salmon contains free ASX [42]. Figure 2.1 represents the relative percentage of ASX and its esters in krill, copepod, shrimp, shell, alga and yeast [42].

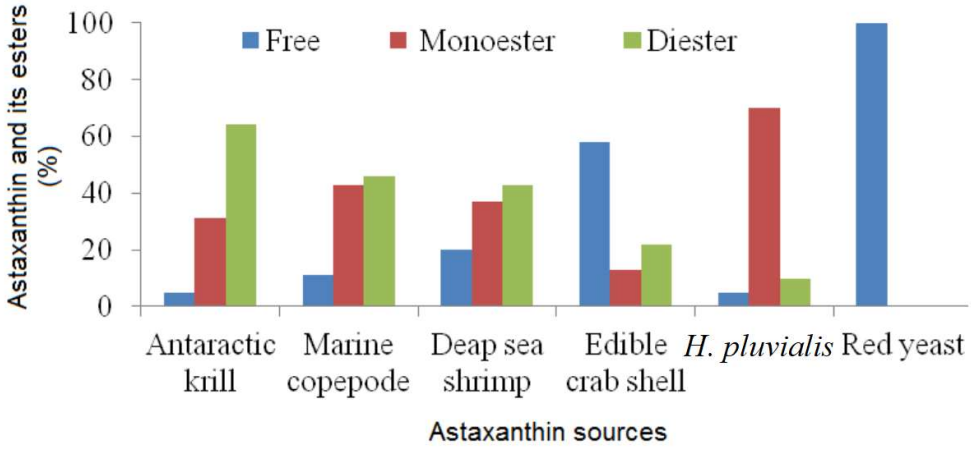

Figure 2.1: Astaxanthin and its esters from various sources [42].

2.4. Extraction methods of lipids and ASX from crustacean by-products

The bulk of studies in extraction from crustacean by-products use conventional chemical extraction methods (alkali, acid and organic solvents). These processes can be expensive, time-consuming and hazardous [44].

2.4.1. Extraction of lipids and ASX using organic solvents

Solvent selection is governed by yield, required purity, the toxicity of the solvent, and the water content and composition of the biomass [8,30]. Organic solvents used in the extraction of lipids and ASX include acetone, n-hexane, isopropanol, methanol, ethanol, petroleum ether, methylene chloride, diethyl ether, chloroform, ethyl acetate, ethyl methyl ketone, benzene and cyclohexane [8,45]. Other solvents that show high yield and selectivity are restricted due to toxicity (dichloromethane, dimethyl sulfoxide and chloroform) [46]. As such, n-hexane and n-heptane are used for carotenoids and lipids extraction at an industrial scale despite the low extraction yields [47]. The most commonly used organic solvents lipid extraction at lab scale are mixtures of chloroform, methanol and water, as in the Bligh and

Dyer procedure. The Bligh and Dyer procedure targets polar lipids from fish tissue and is the standard method in assessment of other solvent extractions with respect to lipid recovery [48]. Carotenoids in by-products can vary in polarity and therefore it is difficult for one solvent which would extract all carotenoids. Non-polar carotenoids (esterified xanthophylls and carotenes) are recovered using non-polar solvents such as hexane, petroleum ether or tetrahydrofuran, while polar carotenoids (xanthophylls such as free ASX) extraction uses polar solvents (acetone, ethanol, and ethyl acetate) [8]. The combination of polar and non-polar solvents has a synergistic effect on the extraction of carotenoids [8,38]. Soxhlet extraction is used to assess the performance of other methods in the recovery of carotenoids [49] although the yield of lipids is lower than that obtained with the Bligh and Dyer method [50].

Sachindra et al.[38] used combinations of organic solvents to optimize carotenoid extraction from shrimp by-products. The shrimp by-products were homogenized with the solvent(s), extract removal, and the process repeated on the residue from the extraction with fresh solvent until the filtrate was colourless. For polar solvents, the phases were separated with petroleum ether, followed by repeated washing extracts with 0.1 % saline solution. The solvent extract phase was then dried through evaporation under vacuum at 40 ˚C. Polar (acetone, methanol, ethyl methyl ketone, isopropanol, ethyl acetate and ethanol), non-polar solvents (petroleum ether and n-hexane), and solvent mixtures of acetone/n-hexane (50:50 vol%) and isopropanol/n-hexane (50:50 vol%) were used. The maximum yield of ASX extracted with isopropanol/n-hexane (50:50 vol%) was 43.9 $\mu g/g_{wet\ waste}$ higher than with pure acetone (40.6 $\mu g/g_{wet\ waste}$) and isopropanol (40.8 $\mu g/g_{wet\ waste}$) [38].

Mezzomo et al. [26] compared extraction methods including ultrasound-assisted, maceration, Chen and Meyers solvent extraction and Soxhlet for total carotenoid extraction [26]. In the Chen and Meyers process, a mixture of petroleum ether, acetone and water (15:75:10 vol%) was added to the sample, and after a 24-hour at 5 °C, the sample was filtered and evaporated under reduced pressure. Maceration is a multi-day cold solvent extraction at room temperature and various solvents (n-hexane, ethanol, acetone, isopropanol, and 50:50 vol% n-hexane/isopropanol were tested in this work. Soxhlet is an atmospheric liquid extraction based on leaching (solid-liquid extraction, with repeated contact of the solid phase with fresh solvent [51] and carried out at the boiling temperature of the solvent [49]. The highest total carotenoid content (198 $\mu g/g_{extract}$) was extracted from freeze-dried, milled, cooked pink shrimp residues using Soxhlet extraction with 50:50 vol% n-hexane/isopropanol. Maceration using acetone gave the second highest total carotenoid content at 188 $\mu g/g_{extract}$ [26].

Brazilian red-spotted shrimp by-products were treated with 60:40 vol% of n-hexane/ isopropanol over 120 min and compared to acetone over 150 min to extract ASX. The ASX yields were 53 $\mu g/g_{dry\ waste}$ for n-hexane/ isopropanol and 34 $\mu g/g_{dry\ waste}$ for acetone. Total carotenoid content (TCC) is defined as the mass of ASX per mass of extract. TCCs were 1.08 $mg/g_{extract}$ with n-hexane/isopropanol and 1.48 $mg/g_{extract}$ with acetone. Lipid extraction was carried out using two different solvent methods; n-hexane at room temperature over a 24-hour period and a modified Bligh and Dyer method adopted from Manirakiza et al. [52] using a mixture of water/isopropanol/ cyclohexane (11:8:10 vol%). The total lipid yield using n-hexane was 33 $mg/g_{dry\ waste}$ and with the modified Bligh and

Dyer method was 49 mg/$g_{dry\ waste}$ [11, 13]**.** Although the modified Bligh and Dyer method gave the high total lipid yield, the use of the toxic solvents limits the application. Based on this work, Brazilian red-spotted shrimp by-products are estimated to contain 5 dry wt% lipids, EPA and DHA at 24 wt% of the total FA content, and 53 µg ASX/$g_{dry\ waste}$ [11,13].

Radzali et al. [29] extracted 86.52 µg/$g_{dry\ waste}$ of ASX from shrimp by-products using acetone-methanol (70:30 vol%) [29]. Dalei and Sahoo [53] extracted astaxanthin from deep-sea shrimp by-products using organic solvents (methanol, ethanol, petroleum ether, chloroform, n-hexane, and acetone). The highest yield of ASX (48.64 µg/g_{waste}) was obtained using acetone [53]. Table 2.3 summarizes the literature on solvent extraction and yield of ASX and lipids from crustacean by-products.

Table 2.3: Summary of various conventional methods using organic solvent for the extraction of lipids and Astaxanthin (ASX) from various crustacean by-product sources.

Crustacean by-product sources	Extraction method	Operational conditions	ASX yield (µg/g_{waste})	Total lipid yield (%)	Ref.
Penaeus indicus (Indian shrimp)	Solvent extraction	Acetone	40.6 [a]	N/A[c]	[38]
Penaeus indicus (Indian shrimp)	Solvent extraction	Petroleum ether	12.1 [a]	N/A	[38]
Penaeus indicus (Indian shrimp)	Solvent extraction	Methanol	29 [a]	N/A	[38]
Penaeus indicus (Indian shrimp)	Solvent extraction	Ethyl methyl ketone	36.8 [a]	N/A	[38]

Crustacean by-product sources	Extraction method	Operational conditions	ASX yield ($\mu g/g_{waste}$)	Total lipid yield (%)	Ref.
Penaeus indicus (Indian shrimp)	Solvent extraction	Ethyl acetate	36.9 [a]	N/A	[38]
Penaeus indicus (Indian shrimp)	Solvent extraction	Ethanol	31.9 [a]	N/A	[38]
Penaeus indicus (Indian shrimp)	Solvent extraction	Acetone/n-hexane (50:50 vol%)	38.5 [a]	N/A	[38]
Penaeus indicus (Indian shrimp)	Solvent extraction	n-hexane	13.1 [a]	N/A	[38]
Penaeus indicus (Indian shrimp)	Solvent extraction	n-hexane/isopropanol (60:40 vol%)	47.86[a]	N/A	[37]
Fermented *Penaeus indicus* (Indian shrimp)	Solvent extraction	n-hexane/isopropanol (60:40 vol%), Fermented over 75 days of storage	32.20-41.85[a]	N/A	[37]
Acid ensiled *Penaeus indicus* (Indian shrimp)	Solvent extraction	n-hexane/isopropanol (60:40 vol%), Acid ensiled over 75 days of storage	43.09-26[a]	N/A	[37]
Acid ensiled *Litopenaeus vannamei* (Mexican shrimp)	Solvent extraction	Petroleum ether/acetone/water (15: 75: 10 vol%) for 3 h, 79 wt% moisture, Acid ensiled	N/A	9.76[b]	[24]

Crustacean by-product sources	Extraction method	Operational conditions	ASX yield ($\mu g/g_{waste}$)	Total lipid yield (%)	Ref.
Litopenaeus vannamei (Mexican shrimp)	Solvent extraction	Petroleum ether/acetone/water (15: 75: 10 vol%) for 3 h, 79 wt% moisture	N/A	9.96[b]	[24]
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp)	Solvent extraction	Petroleum ether/acetone/ water (15:75:10, vol%), at 5 ˚C, 24 h, In natura with 46.3 wt% moisture	N/A	12[b]	[26]
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp)	Solvent extraction	Petroleum ether/acetone/ water (15:75:10, vol%), at 5 ˚C, 24 h, In natura milled	N/A	16[b]	[26]
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp)	Solvent extraction	Petroleum ether/acetone/ water (15:75:10, vol%), at 5 ˚C, 24 h, dried& milled	N/A	18.6[b]	[26]
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp)	Solvent extraction	Petroleum ether/acetone/ water (15:75:10, vol%), at 5 ˚C, 24 h, cooked	N/A	15[b]	[26]
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp)	Solvent extraction	Petroleum ether/acetone/ water (15:75:10, vol%), at 5 ˚C, 24 h, cooked and milled	N/A	13.5[b]	[26]

Crustacean by-product sources	Extraction method	Operational conditions	ASX yield ($\mu g/g_{waste}$)	Total lipid yield (%)	Ref.
Paracoccidioides brasiliensis & Penaeus paulensis (Pink shrimp)	Solvent extraction	Petroleum ether/acetone/ water (15:75:10, vol%), at 5 ˚C, 24 h, cooked, dried and milled	N/A	23.5[b]	[26]
Paracoccidioides brasiliensis & Penaeus paulensis (Pink shrimp)	Soxhlet extraction	n-hexane at boiling point for 8 h, cooked, dried and milled	N/A	19[b]	[26]
Paracoccidioides brasiliensis & Penaeus paulensis (Pink shrimp)	Soxhlet extraction	Isopropanol/ n-hexane (50:50, vol%) at boiling point for 8 h, cooked, dried and milled	N/A	11[b]	[26]
Paracoccidioides brasiliensis & Penaeus paulensis (Pink shrimp)	Soxhlet extraction	Ethanol at boiling point for 8 h, cooked, dried and milled	N/A	68[b]	[26]
Paracoccidioides brasiliensis & Penaeus paulensis (Pink shrimp)	Soxhlet extraction	Acetone at boiling point for 8 h, cooked, dried and milled	N/A	20[b]	[26]
P. brasiliensis and P. paulensis (Pink shrimp)	Soxhlet extraction	Isopropanol at boiling point for 8 h, cooked, dried and milled	N/A	22.5[b]	[26]
Paracoccidioides brasiliensis & Penaeus paulensis (Pink shrimp)	Maceration	n-hexane at room temperature, cooked, dried and milled	N/A	2[b]	[26]

Crustacean by-product sources	Extraction method	Operational conditions	ASX yield ($\mu g/g_{waste}$)	Total lipid yield (%)	Ref.
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp)	Maceration	Ethanol at room temperature for 120 h, cooked, dried and milled	N/A	23.3[b]	[26]
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp)	Maceration	Isopropanol at room temperature for 120 h, cooked, dried and milled	N/A	18[b]	[26]
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp)	Maceration	Isopropanol/n-hexane (50:50, vol%) at room temperature for 120 h, cooked, dried and milled	N/A	9.4[b]	[26]
Pandalus borealis (Northern shrimp)	Solvent extraction	n-hexane /isopropanol (60:40 vol%)	N/A	14.4[b]	[26]
Euphausia superba (Antarctic krill)	Soxhlet extraction	n-hexane at boiling point, freeze-dried (3.4 wt%), 12 h	N/A	16.12	[25]
Farfantepenaeus paulensis (Brazilian redspotted shrimp)	Solvent extraction	60:40 vol% n-hexane/isopropanol	53[b], 1.08 $\mu g/g_{extract}$	5[b]	[11,13]
Penaeus monodon (Tiger shrimp)	Solvent extraction	70:30 vol% acetone/methanol	86.52[b]	N/A	[29]

[a] *Values on a wet weight basis*
[b] *Values on a dry weight basis*
c Not Available

Overall, the range of total lipid yield extracted from various shrimp by-products varied from 1-68 wt% depending on a method and type of residue. The maximum (68 dry wt%) was extracted from pre-treated shrimp by-products (dried, milled and cooked) using Soxhlet with ethanol [26]. As seen in Table 2.3, Sánchez-Camargo et al. [13] proposed a mixture of 60:40 vol% n-hexane and isopropanol to maximize ASX extraction [13]; however, Radzali et al. [29] extracted the highest (86.52 µg ASX/$g_{dry\ waste}$) using 70:30 vol% acetone/methanol [29]. The highest TCC (1080 µg/$g_{extract}$) used 40:60 vol% isopropanol/n-hexane [11].

2.4.2. Oil extraction of ASX

Whole oil (vegetable-based) has been studied as a potential solvent for carotenoid extraction. Chen and Meyers isolated carotenoids from enzymatically hydrolysed crawfish waste by acidifying and heating with soybean oil and ASX extraction using various oils [54]. Effects of extraction conditions, such as oil to waste ratio, time, and temperature on carotenoid extraction from shrimp by-products using different vegetable oils (sunflower, groundnut, gingelly, mustard, soybean, coconut, and rice) showed an oil to waste ratio of 2:1 v/w and a temperature of 70 ˚C over 150 min. Refined sunflower oil extracted the highest carotenoid (26.3 µg/g_{waste}) [7].

Handayani et al. [55] studied the rate and maximum extraction of ASX from 50 g of giant tiger shrimp by-products using palm oil (300 mL). Different temperatures (50-70 ˚C) and particle sizes were studied. ASX has high stability in palm oil at temperatures (30-70 ˚C) due to the tocopherol and phenolics in the oil, which have stabilizing properties. The range

of ASX extracted varied from 48.5-131.74 μg/$g_{dry\ waste}$, with the highest at 70 ˚C and the largest particle size [55].

Pu and Sathivel [56] also studied flaxseed oil to extract ASX from crawfish by-products. There was minimal oxidation of lipids in the flaxseed oil with and without ASX after heating at 30 ˚C. However, the rate of lipid oxidation with ASX was lower than that of flaxseed oil without ASX at higher temperatures (40 to 60 ˚C over 4 h). ASX is an antioxidant, and limits oxidation at temperatures above 30 ˚C. As temperatures approach 50 ˚C and 60 ˚C, ASX begins to degrade, where the oxidation protection effect would become less prominent. Approximately 30.2 μg ASX/$_{crawfish\ waste}$ and 39 μg ASX/$g_{flaxseed\ oil}$ was extracted by using an equal ratio of flaxseed oil to waste at 60 ˚C for 60 min [56].

Sunflower and soybean oil were used to extract pigments through a cold oil (room temperature), and a hot oil extraction (70 ˚C). The carotenoid content recovered was lower with the vegetable oils compared to Soxhlet, maceration, and ultrasound. The TCC and total lipid yield were higher in cold oil compared to hot oil extraction; the stability of the carotenoid in hot oil was lower than in cold oil. TCC was 4.5 μg/$g_{extract}$ and total lipid yield was 32 dry wt% using heated sunflower oil while cold sunflower oil extracted TCC of 6.7 μg/$g_{extract}$ and total lipid yield of 44 dry wt%. Soybean oil was a better solvent than sunflower oil to extract ASX under cold oil extraction due to the thermosensitive unsaturated FAs, such as linolenic acid, present in the soybean oil which increase the interaction between the components in shrimp by-products [26].

Razi Parjikolaei et al. [57] used sunflower oil and methyl ester of sunflower oil (ME-SF) for the extraction of ASX from shrimp by-products and compared to a 60:40 vol% n-

hexane/isopropanol extraction. Temperatures from 25-70 ˚C, solvent to waste ratios of 3-9, waste particle sizes of 0.6 and 2.5 mm, moisture of 86.8 wt%, and stirrer speeds of 120-400 rpm were investigated. The highest ASX yields for both solvents were obtained at 70 ˚C, a solvent to waste ratio of 9:1 v/w, a stirrer speed of 400 rpm, 86.8 wt% moisture, 0.6 mm waste particle size and 3 h extraction time. ASX extracted by ME-SF and sunflower oil was between 60-80 % of solvent extraction efficiency. The highest ASX yields were 41.1 $mg/kg_{wet\ waste}$ for n-hexane/isopropanol (60:40 vol%), 34.2 $mg/kg_{wet\ waste}$ for ME-SF, and 23 $mg/kg_{wet\ waste}$ for sunflower oil [57].

Silva et al. [58] investigated the effects of temperature and moisture (8.70-10.85 wt%) on ASX extraction with palm olein from shrimp by-products. The shrimp drying temperature was varied from 70 to 90 °C to determine the impact on the quality and yield of ASX. The maximum level of ASX yield (31.3 $\mu g/g_{dry\ waste}$) was reported from shrimp waste dried to 8.7 wt% moisture (drying temperature of 70 °C) and extraction temperature of 70 °C. Increasing extraction temperatures from 50 to 70 °C increased ASX yield but increasing drying temperature to 90 °C lowered the ASX yield. Increasing drying temperature from 70 to 90 °C had a negligible effect on the total lipid yield, but the protein in the extract increased [58]. Table 2.4 summarizes the literature on edible oil extraction and ASX yields.

Table 2.4: Summary of edible oil extraction using various vegetable oil for the extraction of astaxanthin (ASX) from various crustacean by-product sources.

Crustacean by-product sources	Operational conditions	ASX yield ($\mu g/g_{waste}$), TCC ($\mu g/g_{extract}$)	Total lipid yield (%)	Ref.
Penaeus indicus (Indian shrimp)	Refined sunflower oil at 70 °C and ratio of 2 v/w for 120 min	26.3[a]	N/A[c]	[7]
Penaeus indicus (Indian shrimp)	Mustard oil at 70 °C and ratio of 2 v/w for 120 min	16.1[a]	N/A	[7]
Penaeus indicus (Indian shrimp)	Groundnut oil at 70 °C and ratio of 2 v/w for 120 min	23.1[a]	N/A	[7]
Penaeus indicus (Indian shrimp)	Gingelly oil at 70 °C and ratio of 2 v/w over 120 min	23.9[a]	N/A	[7]
Penaeus indicus (Indian shrimp)	Soybean oil at 70 °C and ratio of 2 v/w over 120 min	24.8[a]	N/A	[7]
Penaeus indicus (Indian shrimp)	Coconut oil at 70 °C and ratio of 2 v/w over 120 min	24.7[a]	N/A	[7]
Penaeus indicus (Indian shrimp)	Rice bran oil at 70 °C and ratio of 2 v/w over 120 min	24.3[a]	N/A	[7]
Penaeus indicus (Indian shrimp)	Refined sunflower oil at 70°C, 150 min	34.05[a]	N/A	[37]
Penaeus indicus (Indian shrimp)	Refined sunflower oil at 70°C, fermented over 75 days of storage	22.6-31.03[a]	N/A	[37]
Penaeus indicus (Indian shrimp)	Refined sunflower oil at 70°C, acid ensiled over 75 days of storage	19.03-26.18[a]	N/A	[37]
Panaeus monodon (giant tiger shrimp)	Palm oil at 70°C, freeze-dried, 40/60 mesh	48.541[b]	N/A	[55]

Crustacean by-product sources	Operational conditions	ASX yield ($\mu g/g_{waste}$), TCC ($\mu g/g_{extract}$)	Total lipid yield (%)	Ref.
Panaeus monodon (giant tiger shrimp)	Palm oil at 70°C, freeze-dried, 60/80 mesh	83.441[b]	N/A	[55]
Panaeus monodon (giant tiger shrimp)	Palm oil at 70°C, freeze-dried, 80/100 mesh	131.743[b]	N/A	[55]
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp)	Sunflower oil at 70 °C and ratio of 4 v/w over 120 min, cooked, dried and milled	4.5 $\mu g/g_{extract}$	32×10^{-4} [b]	[26]
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp))	Soybean oil at 70 °C and ratio of 4 v/w over 120 min, cooked, dried and milled	3.87 $\mu g/g_{extract}$	25.15×10^{-4} [b]	[26]
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp))	Sunflower oil at 70 °C and ratio of 4 v/w over 120 min, cooked, dried and milled	5.18 $\mu g/g_{extract}$	36.4×10^{-4} [b]	[26]
Paracoccidioides brasiliensis & *Penaeus paulensis* (Pink shrimp))	Soybean oil at 70 °C and ratio of 4 v/w over 120 min, cooked, dried and milled	6.7 $\mu g/g_{extract}$	44×10^{-4} [b]	[26]
Procambarus clarkia (crawfish)	Flaxseed oil at 60 °C, 60 min	30.2[a]	N/A	[56]
Pandalus borealis (Northern shrimp)	Sunflower oil at 70 °C and ratio of 9 v/w over 180 min,	23[a]	N/A	[57]
Farfantepenaeus subtilis (Pink shrimp)	Palm olein at 70 °C and ratio of 36 v/w over 180 min, at 70 °C drying (dehydration, 10.85 wt% moisture)	31.308[a]	N/A	[58]

Crustacean by-product sources	Operational conditions	ASX yield ($\mu g/g_{waste}$), TCC ($\mu g/g_{extract}$)	Total lipid yield (%)	Ref.
Farfantepenaeus subtilis (Pink shrimp)	Palm olein at 70 °C and ratio of 36 v/w over 180 min, at 80 °C drying (dehydration, 9.51 wt% moisture)	28.672[a]	N/A	[58]
Farfantepenaeus subtilis (Pink shrimp)	Palm olein at 70 °C and ratio of 36 v/w over 180 min, at 90 °C drying (dehydration, 8.7 wt% moisture)	22.035[a]	N/A	[58]

[a] *Values on a wet weight basis*
[b] *Values on a dry weight basis*
c Not Available

The highest ASX yields were obtained at 70 ˚C and showed low degradation of products. Oil-based solvents are proposed to serve as a "barrier" against oxidation, reducing the degradation rate of ASX extract. Thus, the use of edible oils for ASX extraction increases the stability of carotenoids, despite the low carotenoid content) [55]. The selectivity of carotenoid extraction is further enhanced when vegetable oils are used compared to traditional extractions; these oils carrying antioxidants, ASX can be used in food formulations to produce dietary supplements [19,21].

2.4.3. Biochemical methods of ASX extraction

Chemical processes which use strong acids and bases for recovery of chitin and proteins from crustacean by-products [59] can impact the properties of other value-added biomolecules such as lipids and proteins and associated carotenoids [60].

Enzymatic processes use enzymes to hydrolysis proteins and minimize undesirable products during the deproteinization of crustacean by-products. Various enzymes have been used to isolate protein from the chitin-protein-mineral complex, such as papain, trypsin, pepsin, and alcalase [15,61–64]. Biochemical methods can be used to extract carotenoproteins, ASX fractions associated with protein [45]. Klomklao et al. [65] investigated the trypsin in the extraction of carotenoproteins from shrimp by-products with an ASX yield of 87.91 μg/g_{waste} using pure trypsin [65]. Extraction of ASX with alcalase, a more efficient enzyme compared to pancreatin recovered 47 μg ASX/$g_{dry\ waste}$, in addition to 100 mg protein hydrolysate/mg protein in waste [66]. Simultaneous extraction of carotenoids with proteins has been proposed to improve the stability of carotenoids in storage [67]. However, Franco-Zavaleta et al. [24] showed over 17 days, ASX in sunflower oil showed better stability compared to ASX in the protein solutions. An egg albumin protein water solution was used as a protein solution [24].

Microbial fermentation has been used to separate protein from the chitin-protein-mineral complex in shrimp by-products [60,68,69]. Proteolytic enzymes, generated from microorganisms during fermentation, separate chitin (solid fraction) and proteins (liquor); the liquor consists of protein hydrolysates, peptides, free amino acids, pigments, phenolics, and antioxidant compounds [70,71]. Table 2.5 summarizes the biochemical approaches to ASX recovery. In addition to oil extraction and biochemical approaches (green alternatives), pressurized processes such as supercritical CO_2 extraction have been proposed to extract carotenoids and lipids from crustacean by-products [45].

Table 2.5: Summary of biotechnological extraction for the recovery of Astaxanthin (ASX) from various crustacean by-product sources.

Crustacean by-product sources	Extraction method	Enzymes	ASX Yield (µg/g waste)	Ref.
Metapenaeus monoceros (Brown shrimp)	Enzymatic hydrolysis	Trypsin	55 %	[62]
Xiphopenaeus kroyeri	Enzymatic hydrolysis	Alcalase and pancreatin	47[a] and 57[a] $\mu g/g_{waste}$	[66]
Penaeus indicus (shrimp)	Fermentation	Lactobacillus plantarum B 4496	31.3 $\mu g/g_{waste}$	[37]
Litopenaeus vannamei, stylirostris and setiferus	Fermentation	Lactobacillus plantarum	115 $\mu g/g_{waste}$	[72]
*Jasus lalandii (*rock lobster)	Enzymatic hydrolysis	Papain	54 $\mu g/g_{waste}$	[73]
Penaeus monodon (giant tiger shrimp)	Enzymatic extraction	Trypsin	87.91 µg/g	[65]
Penaeus monodon (giant tiger shrimp)	Fermentation	Natural probiotic	72.6 %	[69]
Litopenaeus vannamei (shrimp)	Fermentation	Lactobacillus plantarum	2400 $\mu g/g_{waste}$	[74]
Penaeus monodon (giant tiger shrimp)	Autolysis	Endogenous enzymes	63.4 %	[75]
Parapenaeus longirostris	Enzymatic hydrolysis	Barbel and bovine trypsins	80 $\mu g/g_{waste}$	[76]
Litopenaeus vannamei (shrimp)	Autolysis	Endogenous enzymes	826 $\mu g/g_{waste}$	[10]
Penaeus indicus (Indian shrimp)	Enzymatic hydrolysis	Alcalase	82.5 %	[77]

[a] *Values on a dry weight basis (d.w.b)*

2.4.4. Ultrasound, high pressure, and microwave processes

Extraction processes of bioactive compounds include ultrasound-assisted extraction (UAE), high-pressure extraction (HPE), and microwave-assisted extraction (MAE). UAE and MAE have been used in the recovery of carotenoids [78–80] and lipids [81] from plant and animal sources.

2.4.4.1. Ultrasound-assisted extraction

UAE is performed using ultrasound in a liquid medium (solvent) where sound collapses the cell wall of the matrix enhancing mass transfer of target compounds [82]. Operational variables include ultrasonic power, intensity, temperature and a sample to solvent ratio [8,83]. UAE was compared to MAE on shrimp heads. In UAE a 10:1 v/w acetone: waste ratio was used and treated 5 min at 600 W and extracted carotenoids at 234 $\mu g/g_{dry\ waste}$. The yield of carotenoid using MAE with n-hexane/acetone/ethanol, 2:1:1 vol% for 7 min at 30 W was 67.3 $mg/g_{dry\ waste}$ [84]. Studies have suggested that using green co-solvents, such as ionic liquids in UAE, improves the selectivity of bioactive compound extraction and yields [85,86].

Bi et al. [72] extracted ASX from shrimp by-products using UAE with ionic liquids. The ASX yield for 60 min, at room temperature, 75 W, and a solid/solvent ratio (1:40 g/mL) was 92.7 $\mu g/g_{dry\ waste}$, double the value of UAE with ethanol at 46.7 $\mu g/g_{dry\ waste}$ [72]. Deep eutectic solvents, a mixture of choline and other components with functional groups such as carboxylic acids, urea, or polyols were used in UAE to extract ASX from shrimp by-products. ASX yields were 218 $\mu g/g_{dry\ head}$ and 146 $\mu g/g_{dry\ shell}$ in 30 min, at a sample/solvent ratio of 1:15 g/mL and 85 W [85].

Hu et al. [9] investigated three pre-treatment methods (drying in a ventilated dark space; drying in the sun; and drying in the sun after boiling the raw materials) on ASX yield from shrimp and crab shells using UAE with ethanol. The waste-solvent ratio was 1:7 w/v, extraction time of 20 min, and 50 ˚C. The highest yield of ASX was obtained from cooked and sun-dried shells (239.96 $\mu g/g_{dry\ waste}$). Shrimp shells without any pre-treatment were approximately ¼ of this yield; sun-dried and cooked shrimp shells, ventilated-dried shrimp shells and heads had fewer ASX contents (2.69-5.81 $\mu g/g_{dry\ waste}$) [9].

2.4.4.2. High-pressure extraction

HPE or accelerated solvent extraction (ASE), or pressurized liquid extraction (PLE), is used in the food processing industry [87]. The high pressure enhances the penetration of the solvents and improves intermolecular physical interactions and shortens extraction time and the elevated temperature improves diffusion of the solvent into the sample by reducing the viscosity of the solvents [88,89].

Quan et al. [89] investigated the effects of solvents and temperature, pressure, and time on ASX extraction from shrimp by-products in PLE. Pressure played a negligible role in ASX yield, but temperature and extraction time had significant effects. ASX yield of 24 $mg/kg_{wet\ waste}$ was obtained using ethanol as a solvent at 87 ˚C, 4.9 MPa and 14 min [89]. HPE of ASX from shrimp by-products using acetone, dichloromethane, and ethanol at different pressures (0.1-600 MPa) and extraction times (0-20 min) with varying solvent to solid ratios (10-50 mL/g) were studied by Li et al. [90]. When compared to all solvents used, ethanol showed the highest recovery of ASX with a solvent to solid ratio of 20 mL/g over 0.1-600 MPa (71.1 $\mu g/g_{dry\ waste}$ at 200 MPa for 5min). When compared to atmospheric

solvent methods (42.3 μg/$g_{dry\ waste}$) HPE showed a higher yield of ASX (72 μg/$g_{dry\ waste}$) at 400 MPa, with a solvent to solid ratio of 30 mL/g with better antioxidant activity at shorter extraction times (5 min) [90]. Six species of Malaysian shrimp carapaces were treated with 7:3 vol% acetone/methanol in HPE at 210 MPa for 10 min. The *Penaeus monodon* species had the highest TCC of 68.26 μg/mL and yielded the highest ASX of 59.97 μg/$g_{dry\ waste}$. At atmospheric conditions, a 7:3 vol% ratio of acetone and methanol extracted 46.95 of TCC μg/mL and 29.44 μg/$g_{dry\ waste}$ of ASX [88].

2.4.4.3. Microwave-assisted extraction

Like HPE, MAE requires less solvent and shorter extraction time compared to conventional solvent methods [91]. The sample-solvent mixture is heated via irradiation, reducing the thermal degradation of lipids and carotenoids [8]. However, it is still possible that thermal degradation might affect the lipids and the cis-trans isomerization of carotenoids to some degree [8,33]. MAE performed better in carotenoid extraction compared to conventional processes [33]. MAE performance is controlled by microwave power, solid to solvent ratio, and intermittency ratio (α fraction of the radiation time to the total processing time in one cycle) [8]. The MAE of β-carotene and carotenoids from carrot waste was carried out at 180 and 300 W, 75 and 150 mL of solvent volume, and a low intermittency ratio (α = 1/4) [91]. All-E-lycopene (red carotenoid) of tomato peels was extracted using ethyl acetate in MAE at a solid to solvent ratio of 1:20 w/v and 400 W for 60 s [92]. Sardine fish by-products were treated with MAE to extract lipids with a 3:2 vol% n-hexane/isopropanol ratio, compared to distilled water at 800 W. Distilled water showed a higher yield of lipids (80.5 mg/g_{wet}

$_{waste}$) at 10 min [93]. Again, a detailed table summarizes the extraction type and yields are in Table 2.6.

Table 2.6: Summary of other extraction methods for the recovery of astaxanthin (ASX) from various shrimp by-product sources.

Shrimp by-product sources	Extraction method	Operational conditions	ASX Yield	Ref.
Shrimp by-products	HPE	Ethanol containing 0.1 vol% of acetic acid	24 µg/g_{waste}	[89]
Shrimp by-products	UAE	Ionic liquids at room temperature and 75 W for 60 min	92.7 µg/$g_{dry\ waste}$	[85]
Shrimp by-products	UAE	Ethanol at room temperature and 75 W for 60 min	46.7 µg/$g_{dry\ waste}$	[85]
Shrimp by-products	UAE	Deep eutectic solvent at a frequency of 20 kHz and output power of 200 W.	218 µg/g_{head} ,146 µg/g_{shell}	[86]
Shrimp by-products	UAE	Ethanol at a frequency of 20 kHz and an output power of 200 W.	102 µg/g_{head} ,158 µg/g_{shell}	[86]
Aristeus antennatus (red shrimps)	UAE	10:1 v/w acetone/waste at 5 min, 600 W	234[a] µg/g_{waste}	[84]
Aristeus antennatus (red shrimps)	MAE	n-hexane/acetone/ethanol (2:1:1 vol%) at 7 min, 30 W	67.3[a] µg/g_{waste}	[84]
Penaeus Vannamei Boone	HPE	Acetone, dichloromethane and ethanol, 0.1-600 MPa and holding times (0-20 min), solvent/solid (10-50 mL/g)	Highest yield using ethanol with solid ratio of 20 mL/g at 200 MPa for 5min	[90]

Penaeus monodon	HPE	Acetone /methanol (7:3 vol%) at 210 MPa over 10 min	59.97[a] µg/g	[88]
Pandalus borealis	UAE	Ethanol at a frequency of 40 kHz over 20 min	50.32 µg/g	[9]
Procambarus clarkia	UAE	Ethanol at a frequency of 40 kHz over 20 min	239.96 µg/g	[9]

[a] *Values on a dry weight basis (d.w.b)*

2.4.5. Supercritical CO_2 extraction of lipids and ASX

Supercritical fluids have gas-like viscosities and diffusivities, and liquid-like densities and if selected carefully can easily be separated from the product by dropping pressure. As supercritical CO_2 (SC-CO_2) is commonly used for the recovery of high purity thermolabile compounds, such as carotenoids and lipids [94]. The supercritical conditions of CO_2 are at relatively moderate temperatures (above 30 °C) and pressures > 7.38 MPa. SC-CO_2 has a high solvent power and can extract non-polar, low molecular weight slightly polar compounds, and has an affinity to medium molecular weight compounds with high oxygen content [95]. Polar modifiers (co-solvent), temperature, and pressure have been studied for extraction of polar and non-polar carotenoids, and lipids from crustacean by-products [11,96–98].

Brazilian freeze-dried pink shrimp by-products were treated with SC-CO_2 to extract ASX, associated esters, and lipids [13]. The maximum lipid yield was 2.26 dry wt% at 50 °C and 30 MPa and the highest ASX yield was 20.7 µg/$g_{dry\ waste}$ at 43 °C and 37 MPa [13]**.** Sánchez-Camargo et al. [11] used 10 wt% ethanol as a co-solvent with CO_2 to extract lipids and ASX from freeze-dried red-spotted shrimp by-products at 50 °C and 30 MPa and obtained 30.8 µg/$g_{dry\ waste}$ of ASX and 2.9 dry wt% of lipids [11].

In a study of krill by-products, 12.2 dry wt% lipid yield was obtained using SC-CO_2 at 25 MPa, 45 ˚C and 150 min. The residues after extraction contained a higher amount of protein, ash, and non-protein components when compared to the raw krill by-products. These residues were treated with subcritical water hydrolysis to recover amino acids and higher amino acids yields were obtained compared to subcritical water hydrolysis of raw krill by-products [25]. PLs were extracted from Antarctic Krill, using SC-CO_2 extraction followed by solvent extraction using ethanol, n-hexane and acetone. In the first step, neutral lipids were extracted at 45 ˚C, 25 MPa, and CO_2 flow rate of 22 g/min over 150 min. The residues after extraction were treated with organic solvents over a 12-hour period to extract phospholipids. The phospholipid yield using ethanol was 42.7 dry wt%; this was higher than when compared to raw krill treated with the same solvent (37.4 dry wt%). PLs include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI). Approximately 80.4 % of the PLs fraction was PC and EPA dominated the PE fraction [96]. Crawfish tail shells were treated with SC-CO_2 to recover ASX over a range of temperatures, pressures, and moisture content. 10 wt% of ethanol was used as a co-solvent and the average ASX extracted was 177.5 $\mu g/g_{dry\ weight}$ [97]. Table 2.7 summarizes literature in SC-CO_2 extraction of ASX and lipids from crustacean by-products.

Table 2.7: Summary of SC-CO_2 extraction for the recovery of astaxanthin (ASX) and lipids from various crustacean by-product sources.

Crustacean by-product sources	Operational conditions	ASX yield ($\mu g/g_{waste}$)	Total lipid yield (%)	Ref.
Procambarus clarkia (Louisiana crawfish)	CO_2 + 10 wt% ethanol,60 ˚C, 224 bar, 25 wt% moisture	177.58[a]	N/A[b]	[97]
Euphausia superba (Antarctic krill)	CO_2, 45 ˚C, 250 bar, 150 min, freeze-dried, 3.40 wt% moisture	N/A	12.2	[25]
Farfantepenaeus paulensis (Brazilian redspotted shrimp)	CO_2, 43˚C, 370 bar, 200 min, ground, freeze-dried	20.7[a]	1.93[a]	[13]
Farfantepenaeus paulensis (Brazilian redspotted shrimp)	CO_2, 50 ˚C, 300 bar, 200 min, ground, freeze-dried	19.4[a]	2.26[a]	[11]
Farfantepenaeus paulensis (Brazilian redspotted shrimp)	CO_2+5 wt% ethanol, 50 ˚C, 300 bar, 100 min, ground, freeze-dried	26[a]	1.96[a]	[12]
Farfantepenaeus paulensis (Brazilian redspotted shrimp)	CO_2+10 wt% ethanol, 50 ˚C, 300 bar, 100 min, ground, freeze-dried	30.8[b]	2.9[b]	[12]
Farfantepenaeus paulensis (Brazilian redspotted shrimp)	CO_2+15 wt% ethanol, 50 ˚C, 300 bar, 100 min, ground, freeze-dried	35[a]	4.67[a]	[12]
Euphausia superba (Antarctic krill)	CO_2, 4 ˚C, 600 bar, 150 min, freeze-dried	N/A	12.34	[98]
Pandalus borealis Kreyer (Northern shrimp)	CO_2, 50 ˚C 150 bar, 20 min, ground, freeze-dried	11000[a]	0.68[a]	[6]

Crustacean by-product sources	Operational conditions	ASX yield ($\mu g/g_{waste}$)	Total lipid yield (%)	Ref.
Pandalus borealis Kreyer	CO_2, 40 ˚C, 350 bar, 90 min, ground, freeze-dried	137000[a]	10.89[a]	[6]
Paracoccidioides brasiliensis & Penaeus paulensis (Pink shrimp)	CO_2, 50 ˚C, 300 bar, 180 min, cooked, dried and milled	N/A	3[a]	[99]
Paracoccidioides brasiliensis & Penaeus paulensis (Pink shrimp)	CO_2 +2 wt% n-n-hexane/isopropanol, 50 ˚C, 300 bar, 180 min, cooked, dried and milled	N/A	4.2[a]	[99]
Paracoccidioides brasiliensis & Penaeus paulensis (Pink shrimp)	CO_2 +5 wt% n-hexane/isopropanol, 50 ˚C, 300 bar, 180 min, cooked, dried and milled	N/A	4.3[a]	[99]
Paracoccidioides brasiliensis & Penaeus paulensis (Pink shrimp)	CO_2 + 2 wt% sunflower oil, 50 ˚C, 300 bar, 180 min, cooked, dried and milled	N/A	0.004[a]	[99]
Paracoccidioides brasiliensis & Penaeus paulensis (Pink shrimp)	CO_2 + 5 wt% sunflower oil, 50 ˚C, 300 bar, 180 min, cooked, dried and milled	N/A	0.005[a]	[99]
Penaeus monodon (Tiger shrimp)	CO_2 + pure methanol, 60 ˚C, 200 bar, 120 min, freeze-dried, ground	82.51[a]	N/A	[29]
Penaeus monodon (Tiger shrimp)	CO_2 + pure ethanol, 60˚C, 200 bar, 120 min, freeze-dried, ground	84.51[a]	N/A	[29]

Crustacean by-product sources	Operational conditions	ASX yield ($\mu g/g_{waste}$)	Total lipid yield (%)	Ref.
Penaeus monodon (Tiger shrimp)	CO_2 + water, 60 ˚C, 200 bar, 120 min, freeze-dried, ground	5[a]	N/A	[29]
Penaeus monodon (Tiger shrimp)	CO_2 +50 vol% methanol, 60 ˚C, 200 bar, 120 min, freeze-dried, ground	17.49[a]	N/A	[29]
Penaeus monodon (Tiger shrimp)	CO_2 +50 vol% ethanol, 60˚C, 200 bar, 120 min, freeze-dried, ground	15.53[a]	N/A	[29]
Penaeus monodon (Tiger shrimp)	CO_2 +70 vol% methanol, 60 ˚C, 200 bar, 120 min, freeze-dried, ground	30.74[a]	N/A	[29]
Penaeus monodon (Tiger shrimp)	CO_2 +70 vol% ethanol, 60 ˚C, 200 bar, 120 min, freeze-dried, ground	51.79[a]	N/A	[29]
Pandalus borealis (Northern shrimp)	CO_2, 55 ˚C, 400 bar, 180 min, dried ground	23[a]	N/A	[28]
Pandalus borealis (Northern shrimp)	CO_2 +5 wt% ethanol, 55 ˚C, 400 bar, 180 min, dried ground	50.8[a]	3.2[a]	[28]
Pandalus borealis (Northern shrimp)	CO_2 +5 wt% sunflower oil, 55˚C, 400 bar, 180 min, dried ground	25[a]	4[a]	[28]
Pandalus borealis (Northern shrimp)	CO_2 + 5 wt% methyl ester of sunflower oil, 55 ˚C, 400 bar, 180 min, dried ground	38[a]	N/A	[28]

Crustacean by-product sources	Operational conditions	ASX yield ($\mu g/g_{waste}$)	Total lipid yield (%)	Ref.
Pandalus borealis (Northern shrimp)	CO_2+15 vol% ethanol, 57 ˚C, 216 bar, 120 min, freeze-dried	82.51[a]	N/A	[100]

[a] *Values on a dry weight basis*

[b] Not Available

2.5. Design of SC-CO_2 processes in extraction of lipids/ASX from crustacean by-products

As demonstrated above, the extraction efficiency of lipids and carotenoids depends not only on the matrix, but also type of extraction method, extraction time, operational conditions (temperature and pressure), and types of solvent and ratios of solvent: solid [30]. The extraction steps include the solubilization of compounds in the solid matrix, the solubilization in the solvent, and the internal and external diffusion of the solubilized compounds. Thus, either the solubilization or the diffusion step or combination controls performance [101]. The solvent power (solvent density) of SC-CO_2 can be customized by manipulating the density of CO_2 [33,101]. Increasing the pressure substantially increases the solvent power and lower volatility (higher molecular weight) and/or more polar compounds can be extracted [33]. Polar compound extraction can be increased by adding polar modifiers or co-solvents to SC-CO_2 [95]. In addition to the supercritical fluid density, solute vapour pressure affects the solute solubility. Increases in temperatures enhance the solute vapour pressure but decrease the supercritical fluid density [101,102].

Fundamental knowledge of thermodynamic data (solubility and selectivity) and overall extraction curves, representing kinetic data, is essential to define parameters for process

design, such as equipment dimensions, solvent flow rate and particle size. Supercritical extraction curves are generally represented as a graph of accumulated extracted mass *versus* time of extraction [99,101]. Extraction curves are divided into three distinct sections : (1) Constant Extraction Rate (CER) period, where convection is the dominant mass transfer resistance; (2) Falling Extraction Rate (FER) period, where diffusion and convection control mass transfer; and (3) Diffusion-Controlled (DC) period, where the mass transfer is controlled by internal diffusion into the solid particles [94,103,104].

The solubility of solutes has an essential role in determining the extraction rate [13,27]. Fuente et al. [105] measured the solubility of ASX in SC-CO_2 as a function of temperature (40-60 ˚C) and pressure (10-42 MPa). The solubility of ASX ranged from 1.1×10^{-8} (molar fraction) at 40 ˚C and 10 MPa to 1.2×10^{-6} (molar fraction) at 60 ˚C and 39 MPa. The solubility of ASX increased with pressures at a constant temperature, and with an increase in temperatures between 10-42 MPa [105]. Youn et al. [32] measured the solubility of ASX in CO_2 from 30-60 ˚C and 8-30 MPa. As temperature and pressure increased, the solubility of ASX increased from 0.42×10^{-5}-4.89×10^{-5} (molar fraction). The solubility of ASX increased with an increase in pressure at a constant temperature as the fluid density increased, but from 30-60 ˚C and 8-30 MPa increases in temperature had a larger impact on solubility compared to pressure changes. Although both temperature and pressure are effective in increasing the density and solubility, an increase in pressure rather than in temperature is preferred for higher solubility of ASX in SC-CO_2 due to the thermal degradation of ASX at high temperature [32].

Sánchez-Camargo et al. [13] studied the SC-CO_2 extraction of ASX and lipids from Brazilian freeze-dried pink shrimp by-products. Extraction curves were obtained at pressures from 20-40 Pa at 50 ˚C, and at temperatures from 40-60 ˚C at a pressure of 40 MPa. At 50 ˚C, the solubility increased with an increase in pressure to 30 MPa, at 30 MPa (cross-over pressure) the solubility did not change with an increase in temperature**.** At pressures below 30 MPa, the solubility of the lipids decreased with an increase in temperature but above the cross-over pressure, solubility increased with an increase in temperature. Solubility was constant at the cross-over pressure regardless of temperature [13].

Yang et al. [27] observed the same behaviours in the solubility of lipids from Pacific white shrimp head waste**.** Increasing the pressure from 20 to 40 MPa at 45 °C increased the solubility. At 30 MPa and 45 °C, an increase in the flow rate of CO_2 from 0.5 to 1.5 L/min increased the solubility due to a higher convective mass transfer coefficient. However, at very high flow rates (>1.5 L/min), the reduction in contact time outweighed the enhanced mass transfer [27].

Mezzomo et al. [99] performed experiments at 20 MPa and 60 ˚C for SC-CO_2 extraction of lipids and ASX from pink shrimp by-products. At a moisture content of 46.30 wt%, the CER time was reduced from 67 min to 31 min as the flow rate of the CO_2 was increased from 8.3 g/min to 13.3 g/min. The CER and FER periods decreased again due to the increase in convection mass transfer coefficient. The CER period which is mostly controlled by convection as such the decrease in water to 11.21 wt% from 46.3 wt%, lowered the CER time [99].

2.5.1. Effects of temperature and pressure on lipid/ASX yields

Sánchez-Camargo et al. [13] did not observe an impact of pressure over a range of 20-40 MPa or temperature from 40-60 ˚C on total lipid yield (from Brazilian freeze-dried pink shrimp by-products)**.** However, ASX yields decreased at the lower pressures (20-25 MPa) as temperature increased, while at higher pressures (30-40 MPa), an increase in temperature marginally increased ASX [13]**.**

A neutral lipids-rich extract was recovered from krill *(Euphausia superba)* residues using SC-CO_2 from 35-45 ˚C and 15-25 MPa at an extraction time of 2.5 h. Increasing either pressure or temperature increased the total lipid yield and the amount of oil per mass of CO_2. The total lipid yields increased from 4-6 dry wt% (at 35-45 ˚C and 15 MPa) to 10-11.5 dry wt% (at 35-45 ˚C and 25 MPa) [96]. Yang et al. [27] evaluated the effects of pressures (20-50 MPa) and temperatures (35-50°C) at 1.5 L/min of CO_2 flow rate on the yield of lipids extracted from Pacific white shrimp head waste. Increasing the pressure from 30 to 40 MPa, at 45 °C, resulted in higher yields of lipids compared to total lipid yields obtained at both 20 MPa and 50 MPa [27].

The effects of temperature (40-60 °C) and pressure (10-30 MPa) on total lipid yield were evaluated at 13.3 g CO_2/min for pink shrimp by-products (11.21 wt% moisture). Increasing pressure at a constant temperature increased the total lipid yield. A temperature increase from 40 to 60 °C, at constant pressure (10 MPa), lowered the total lipid yield from 1.27 to 0.50 dry wt%. However, at pressures >20 MPa, the total lipid yield increased relative to lower pressures at the same temperature. This study proposed that the cross-over pressure for the extract/CO_2 system was from 18 to 20 MPa. The temperature had little effect on

TCC from 10 to 20 MPa, but an increase in temperature at 30 MPa increased TCC. The yield increased to 1223 µg of ASX/$g_{extract}$ at 30 MPa and 60 °C, compared to 3.48 µg of ASX/$g_{extract}$ at 10 MPa and 40 °C. The effects of temperature under and above cross-over pressure on TCC had the same trend on total lipid yield [99].

Razi Parjikolaei et al. [28] evaluated the effects of pressure (20-40 MPa) and temperature (35-55 ˚C) on ASX extracted from Northern Shrimp. At 55 ˚C, the ASX yield increased from 3.5 to 23.2 mg/$kg_{dry\ waste}$ as pressure increased from 20 to 40 MPa. At 20 MPa, increasing temperature negatively affected the ASXA yield while at 40 MPa, ASX yield increased with increasing temperature. The highest ASX yield was 23 mg/$kg_{dry\ waste}$ at 40 MPa and 55 °C [28]. Extraction of ASX from Tiger Shrimp using SC-CO_2 with 15 vol % ethanol from 40 to 80°C, 15-25 MPa, and extraction flow rate of 1 to 3 mL/min was studied by Radzali et al. [100]. ASX yield and free ASX concentration increased from 40 to 60 °C for 15-25 MPa. At 80 °C, the ASX yield decreased due to the degradation of the extract. Increasing pressure from 15 to 20 MPa increased the ASX yield. Since the density and solvent power of CO_2 increases with an increase in pressures, the solubility of astaxanthin in CO_2 increases [100].

Increasing pressure increases yields of lipids and ASX due to increases in solvent density and the solubility of the carotenoid-enriched extract. An increase in temperature increases the solute vapour pressure but decreases the solvent density; below the cross-over pressure, the lipid and ASX yields decrease due to the dominant effect of the solvent density over the solute vapour pressure. Above the cross-over pressure, the enhanced solute vapour pressure has a larger effect on the solubility with an increase in temperatures compared to

the reduced solvent density; therefore, increasing temperature increases the lipid and ASX yields.

2.5.2. Effects of co-solvents on lipid and ASX yields

Ethanol was varied from 5 to 20 wt% to extract ASX from crayfish by-products using SC-CO_2. At 15 wt% ethanol, the maximum yield of ASX was extracted at 60 ˚C and 20 MPa over 15 min [31]. Sánchez-Camargo et al. [11] have shown that 10 wt% ethanol in SC-CO_2 increased the ASX yield by 60.8 % compared to extraction without ethanol. The study proposed ethanol enabled the formation of hydrogen bridges and the release of pigments due to the expansion of the pores of the matrix [11]. Ethanol as a co-solvent in the extraction of ASX and n-3 FAs (EPA + DHA) from red-spotted shrimp by-products were studied at 50 ˚C and 30 MPa (based on the maximum yield using pure SC-CO_2) [13]. Increasing the amount of ethanol from 5 to 15 wt% increased lipid recoveries from 39.7 to 93.8 %, higher than the total lipid yields in SC-CO_2 without co-solvents (47 %) (all recoveries compared to the modified Bligh and Dyer methodology) [12].

The polar nature of the CO_2/ethanol mixture increased the extraction of polar lipids (PLs and glycolipids). Total lipid yields increased from 1.96 dry wt% with 5 wt% ethanol to 4.63 dry wt% with 15 wt% ethanol. The yield of ASX rose (26.0 to 34.8 $\mu g/g_{dry\ waste}$) with an increase in ethanol (5-15 wt%). Compared to supercritical extraction with only CO_2 the ASX yield increased by 74.6 % with 15 wt% ethanol. TCC was a maximum (1325 $\mu g/g_{extract}$) in 5 wt% ethanol and decreased with an increase in ethanol. This study proposed the reduction in TCC was due to an increase in total lipid yields with more polar compounds [12].

Pacific white shrimp head waste was treated with SC-CO_2/ethanol to separate lipids. As ethanol increased from 4 to 8 wt%, there was an increase in total lipid yield (30 MPa, 45 °C and 1 L/min), but at 12 wt% ethanol, the total lipid yield decreased [27]. Radzali et al. [28] studied the effects of various co-solvents on the performance of SC-CO_2 at 60 ˚C and 29 MPa for recovery of ASX and other carotenoids from shrimp by-products. Co-solvents included ethanol, water, methanol 50 vol% ethanol, 50 vol% methanol, 70 vol% ethanol, and 70 vol% methanol. The highest yield of carotenoid (84.02 $\mu g/g_{dry\ waste}$) was obtained using ethanol, with 58.03 $\mu g/g_{dry\ waste}$ of free and esterified astaxanthin. As astaxanthin is less polar than water, ethanol and methanol performed better than water [29]. 5 wt% of ethanol-SC-CO_2 increased ASX yield from 23.2 to 50.8 $\mu g/g_{dry\ waste}$ at 55 °C and 40 MPa, but the total lipid yield did not considerably increase [28].

Other co-solvents with promising results in extraction research are vegetable oils. These edible oils act as a barrier to oxygen which delays the oxidation time and reduces degradation of the ASX in the extract. ASX is also soluble in vegetable oils [56,57]. Furthermore, the extract (including lipids and carotenoids) obtained from oil extractions can supplement aquaculture feed [56].

Mezzomo et al. [99] studied sunflower oil as a co-solvent in SC-CO_2 at 60 °C and 30 MPa to enhance the selectivity of the ASX extraction from shrimp by-products. Organic solvents (2 and 5 wt% of n-hexane/isopropanol, 50:50 vol%) as co-solvents were also added to increase the solubility of the solute in CO_2 at the same conditions. Without co-solvents, the total lipid yield was 3 dry wt%, at 2 wt% n-hexane/isopropanol (50:50 vol%) the total lipid yield increased to 4.2 dry wt%, at 5 wt% co-solvent there was no appreciable increase in

lipid yield (4.3 dry wt%). TCC decreased with the addition of the co-solvents (from 1223 to 24 $\mu g/g_{extract}$). The lipid and carotenoid yields with sunflower oil as a co-solvent were lower compared to other organic co-solvents. The selectivity of the sunflower oil was evaluated by calculating the ratio of TCC to total yield. The ratios were 1920 for 2 wt% of sunflower and 5.5 for 2 wt% of n-hexane/isopropanol (50:50 vol%) at 30 MPa/60 °C. This demonstrates the carotenoid can be more selectively extracted using sunflower oil as co-solvent, although a limited amount of lipids was extracted [99].

Razi Parjikolaei et al. [57] used sunflower oil and its methyl ester as co-solvents, at pressures of 20-40 MPa, temperatures of 35-55 ˚C and a flow rate of 20 g/ min for 180 min on the extraction of ASX from shrimp by-products. At 5 wt% of sunflower oil, 25.4 mg ASX/$kg_{dry\ waste}$ and at 5 wt% of its methyl ester, 38 mg ASX/$kg_{dry\ waste}$, were extracted at 55 °C and 40 MPa. When compared to 5 wt% of ethanol (51 mg/$kg_{dry\ waste}$), the yield with sunflower oil was lower under the same conditions [57].

2.5.3. Moisture content and flow rate on lipid/ASX extraction

Charest and colleagues [97] studied ASX extraction from crawfish shells at 10 wt% ethanol in SC-CO_2. As moisture content increased, more ASX was extracted. Other variables such as temperature, pressure, cooking by steam, and particle size did not impact the extraction, but the temperature did have an effect on ASX yield when water was present [97]. The effect of the flow rate (1-3 mL/min) on the yield of ASX from crayfish waste was studied in the ethanol/SC-CO_2 system between 20-35 MPa and 40-60 ˚C. The flow rate which gave high yields (60 ˚C and 20 MPa over 15 min) was 2 mL/min [65]. The highest yield of lipids was obtained at 20 MPa, 60 ˚C and 13.3 g/min with moisture at the lower range (11.2 wt%).

Higher water content results in lower solubilization of lipids in the solvent and the total lipid yield decreases [99].

Yang et al. [27] varied the CO_2 flow rate from 0.5 to 1.5 L/min and observed the total lipid yield increased (at 30 MPa and 45 °C). A further increase in flow rate (>1.5 L/min) decreased the total lipid yield [27]. ASX from Tiger Shrimp, using SC-CO_2 with 15 vol% ethanol was a maximum at a flow rate of 1.89 mL/min. As the flow rate increased to 3 mL/min, shorter contact time lowered the ASX yield. The maximum yield of carotenoid was 58.50 $\mu g/g_{dry\ waste}$ with free ASX yield, 12.20 $\mu g/g_{dry\ waste}$ at 21.57 MPa, 56.88 °C and 1.89 ml/min over 120 min extraction time [100]. High CO_2 flow rate results in high mass transfer coefficients but lowers contact time between solvent and solute; thus, the higher flow rate lowers the yields.

2.6. Operational condition effects of SC-CO_2 extraction on carotenoid and FAs profiles

Proteins in esterified ASX enhance the polarity, while ASX esterified with FAs has lower polarity. Esterified ASX with lower polarity has a high solubility in CO_2 at low pressures. SC-CO_2 performs well when extracting esterified ASX, which is found mostly in shrimp extracts. Mezzomo et al. [99] demonstrated that the dominant carotenoids were free and esterified ASX. Increasing pressure increased esterified ASX concentration at constant temperatures [99].

Sánchez-Camargo et al. [13] produced an extract where 40 wt% was SFAs at 40 ˚C and 30 MPa but further increases from 37 to 40 MPa decreased SFAs. PUFAs rose from 18 to 30 wt% of total FAs as pressure increased to 37 MPa (at 57 ˚C). Increases in temperature

(43-57 ˚C) at 37 MPa increased the PUFA amounts further. Similarly, n-3 FAs, EPA and DHA, increased with pressure and temperature where a maximum of 10 wt% (of total lipid) EPA + DHA was extracted by SC-CO_2 at 30 MPa and 50 ˚C [13]. In another study, unsaturated FAs yield increased to 64.69 wt% of total FAs at 45 °C as pressure was increased to 30 MPa for Pacific white shrimp head by-products using an ethanol-SC-CO_2 mixture. Increasing pressure over 30 MPa decreased unsaturated FAs. Increasing temperature from 35 to 45 °C increased the unsaturated FAs which then decreased 45 to 50 °C. Conversely, SFAs decreased with increasing temperatures from 35 to 45 °C, but marginally increased >45 °C [27].

Treyvaud Amiguet et al. [6] extracted oils from Northern shrimp by-products at 15 MPa and 50 ˚C, and 35 MPa and 40 ˚C. The total lipid yields increased with extraction time and at the lower pressure, the yield was 11 mg/$g_{dry\ waste}$ which included 620 mg FAs/g_{oil}. At 35 MPa, the extraction time increased from 20 to 90 min, and the total lipid yield increased to 137 mg/$g_{dry\ waste}$ with 795 mg FAs/g_{oil} including EPA, 78 mg/g_{oil} and DHA, 79.7 mg/g_{oil} [6].

Sánchez-Camargo et al. [12] studied the effect of ethanol/SC-CO_2 system on FA compositions extracted from freeze-dried red-spotted shrimp by-products. Adding ethanol decreased the SFAs and increased the unsaturated FAs at 30 MPa and 50 ˚C compared to pure CO_2. As ethanol increased, the MUFA was constant, but the n-3 FAs increased. The levels of EPA increased from 5.91 to 11.48 wt% of total FAs and of DHA increased from 4.29 to 12.24 wt% of total FAs when ethanol increased from 5-15 wt% [12].

2.7. Comparison of supercritical CO_2 extraction and solvent extraction

Conventional extractions, using organic solvents are widely used but have a number of disadvantages including operational costs, handling and management of toxic solvent, solvent recovery costs, and product quality. For example, in Soxhlet extraction, samples must be heated to the boiling point which may impact the quality of the lipids and carotenoids recovered [8,33]. SC-CO_2 extraction is characterized by mild extraction temperatures and low energy requirements for solvent recovery. However, this process is not suitable for samples containing large amounts of water and polar compounds [8]. Despite these obstacles, there are many clear advantages such as high diffusivity of supercritical fluids (10^{-4} cm^2/s) compared to that of the liquid solvents (10^{-5} cm^2/s), ability to tune solvent power by altering temperature and/or pressure to obtain carotenoids and lipids with high purity, separation of dissolved solutes from CO_2 by decreasing pressure, low temperatures for recovery of heat-sensitive materials, no need for hazardous solvents or only a few millilitres of organic solvents in supercritical fluid extraction, and scalability [8,33].

When compared to various solvent methods, lipid recovery from shrimp by-products using SC-CO_2 at different operating conditions is from 22-93.8 % of lipid recovery by solvent methods. When co-solvents are added, the SC-CO_2 exceeds solvent recovery by 50 %. ASX recoveries by SC-CO_2 are 36-97.1 % relative to traditional solvent processes [11–13,26,28,29,99]. Compared to more methods such as n-hexane extraction at 50 ˚C and 30 MPa, the lipid recovery of only SC-CO_2 was 64 % [12].

2.8. Conclusions

Crustacean by-products have been recognized as a source of nutraceuticals such as lipids and ASX [8,40,43,45]. The emergence of innovative valorization processes has made recovery of these products from fish processing by-products much more sustainable. A number of researchers have targeted carotenoid and lipid extraction from shrimp by-products in particular.

Several crucial properties affect the efficiency of lipid and carotenoid extraction. These include the moisture content of the feedstock, the nature of feedstock, the lipids and carotenoid compositions, pressure, temperature as well as the solvents used. An appropriate solvent must be chosen to ensure effective optimal ASX and lipid extraction. In the absence of precise recommendations for a specific solvent or solvent mixture, a combination of polar and non-polar solvents is often employed. These combinations enable simultaneous extraction of both polar and non-polar carotenoids and lipids. Conventional processes give high extraction yields, but ASX and lipids extracted with organic solvents may have barriers to market in cosmetic and pharmaceutical products because they have been exposed to toxic solvents during the processing phase [13,106].

SC-CO_2 extraction can be regarded as a more environmentally friendly, low-temperature method for isolating bioactive compounds. SC-CO_2 used in thermo-labile compounds such as ASX and lipid extraction not only eliminates disadvantages of other processes but also offers a promising alternative for the food processing and pharmaceutical industry due to not lack of toxic solvents [33]. To enhance the extraction yield of carotenoids, various co-solvents are used in the extraction process. The most common co-solvent for SC-CO_2

extraction is ethanol, which increases the yields of lipids and carotenoids. Higher amounts of both non-polar and polar substances are extracted when polar co-solvents are used in supercritical extraction; this increases the polarity of CO_2. However, the addition of ethanol also makes solvent SC-CO_2 extraction far less selective.

In response to these challenges, edible oils have been introduced as a potential alternative to organic solvents. These oils have the potential to be a much safer option for ASX extraction. These solvents have been shown to increase yields of ASX from shrimp by-products. Edible oil-based solvents act as a barrier against oxygen, retard the oxidation time and degradation rates of ASX extract, and increase the stability of carotenoids. Improvements in this process, using oils as green co-solvent in SC-CO_2 extraction, is still required to achieve a higher recovery of polar carotenoids and lipids; this green technology can be viewed as a promising approach for the food processing and pharmaceutical industries. However, developing new strategies in supercritical fluid extraction, using cost-effective green co-solvents to augment the production of ASX and lipids from natural sources for large scale industrial applications, is still in its infancy.

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Chapter 3 Soxhlet extraction of lipids/astaxanthin

Evaluation of Conventional Solvent Processes for Lipid and Astaxanthin Extraction from Shrimp Processing By-products

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Abstract

Shrimp by-products from processing are typically disposed of as "waste" into landfills or the oceans, representing an environmental and economic cost. However, shrimp processing by-products are a source of valuable biomaterials/bioactives such as lipids and astaxanthin (ASX). As such, extracting lipids/ASX would reduce the environmental burden and enhance the industry's finances. However, in order to determine the feasibility, the quality and quantity of lipids/ASX in the by-products as a function of extraction conditions and pre-treatment is required. This work provides, for the first time, a comprehensive analysis of the quality and yields of lipid/ASX extract from Atlantic shrimp by-products as a function of the extractant solvent and pre-treatment (drying) of the by-products. This

information on the quantity/quality of the extract in terms of lipids/FAs and ASX compositions is required for product application (nutritional, medical, etc.). In general, a mixture of polar/non-polar solvents maximized lipid/ASX yields. The extract quality can be tuned with a proper solvent mixture to favour lipid yield, ASX yield, or a balance of both depending on the final application. ASX yields varied from 57-88 $\mu g/g_{waste}$ depending on Soxhlet solvent(s) for wet shrimp by-products to 118-218 $\mu g/g_{waste}$ for the freeze-dried. Lipid extracts are rich in omega-3 fatty acids and the composition of lipid classes varied with solvent(s) used and pre-treatment. Overall, pre-treatment to remove water decreased lipid yield but increased ASX yield/quality.

Keywords: Astaxanthin, Lipids, Shrimp by-products, Soxhlet extraction.

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- writing the paper
- performing all the laboratory testing and analyses (except where noted)
- conducting all data processing and interpretation of results
- performing all literature searches required for background information
- writing the paper and performed all literature searches required for background information

Dr. Hawboldt and Dr. Cheema provided technical guidance and editing of the manuscript.

3.1. Introduction

The Canadian fishery industry plays a key role in employment, food security, and economic growth [1]. Approximately 84 % of the total commercial fish landings in Canada occur in the Atlantic region. Shrimp is one of the major crustacean species in this region [2]. In 2020, the total shrimp landing in the Atlantic region was 66,106 tonnes which was valued at $237,028 (approximately 13 % of the total shellfish landed value) [3]. Approximately between 45-60 wt% of the body mass of shrimp are discarded as "waste" during harvesting and processing [4–13]. This translates to approximately 163,000-220,000 tonnes/year in 2019 in Canada [1]. Typically, this "waste" has gone to landfill sites or into the sea resulting in an environmental burden to the ocean and land [14]. The disposal as a "waste" is also a loss of profit as the by-product is a source of valuable bioactive materials and biomaterials. Shrimp processing discards are made up of approximately 35–50 wt% proteins, 10-35 wt% minerals, and 15-30 wt% chitin [15–17]. This represents that approximately 16,336-108,907 tonnes/year of "lost" value-added biomaterials/bioactive are wasted for Canada.

Shrimp processing discards are also rich in ASX [1,9,18,19] a major class of carotenoid pigments, phospholipids and omega (n)-3 fatty acids (FAs) [4,11,20–22]. In previous work, Northern shrimp by-products (*Pandalus borealis* Kreyer*)* were shown to contain 8 wt% eicosapentaenoic acid (EPA) and 10.7 wt% docosahexaenoic acid (DHA) of the total FA fraction [4]. Previous work has reported lipid content from 2.3 wt% (wet basis) to 8.12 wt% (dry basis) and 148 to 284 $\mu g/g_{waste}$ for ASX depending on the method of extraction and pre-treatment [23,24]. However, data varied considerably in extracted masses due to

variability in solvent composition, extraction time, method of extraction, etc. A review of lipid and ASX levels from various shrimp species and other crustaceans are outlined in a prior literature review [25]. Lipids and carotenoids are valuable feedstocks for the food and pharmaceutical industries. Given the decreasing feedstock of many fish species and impacts on the ocean, it is obvious we must extract more from less. Recovery of these compounds could not only decrease environmental impacts associated with shrimp harvesting but also result in economic benefits [4–13].

Processes for extraction of lipids and ASX from biomass include solvent-based methods (Soxhlet extraction, accelerated solvent extraction, and supercritical fluid extraction), ultrasonic, pulsed electric field assisted extraction and enzymatic processes [6,25]. Each extraction process has pros and cons. The most common process at the lab scale is organic solvent-based extraction as it results in an overall high quantity and quality lipid and ASX extracts. The use of solvent(s) can vary based on the type/amount of carotenoids present in the shrimp by-products. Non-polar solvents such as hexane are effective in the recovery of non-polar carotenoids (esterified ASX and carotenes), while polar solvents (acetone and ethanol) are used for polar carotenoids such as free ASX [6]. Sachindra et al. [7] showed a synergistic effect of polar/non-polar solvent mixtures for the extraction of carotenoids [7]. In the solvent extraction process, solid samples are exposed to fresh solvent with extraction efficiency aided through physical mixing or increased temperature to improve mass transport. Solids are subsequently separated from the extract phase via filtration. Extraction efficiency can be further improved by repetition of the extraction step on residual solids with fresh solvent. These process steps are repeated at room temperature until the extract

is colourless [7,8,26] (i.e., carotenoid extraction is a visual indicator of extraction completion). In the Soxhlet method, the extractions occur at the boiling temperature of the solvent(s). Fresh solvent is added to a boiling flask without direct contact with solid samples, eliminating the need to separate the solid phase from the extract phase. The solvent is recycled through the system in cycles, eliminating the need to add fresh solvent each cycle. Another advantage is there is no direct contact with a potentially toxic solvent over the 5-7 h extraction period.

In the sixteen studies focusing on extraction of lipids and ASX from shrimp by-products [4,7–11,16,23,27–34] four papers focused on organic solvent-based methods for ASX recovery [7,8,23,30]; two papers investigated a variety of organic solvents: one study investigated the most efficient solvent process in terms of ASX extraction from wet shrimp by-products [7], and the second compared different organic solvents in different processes (solvent, Soxhlet, maceration) and compared with edible oil for lipid and ASX recovery from cooked/dried shrimp by-products [8]. While these studies provide data, the focus has either been on the yield or quality of the ASX, and none have addressed the lipid distribution as a function of the extraction method. The lipid composition will impact not only ASX solubility and reactivity but also the value and final application of the extract (depending on n-3 FAs, PLs etc.); thus, the total extract (lipid+ASX) quality and yield must be established as a function of extraction conditions. As outlined above, the Soxhlet process has advantages relative to the solvent process but there are only a handful of studies of Soxhlet in lipid and ASX recovery [4,8].

Any preprocessing (i.e., water removal) done on the shrimp by-products prior to extraction will also impact the quantity and quality of the extract. A "dried" product has lower mass transfer resistance (potentially higher recovery) but could impact the nutritional quality of the ASX recovered. There have been studies on the impact of drying wet biomass on lipid extraction from microalgae. In studies by Kanda et al. and Liu et al. [35,36], the lipid yield increased with decreasing water content while Halim et al. [37] observed no impact of water content on lipid recovery [37]. Medina et al. [38] showed an increase in lipid yield with increasing water content. There was little discussion of the impact on lipid composition [38]. These studies highlights that generalities cannot be made with respect to lipid yields and water content.

The objective of this study was to evaluate Soxhlet extraction of lipids and ASX from Atlantic shrimp processing by-products (*Pandalus Borealis*) using organic solvents/solvent mixtures with different polarities. In this work, a comprehensive analysis of lipid/FA compositions, and the yield/quality of the extract was studied as a function of solvent and water content. These results can be used as a reference for; the impact of temperature and time on yield/quality, evaluation/development of scalable extraction processes (supercritical CO_2, edible oil extraction of ASX etc.), and to compare the quality of different lipid/ASX mixtures in food/medical applications. The performance of the Soxhlet process was compared with published solvent processes [7,8]. The impact of drying on lipid/ASX yields and quality from shrimp by-products was studied in this work by using "wet" and freeze-dried shrimp by-products.

3.2. Materials and methods

3.2.1. Materials and chemicals

Shrimp by-products from the processing of *Pandalus Borealis* were shipped from the St. Anthony Basin Resources Incorporated (SABRI) shrimp processing plant. The plant is located on the Northern Peninsula of Newfoundland and Labrador and the shrimp by-products were transported to the laboratory frozen (- 4 ˚C) and stored at - 30 ˚C until use. The standard ASX (≥92%, A9335) used for UV-vis analysis to obtain calibration curve was purchased from Sigma–Aldrich (CAS registry No. 472-61-7). Hexane (CAS registry No. 110-54-3), acetone (CAS registry No. 67-64-1), anhydrous ethanol (100 %) and isopropanol used in Soxhlet extraction were of ACS grade and purchased from Fisher Scientific and ACP Chemicals Inc.

3.2.2. Preparation of shrimp by-products

Clean, frozen shrimp by-products were ground in a lab mixer (Black & Decker, food processor, FP5050SC, China) before use. The ground shrimp by-products are wet biomass with 67.72 wt% water content (wet residues) measured using moisturizer (Mettler Toledo, HB43-S). Freeze-dried (FD) residues were prepared from the ground shrimp using a freeze drier (Labconco® Freeze Dry Systems, 6 L Benchtop Models, US) at -52 ˚C for 72 hours and then kept in a desiccator under -20 ˚C. The resulting FD residues were homogenized by a laboratory mortar and pestle to produce fine particles with a water content of 11.97 wt%.

3.2.3. Soxhlet extraction of lipids and ASX

Approximately 2 g of the ground residues were placed in a thimble-holder that was gradually filled with condensed fresh solvent from a 250 mL boiling flask (Fig. 3.1). Solvent in the flask is heated at its boiling temperature (49.8-64.40 ˚C). As the liquid overflows the holder, the extract is siphoned and fed back into the flask. This operation is repeated, and the extract is recirculated through the residues over 5-7 h. The organic solvents compared were: hexane, ethanol, hexane/acetone (40:60 vol%), hexane/acetone (50:50 vol%), hexane/isopropanol (50:50 vol%) and hexane/isopropanol (60:40 vol%). All experiments were carried out in duplicate or triplicate to extract lipids and ASX from both wet and FD residues.

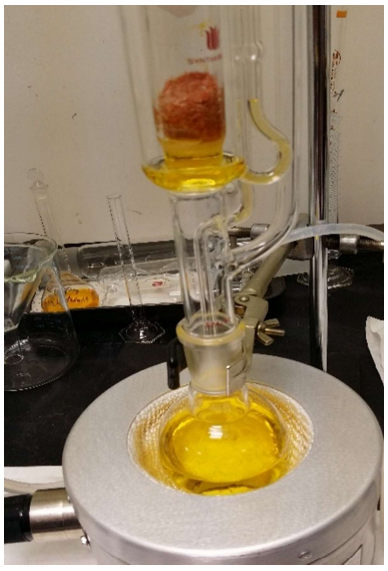

Figure 3.1: Soxhlet process

The extract consisting of lipids, ASX and other soluble compounds was concentrated (Fig.3.2) under vacuum at 56 ˚C using a rotatory evaporator (Buchi® Rotavapor® R-300

evaporator with I-300 Pro interface, vacuum pump Buchi® V-300 and water heating bath Buchi® B-300 base). To completely remove any traces of water and solvents, the extract was dried in the oven at 40 ˚C for 1-3 h to a constant weight, and then cooled in a desiccator before weighing the total lipid mass. To measure the ASX in the extract, the concentrated extract was re-dissolved in a mixture of hexane and acetone (80:20 vol%) before further analysis.

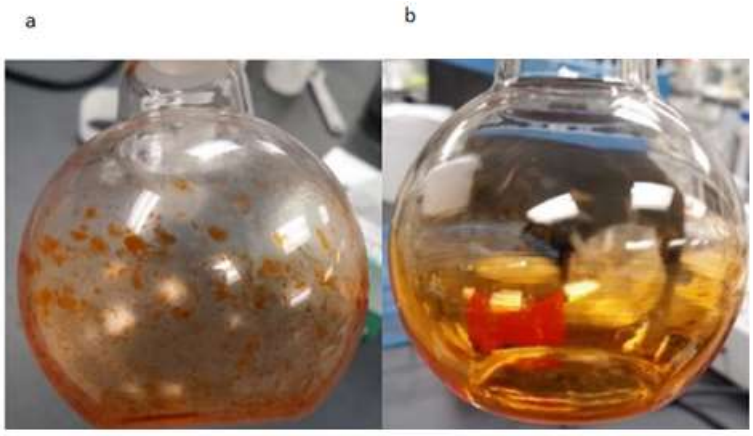

Figure 3.2: Concentrated extracts from wet (a) and freeze-dried shrimp by-products (b) using hexane/acetone (40:60 vol%) in Soxhlet.

3.3. Extract quantity and quality

The yield was quantified in terms of total lipid yield (dry wt%) and ASX yield ($\mu g/g_{waste}$), while quality was evaluated as total carotenoid content (TCC) in the extract ($mg/g_{extract}$). TCC in this study represents the total ASX (free/esterified forms) amount in the extract and ASX yield represents the total ASX amount in waste. ASX concentration in the extract was measured using UV-Vis-NIR spectrophotometer. Lipids were analyzed using an Iatroscan Mark VI TLC-FID for various lipid classes, and FA profiles were analyzed with an Agilent GC-FID equipped with an autosampler at the Ocean Science Centre (Memorial University of Newfoundland and Labrador).

3.3.1. Total lipid yield calculation

The concentrated extract from the solvent evaporation phase was dried in the oven at 40 ˚C for 1-3 h to a constant weight, and cooled in a desiccator before measuring the total lipid mass. The total lipid yield was based on dry wt% (Eq.3-1).

$$\textit{Total lipid yield, dry wt\%} = \frac{m_{extract}}{mass\ of\ \ dry\ biomass} \times 100 \qquad \textit{3-1}$$

Where m is mass of extract (g); mass of dry biomass (g) is calculated using shrimp by-product mass (g) and its water content.

3.3.2. ASX yield and TCC calculations

To measure ASX in the extract, the concentrated extract was re-dissolved in a mixture of hexane and acetone (80:20 vol%) before analysis on UV-Vis-NIR spectrophotometer. The concentration of ASX (mg/L) is determined by measuring absorbance between 475-488 nm using UV-Vis-NIR spectrophotometer (Agilent Cary 6000i) at CCART-SIRI/MUN Materials Characterization Facility. A calibration curve of standard ASX solutions (0.5-40 mg/L) in each solvent was determined by measuring the absorbance of the standards at 475 nm (the λ_{max} of ASX) against 80:20 vol% hexane/acetone as blank. Samples were treated in the same way and concentrations of ASX were determined from the calibration curve. TCC represents the extract quality and was calculated via the following formula:

$$\textit{TCC (mg/g}_{extract}\textit{)} = \frac{C_{ASX} \times V}{m_{extract}} \qquad \textit{3-2}$$

Where C_{ASX} is concentration (mg/L); V (L) is volume of the extract diluted in solvent for analysis (solvent extract solution volume); m is mass of extract (g). The ASX content in the shrimp by-products reported as ASX yield, $\mu g/g_{waste}$, wet weight basis was calculated as follows:

$$ASX\ yield\ (\mu g/g_{waste}) = \frac{C_{ASX} \times V}{m} \qquad 3\text{-}3$$

Where C_{ASX} is concentration (mg/L); V (mL) is volume of extract diluted in solvent; m is mass of shrimp by-products (g). All ASX yields in this work are expressed in wet weight basis, which means ASX yield is calculated with respect to the whole mass of the shrimp by-products (~ 2 g).

A series of solvent studies in the Soxhlet were carried out and the process yielding the highest lipid and/or ASX was used as the "baseline" and compared to other solvent yields:

$$Recovery\ (\%) = \frac{ASX\ yield}{Highest\ ASX\ yield\ using\ Soxhlet} \times 100 \qquad 3\text{-}4$$

3.3.3. Lipids/ FAs profile analysis

Lipid compositions of the extract were analyzed using thin layer chromatography (TLC) equipped with flame ionization detection analysis (FID) (Mark VI Iatroscan with silica coated chromarods). The lipid classes include straight chain hydrocarbons (HCs), steryl esters (SEs), ethyl esters (EEs), methyl esters (MEs), ethyl ketones (EKs), methyl ketones (MKs), glycerol ethers (GEs), triacylglycerols (TAGs), free fatty acids (FFAs), alcohols (ALCs), sterols (STs), diacylglycerols (DAGs), acetone mobile polar lipids (AMPLs), and phospholipids (PLs).

FA compositions of the extract were analyzed on a GC-FID (HP 6890) equipped with an autosampler (7683). All results of lipid and FA compositions were expressed in percentage weight (wt%) of total lipid and FA classes.

Results of lipid and ASX extraction experiments carried out in duplicate, or triplicate were expressed as the mean ± relative standard deviation. Data of some experiments which was not replicated was reported without standard deviation.

3.4. Results and discussion

3.4.1. Total lipid yield, TCC and ASX yield

Total lipids, ASX yields, and TCC for organic solvent mixtures are summarized in Table 3.1 for wet residues and Table 3.2 for FD residues.

For wet residues, total lipid yield varied from 2.32-8.69 dry wt%, TCC from 0.19 - 2.40 mg ASX/$g_{extract}$, and ASX yield from 1.61-57.74 µg ASX/g_{waste}. For FD residues, total lipid yield varied from 1.29-4.32 dry wt%, TCC from 5.66 -12.95 mg ASX/$g_{extract}$, and ASX yield from 117.54-254.71 µg ASX/g_{waste}. The highest lipid yield (8.69 dry wt%) was extracted with ethanol from wet residues; however, it should be noted that hexane/acetone (40:60 vol%) was only slightly lower and within the standard deviation. The highest quality of extract (TCC) was 12.95 mg ASX/$g_{extract}$ using hexane/isopropanol (60:40 vol%) with FD residues, again hexane/isopropanol (50:50 vol%) was only slightly lower and within standard deviation.

Table 3.1: Total lipid yield, total carotenoid content (TCC) and astaxanthin (ASX) yield of the extracts recovered from wet residues using Soxhlet.

Extraction media, solvent type	Polarity[a] Index	Total lipid yield, dry wt %[b]	TCC, mg ASX/$g_{extract}$	ASX yield (µg ASX/$g_{dry\ waste}$[b])
Hexane	0.1	2.32 ± 0.09	0.19 ± 0.078	4.63 ± 1.91
Hexane/isopropanol (60:40 vol%)	1.6	3.46 ± 0.05	5.87 ± 1.53	244.25 ± 44.14
Hexane/isopropanol (50:50 vol%)	2.2	5.25 ± 0.38	4.22 ± 0.50	191.33 ± 18
Hexane/acetone (50:50 vol%)	2.7	5.38 ± 0.52	4.62 ± 0.53	252.13 ± 16.39
Hexane/acetone (40:60 vol%)	3.1	8.69 ± 0.38	2.25 ± 0.85	280.99 ± 12.64
Ethanol	5.2	8.41 ± 0.20	2.40 ± 0.04	182.84 ± 23.64

[a]: Ref. [37]

[b]: total lipid yield expressed in terms of the dry biomass of waste calculated using the shrimp by-product mass (~2 g) and its water content (67.72 ± 4.27 wt %).

Table 3.2:Total lipid yield, total carotenoid content (TCC) and astaxanthin (ASX) yield of extracts recovered from freeze-dried (FD) residues using Soxhlet.

Extraction media, solvent type	Polarity[a] Index	Total lipid yield, dry wt %[b]	TCC, mg ASX/$g_{extract}$	ASX yield (µg ASX/$g_{dry\ waste}$) [b]
Hexane	0.1	1.29 ± 0.38	12.44 ± 1.27	135.27 ± 2.92
Hexane/isopropanol (60:40 vol%)	1.6	1.76 ± 0.20	12.95 ± 2.12	233.78 ± 15.76
Hexane/isopropanol (50:50 vol%)	2.2	1.83 ± 0.16	11.93 ± 0.71	199.70 ± 6.39
Hexane/acetone (50:50 vol%)	2.7	1.75 ± 0.41	11.04 ± 0.50	207.02 ± 7.47
Hexane/acetone (40:60 vol%)	3.1	2.57 ± 0.36	9.74 ± 1.95	274.48 ± 56.99
Ethanol	5.2	4.32 ± 0.94	5.66 ± 0.92	216.56 ± 20.50

[a]: Ref. [37]

[b]: total lipid yield expressed in terms of the dry biomass of the waste calculated using the shrimp by-product mass (~2 g) and its water content, 11.97 ± 0.78 wt %.

The total lipid yield from the wet residues using hexane/acetone (40:60 vol%) in Soxhlet is in good agreement with the literature using the same shrimp species (*Pandalus borealis*) [23,24]. This work showed higher ASX yields compared to published work (Table 3.3) for the same species of shrimp by-products treated with hexane/isopropanol (60:40 vol%) [12,23].

Table 3.3: Comparison of astaxanthin (ASX) yields recovered from the same shrimp species (Pandalus borealis) with the literature.

Shrimp by-products/pre-treatment	ASX yield (μg ASX/g_{waste}, wet weight basis)	Ref.
Mixture of ground heads, tails and shells, 67.72% moisture	87.98 ± 0.35[a]	This work
Cooked/ground shells, 74.14% moisture	73.56 ± 7.23	[23]
Mixture of ground heads, tails and shells, 87% moisture	41.1	[12]

[a]: ASX yield expressed in terms of the whole mass of the shrimp by-products (~ 2 g).

3.4.2. Polarity effect of solvent/solvent mixture on lipid yields and TCC values

The lowest lipid yield was using hexane from wet (2.32 dry wt %) and FD (1.29 dry wt %) residues. Hexane, a non-polar solvent, favors nonpolar compound extraction. This observation is similar to work in red-spotted shrimp (*Farfantepenaeus paulensis*) [11].

Total lipid yields and TCC results for wet residues show an increase in the polarity index (PI) of the extraction medium (solvent or solvent mixtures) from 1.6 to 5.2, doubles total lipid yields but TCC values were halved. The same pattern was also observed for FD residues (Table 3.2). This is likely due to the fact that the shrimp are high in polar lipids such as PL (Table 3.5) and free/esterified ASX [8,10,24,39–41]. The esterified or stabilized form of ASX is formed through an esterification of the free ASX with FAs, forming monoesters

or diesters of ASX or with proteins (carotenoprotein complex) [41,42]. As such, the esterified ASX can vary in polarity index depending on the linked molecule (FAs vs protein). Therefore, polar solvents (ethanol, isopropanol and acetone) will extract free ASX due to the lower molecular chain and the presence of free oxygenated groups in the free ASX, but extraction is not as favourable for the esterified form of ASX linked with FAs. In this study, increasing the polarity of solvent/solvent mixtures results in a decrease in ASX recovery (Table 3.1 and 3.2). This is likely due to the fact that esterified ASX (less polar) dominates free ASX and probably is linked with FAs as observed by Mezzomo et al. [8]

Higher ASX yield was observed when a polar solvent was added to hexane. The TCC for wet residues (Table 3.1) increased from 1.5-2 times by adding 40-50 vol% of isopropanol in hexane. Similarly, TCC increased by 10-20 times by mixing 50-60 vol% of acetone in hexane. Non-polar solvents such as hexane are not as effective in the extraction of large molecules such as ASX (free and esterified ASX), especially in the presence of water (in which hexane is not miscible). As such, we see an increase simply due to the poor performance of hexane as a single solvent.

3.4.3. Lipid and FA compositions of the extracts

Table 3.4 summarizes the fatty acids distribution of the lipid fraction of the extracts. The ratio of PUFA:SFA was greater than one. The PUFAs were rich in n-3 FAs, mainly EPA (13.33-18.02 wt%) and DHA (8.89-14.21 wt%) (Table 3.4); these values were in good agreement with the literature values of 10.40 wt% for EPA and 9.50 wt% for DHA in shrimp oil extracted from shrimp (*Pandalus borealis*) processing water [43], and 11.69 wt% for EPA and 12.24 wt% for DHA in shrimp (*Farfantepenaeus paulensis*) by-products [44].

Jiao et al. [43] showed shrimp oil extracted from shrimp (*Pandalus borealis*) processing water is higher in n-3 FAs, compared to n-6 FAs (n-3:n-6 FA of 14.82) [43]. In this study, the n-3:n-6 FA ratio ranged from 5.24-12.15. The lipid class distributions of the extracts using Soxhlet are summarized in Table 3.5. The major lipid classes are TAG, FFA and PL. PL varied between 50.25 to 68.62 wt%, with the maximum amount using hexane/acetone (40:60 vol%) from wet residues. PL in extracts from FD residues was lower but comparative (31.41-35.92 wt%). FFA and ST were higher for FD residues compared to wet residues. The impact of freeze-drying is complex but in general, studies showed that freezing rates impact the microstructure/integrity of the cellular matrix. Cell structure can be damaged depending on the growth rate of an ice crystal which lyses and compresses cells to impact lipid extraction [45,46]. Furthermore, in aqueous/lipid media, freeze-drying (in general), and the freezing rate (in particular) impacts lipid compositions [45]. However, given the complex nature of the shrimp by-products, a comprehensive analysis of the impact on freeze-drying is outside the scope of this work.

Table 3.4: FA profiles, wt % of the extracts obtained using different solvents/solvent mixtures from wet and freeze-dried (FD) residues.

	Hexane		Hexane/acetone (50:50 vol%)		Hexane/acetone (40:60 vol%)		Ethanol		Hexane/isopropanol (60:40 vol%)	Hexane/isopropanol (50:50 vol%)
FAs, wt %	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	Wet shrimp residues
C14:0	3.17± 0.73	3.35± 0.53	1.92	3.23	2.19± 0.48	2.81± 0.23	3.13± 0.05	2.02	2.89± 0.04	2.88
C16:0	15.93± 0.50	12.12± 1.25	11.75	12.78	14.85± 2.05	12.42± 1.63	12.26± 0.24	12.51	12.33± 0.03	12.26
C16:1n7	9.04± 1.10	9.91± 1.05	6.74	10.60	7.67± 0.73	8.74± 0.90	8.86± 0.17	6.82	8.13± 0.04	8.59
C18:0	4.58± 0.08	2.16± 0.66	2.53	1.91	3.00± 0.53	2.48± 0.58	1.52± 0.08	2.25	2.05± 0.01	2.03
C18:1n9	17.69± 3.10	15.26± 0.60	14.87	14.26	15.95± 0.25	14.12± 1.17	15.59± 0.05	15.38	15.42± 0.09	15.56
C18:1n7	5.07± 0.41	4.70± 0.28	9.17	6.55	6.00± 0.58	5.16± 0.63	5.43± 0.12	7.80	5.75± 0.01	5.67
C18:2n6	3.29± 1.72	1.29± 0.09	1.31	1.59	1.39± 0.24	1.45± 0.48	1.39± 0.00	1.21	1.43± 0.01	1.43

	Hexane		Hexane/acetone (50:50 vol%)		Hexane/acetone (40:60 vol%)		Ethanol		Hexane/isopropanol (60:40 vol%)	Hexane/isopropanol (50:50 vol%)
FAs, wt %	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	Wet shrimp residues
C18:3n6	0.059± 0.10	0.05± 0.04	0.06	0.09	0.01± 0.01	0.01± 0.02	0.09± 0.00	0.06	0.07± 0.00	0.09
C18:3n3	0.78± 0.40	0.53± 0.02	0.24	0.41	0.42± 0.05	0.51± 0.09	0.46± 0.02	0.24	0.45± 0.01	0.47
C20:1n9	3.02± 0.56	5.98± 0.98	4.60	5.37	4.02± 0.48	5.24± 0.48	4.49± 0.11	4.75	4.35± 0.05	4.21
C20:4n6	0.75± 0.61	1.10± 0.18	1.69	1.12	1.11± 0.40	0.76± 0.69	1.31± 0.03	1.55	1.37± 0.01	1.40
C20:5n3, EPA	15.28± 0.14	13.33± 2.36	18.02	15.51	16.85± 0.63	14.74± 3.44	17.96± 0.26	17.76	17.86± 0.00	18.22
C22:5n3	0.39± 0.33	0.77± 0.42	0.52	0.64	0.70± 0.39	0.78± 0.35	1.18± 0.02	0.60	1.14± 0.01	1.20
C22:6n3, DHA	9.21± 0.60	8.89± 0.69	14.21	11.23	12.75± 0.44	10.38± 1.86	12.06± 0.43	13.43	12.66± 0.03	12.41
Σ SFA	26.16± 1.79	18.66± 1.47	17.03	18.67	21.65± 2.66	18.94± 2.20	18.10± 0.41	18.03	18.48± 0.12	18.52

	Hexane		Hexane/acetone (50:50 vol%)		Hexane/acetone (40:60 vol%)		Ethanol		Hexane/isopropanol (60:40 vol%)	Hexane/isopropanol (50:50 vol%)
FAs, wt %	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	FD shrimp residues	Wet shrimp residues	Wet shrimp residues
Σ MUFA	41.48± 1.68	51.27± 2.75	44.14	46.34	41.96± 0.51	49.16± 4.87	43.24± 0.24	44.05	42.45± 0.24	41.93
Σ PUFA	31.55± 2.31	28.98± 3.96	38.05	34.01	35.37± 2.51	31.04± 6.71	37.87± 0.70	37.03	38.32± 0.10	38.75
n-3 FAs	26.67± 0.98	25.24± 3.52	33.67	29.68	32.28± 1.58	27.95± 5.99	33.55± 0.67	32.90	34.06± 0.00	34.31
n-6 FAs	5.20± 0.34	2.82± 0.21	3.60	3.82	2.48± 0.71	2.73± 0.41	3.04± 0.38	3.54	3.27± 0.09	3.40

Table 3.5: Lipid compositions (wt%) of the extracts obtained using different solvents/solvent mixtures from wet and freeze-dried (FD) residues.

	Wet residues					FD residues		
	Hexane	Hexane/isopropanol (60:40 vol%)	Hexane/isopropanol (50:50 vol%)	Hexane/acetone (40:60 vol%)	Ethanol	Hexane	Hexane/acetone (40:60 vol%)	Ethanol
TAGs	8.99 ± 0.26	14.77 ± 2.07	12.98 ± 2.42	6.80 ± 0.25	15.99 ± 1.97	31.36 ± 0.07	12.65	2.39
FFAs	8.75 ± 0.87	5.86 ± 0.64	3.03 ±0.43	5.93 ± 0.06	2.84 ± 0.70	0 ± 0	10.74	19.0 3
PLs	54.13 ± 0.33	50.25 ± 5.87	56.94 ±6.90	68.62 ± 7.03	51.91 ± 3.16	31.41 ± 6.93	33.93	35.92
STs	4.24 ± 022	8.72 ± 1.00	8.08 ±0.03	8.98 ± 0.08	9.25 ± 0.20	40.38 ± 3.56	22.16	37.85
AMPLs	9.86 ± 0.56	12.31 ± 1.40	0 ± 0	4.7 ± 0.14	10.96 ± 0.50	4.93 ± 0.02	13.89	4.02

[a] triacylglycerols (TAG), free fatty acids (FFA), phospholipids (PL), sterols (ST), and acetone mobile polar lipids (AMPL).

3.4.4. Soxhlet method performance in ASX recovery

The performance of Soxhlet was compared to the published results using solvent extraction [7,26]. Soxhlet had higher ASX yield values compared to the literature (Fig. 3.3) using the same solvents as the solvent extraction method. Soxhlet with hexane/isopropanol (60:40 vol%) extracted 87.98 µg ASX/g_{waste}, 87.30 µg ASX/g_{waste} with hexane/acetone (50:50 vol%) and 57.74 µg ASX/g_{waste} with ethanol from the wet residues which are twice than the solvent extraction methods [7,26].

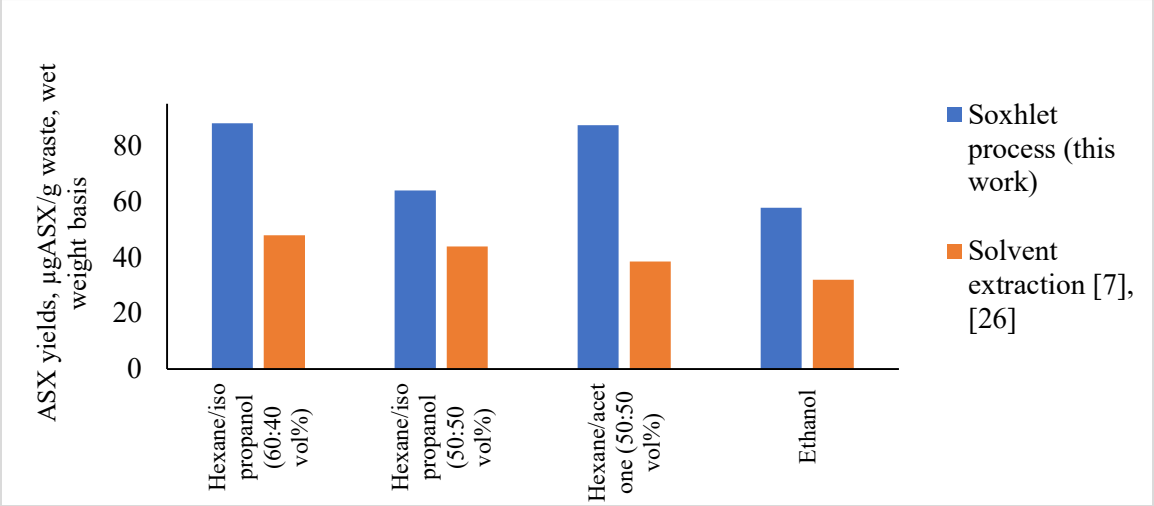

Figure 3.3: Comparison of astaxanthin (ASX) yields recovered from wet shrimp by-products using Soxhlet with solvent extraction [7, 26].

In a study by Mezzomo et al.[8], different extraction processes (maceration, Soxhlet and etc.) were compared, and the Soxhlet process recovered higher ASX yields [8]. Mezzomo et al.[47] proposed this was a result of the high solubilization and diffusion of components from the raw material in the Soxhlet process due to (1) high temperature reducing the solvent viscosity/surface tension and consequently solvent/solute interactions, and (2) numerous cycles of solvent [8,47]. Table 3.6 compares lipid and ASX yields in this study with the literature using the same Soxhlet method.

Table 3.6: Total lipid and astaxanthin (ASX) yields using Soxhlet method on different species shrimp by-products for different pre-treatment processes.

Solvent/extraction medium	Pre-treatment	Shrimp species	Total lipid yield, dry wt %	ASX yield, μg ASX/g_{waste}, dry weight basis	Ref.
Hexane	Freeze-drying	*Pandalus borealis*	1.29 ± 0.38[a]	135.27 ± 2.92[a]	This study
	Cooking (water bath at 100 ˚C for 10 min) and drying (at 60 ˚C for 5 h in an oven)	*Paracoccidioides brasiliensis/ Penaeus* paulensis	19 ± 2	5.5	[8]
	Freeze-drying	*Farfantepenaeus paulensis*	3.3 ± 0.1	N/A[b]	[11]
Hexane/isopropanol (50:50 vol%)	Freeze-drying	*Pandalus borealis*	1.83 ± 0.16[a]	199.70 ± 6.39[a]	This study
	Cooking (water bath at 100 ˚C for 10 min) and drying (at 60 ˚C for 5 h in an oven)	*Paracoccidioides brasiliensis/ Penaeus* paulensis	11 ± 1	21 ± 1	[8]
Ethanol	Freeze-drying	*Pandalus borealis*	4.32 ± 0.94[a]	216.56 ± 20.50[a]	This study
	Cooking (water bath at 100 ˚C for 10 min) and drying (at 60 ˚C for 5 h in an oven)	*Paracoccidioides brasiliensis/ Penaeus* paulensis	68 ± 6	17 ± 1	[8]
Hexane/isopropanol (60:40 vol%)	Freeze-drying	*Pandalus borealis*	1.76 ± 0.20[a]	218.39 ± 28.89[a]	This study
	Freeze-drying	*Farfantepenaeus paulensis*	5.3 ± 0.2	53 ± 2	[11]

[a]: total lipid/ASX yields expressed in terms of the dry biomass and its water content (11.97 ± 0.78 wt %).
[b]: N/A, not available

Total lipid yields in this study are approximately 30% of yields in Sánchez-Camargo et al. (2011) for the same solvent(s) and 5-10% of Mezzomo et al. (2011). The variability in lipid/ASX yields may be a result of different drying methods and biomass feedstocks, and particle sizes. Mezzomo et al. (2011) observed cooking improved lipid recovery, especially when combined with drying. This is likely due to better separation of the lipid compounds from the solid matrix during cooking, and also removing water through drying in an oven instead of freeze-drying [8]. As mentioned earlier, freeze-drying impact on lipid recovery can be complex; freezing rates impact intracellular ice crystal formation and the potential damage to cell structure which can, in turn, impact the lipid distribution in the solid phase [45,46]. A study showed cooking prior to freezing can reduce cell structure damage [46]. The ASX yields in this study were 4-25 times higher in this work compared to the referenced work [8,11]. This difference could be due to thermal degradation of ASX due to high temperatures used in cooking (100 ˚C) and long drying times (60 ˚C for 5 h) as the free ASX presents in shrimp by-products [8] which is unstable and very sensitive to oxidation, degradation, and isomerization [48].

3.4.5. Water content on lipid and ASX recovery

The shrimp by-products from the processing plant were approximately 67 wt% water. As shown above, water will impact lipid and ASX yields and overall extraction costs. If the water is left in costs could increase due to larger extraction vessels, pumps etc. However, water removal can also be costly depending on what equipment the processing plant has available. In this study, freeze-drying was used to reduce the water content to 11.97 wt%. FD was used in this study as it is a common unit operation at many fish processing plants.

ASX yields for wet and FD residues are compared in Fig 3.4. Note the yields are normalized to a dry basis.

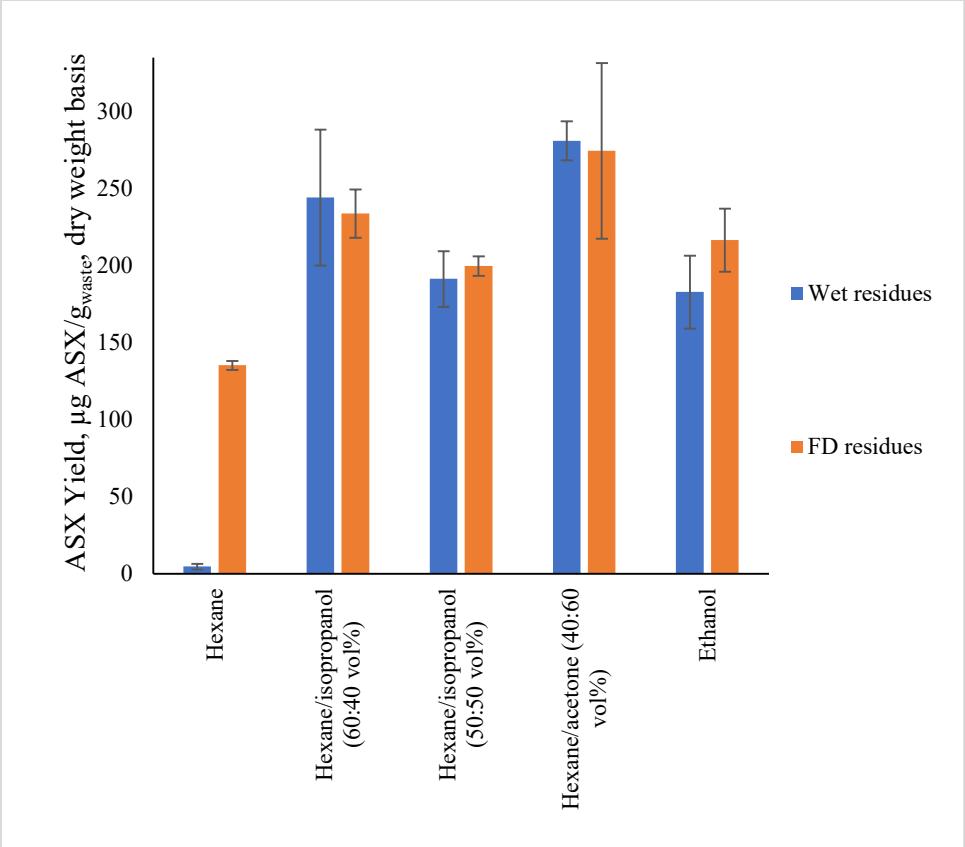

Figure 3.4: Water content impact on astaxanthin (ASX) yield using Soxhlet as a function of solvent.

Compared to FD extraction, the wet shrimp showed lower ASX yields when hexane or ethanol was used as an extraction solvent in Soxhlet. The yields for wet residues were slightly lower for the nonpolar/polar solvent mixtures. Similar results were observed for the extraction of carotene [49]. The lower wet extraction recoveries are likely due to the water in part acting as a barrier to mass transfer by blocking micropores, restricting the accessibility of solvents. This would be especially evident for non-polar solvents such as hexane (a hydrophobic solvent) [33,50]. Since hexane is not water-miscible and ASX is fat-

soluble, hexane is not as effective in extracting ASX [7,51], and less ASX compounds (free and esterified ASX) are available to hexane in wet residues compared to other solvents/solvent mixtures. This, in part, explains the much more substantial impact of freeze-drying in ASX yield when hexane was the solvent.

Freeze-drying improved the quality of the TCC extracts (Fig. 3.5). TCC values were 2-4 times higher for FD residues compared to wet residues. Again, with respect to TCC, the impact of freeze-drying was much more substantial in hexane performance (approximately 65 times higher compared to wet residues).

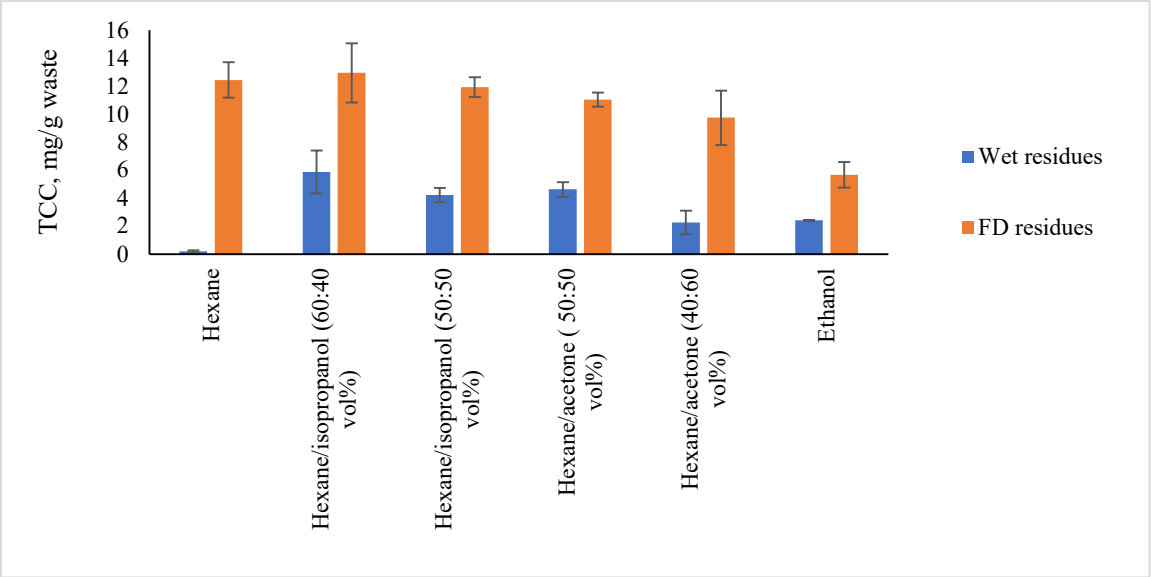

Figure 3.5: Water content impact on total carotenoid content (TCC) values using Soxhlet as a function of solvent.

This data further strengthens that the esterified ASX in the extracts is linked with FAs and dominates free ASX. This would, in part, explain the impact of freeze-drying on hexane performance. The data also shows high ASX yields can be achieved from wet residues

using polar/non-polar solvent mixtures (40:60 vol% hexane/acetone or 60:40 vol% hexane/isopropanol) without any drying methods (lower equipment/energy costs).

In contrast, the higher the water content, the higher the total lipid yield. As noted, the lipid fractions from shrimp by-products are mostly PLs (54-68 wt%). PLs are soluble in both water and oil due to its hydrophilic head and hydrophobic tails; thus, the presence of water increases the polarity of the extraction medium and, consequently the solubility of PL in the extraction medium [52]. Thus, the solvent mass transfer properties and the lipid diffusion rate in such polar extraction medium are enhanced compared to those in non-polar extraction medium (Fig. 3.6). Moreover, as mentioned previously, freeze-drying can impact lipid distributions through cell structure damage, consequently lipid yield.

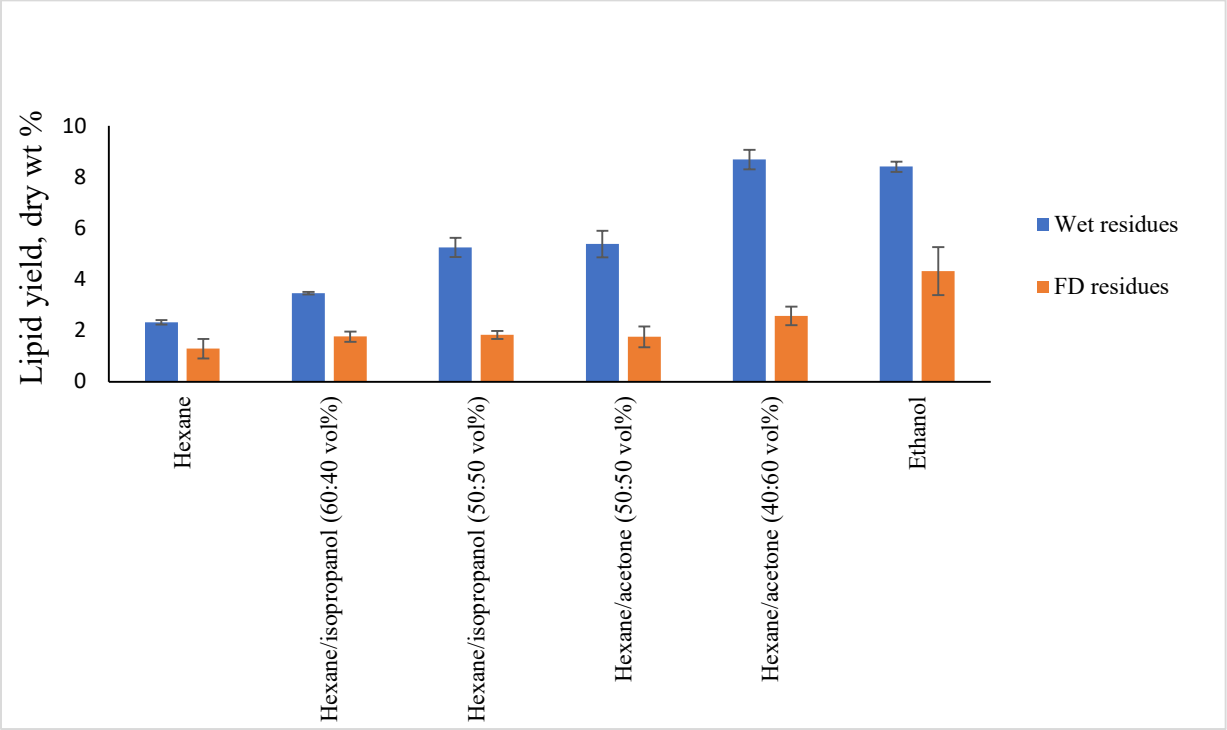

Figure 3.6: Water content impact on lipid yield using Soxhlet as a function of solvent.

3.4.6. Optimization of extraction methods for high lipids, high ASX or high ASX/lipids

The highest yield of lipids was extracted from wet residues using either 40:60 vol% hexane/acetone (8.69 dry wt%) or ethanol (8.41 dry wt%). The hexane/acetone (40:60 vol%) gave the highest yield of ASX from FD residues (254.71 µg ASX/g_{waste}), although the highest quality (TCC) was achieved from FD residues using hexane/isopropanol (60:40 vol%).

The performance of all solvents/solvent mixtures used in ASX recovery (in terms of ASX yield, dry weight basis) for wet and FD residues was compared with the 40:60 vol% hexane/acetone using Eq. 3-4. For wet residues, hexane/acetone (50:50 vol%) and hexane/isopropanol (60:40 vol%) were approximately 87-90 % of ASX extracted with 40:60 vol% hexane/acetone. For FD residues, hexane/isopropanol (60:40 vol%) ASX yield was approximately 85 % of 40:60 vol% hexane/acetone.

Among the Soxhlet extraction processes in this study, ethanol showed both high lipid and ASX yield from FD residues with total lipid yield, 4.32 dry wt%, and ASX yield, 197.23 µg ASX/g_{waste}, and TCC, 5.66 mg ASX/$g_{extract}$. The 40:60 vol% hexane/acetone mixture with FD residues also showed high yields and quality (total lipid yield, 2.57 dry wt%, ASX yield, 254.71 µg ASX/g_{waste}, and TCC, 9.74 mg ASX/$g_{extract}$).

3.5. Conclusions

This study provides important data regarding lipids and ASX extraction from the Atlantic shrimp by-products (*Pandalus borealis*). Various solvents/solvent mixtures with a variety of polarities were used in Soxhlet to evaluate the lipid and ASX yield as a function of

solvent(s). Our findings are critical because the "optimal" lipid/ASX ratios and lipid distributions are a function of application. The polarity of organic solvents and water content play a vital role in lipid and ASX extraction. This study validates solvent and/or solvent mixtures with higher polarity were effective in lipid extraction, but decreased the extract quality (TCC, ASX content in the extract). A non-polar solvent such as hexane is not as effective in the extraction of large molecules such as ASX (free and esterified ASX), especially in the presence of water. Our findings further demonstrate that the amount of water in the residue impacts the quality of extract and the costs associated with extraction. In this study, freeze-drying decreased lipid yield, likely due to the fact that the lipid fraction with high polar lipid percentage can extract more easily in water medium and also freeze-drying can impact lipid distributions through cell structure damage. In contrast, the freeze-drying impact was positive in ASX yield and TCC.

The highest recovery of ASX was achieved with hexane/acetone (40:60 vol%) from FD residues and the highest lipid recovery with the same solvent mixture but with wet residues. The highest extract quality (the highest TCC value) was obtained using hexane/isopropanol (60:40 vol%) with FD residues. The highest recovery for both lipids and ASX was ethanol, followed by 40:60 vol% hexane/acetone with FD residues. Lipid fractions extracted in this study are rich in PLs, n-3 FAs and ASX which are valuable for the food and pharmaceutical industries.

This work is a comprehensive analysis of solvent effectiveness in lipid and ASX extraction, and the impact of freeze-drying on yields. This information would be applicable to other shrimp species and play a vital role for researchers in the utilization of lipids and ASX

from marine sources in the food, pharmaceutical, and cosmetic industries. Removal of the lipids/ASX from the shrimp residue still leaves a solid residue rich in proteins and chitins. By removing the high-value ASX, we can then use other processes such as demineralization and hydrolysis to recover the proteins and chitin for animal feed and materials, driving towards a zero effluent process.

Conflict of interest statement

We know of no conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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Chapter 4 Edible oil extraction of astaxanthin

Extraction of Astaxanthin from Atlantic Shrimp By-products Using Waste Fish Oil: Process Optimization and Operational Parameter Effects

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Abstract

Shrimp processing by-products have high levels of astaxanthin (ASX) relative to other crustaceans. ASX has a multitude of application in industries ranging from aquaculture, pharmaceutical and food. Edible waste oils are a potentially "green" solvent that could replace organic solvents and act to prevent degradation of the extracted ASX. This study investigates waste fish oil as a solvent for ASX extraction from Atlantic shrimp by-products (*Pandalus borealis*). Time, temperature and oil:waste ratio were studied to determine optimal operating conditions to maximize yields using the Box–Behnken design. This study observed that the higher the water content in the residues, the lower the ASX amount in the extract. As extended extraction times or high temperatures the ASX yield decreased, this is due to degradation of ASX with time/temperature. The optimal conditions to maximize the yield of ASX from both wet and freeze-dried shrimp by-products were 65 ˚C, 9:1 v/w and 1.5 h. The maximum ASX extracted was 25.62 $\mu g/g_{waste}$ for wet residues and 123 $\mu g/g_{dry\ waste}$ for freeze-dried residues. ASX extractions were 40-60 % lower compared to Soxhlet, however the fish oil extracts were higher in triacylglycerols and omega-6 fatty acids. Wet residues had lower free fatty acids and phospholipids relative to freeze-dried extracts.

A version of this paper has been submitted to the *Journal of Cleaner Production*; the manuscript is under review. The lead author is Sara Ahmadkelayeh and co-authors are Dr. Kelly Hawboldt and Dr. Sukhinder Kaur Cheema. Miss Ahmadkelayeh's contributions to this paper include the following:

- writing the paper
- performing all the laboratory testing and analyses (except where noted)
- conducting all data processing and interpretation of results
- optimizing all data using Design-Expert software
- performing all literature searches required for background information
- writing the paper and performed all literature searches required for background information

Dr. Hawboldt and Dr. Cheema provided technical guidance and editing of the manuscript.

4.1. Introduction

Carotenoids are high-value compounds found in a variety of marine species. Astaxanthin (ASX; $C_{40}H_{52}O_4$), a red-orange carotenoid pigment, is of particular interest due to several associated health benefits [1–6] and its concentration can be high relative to other bioactives in crustaceans [7,8]. ASX-based bioactives have applications in anti-cancer, immunomodulating, anti-diabetic, and anti-inflammatory conditions, and have been used in many aquaculture, cosmetic, pharmaceutical and food industries [1,2,8–15]. ASX has the potential as a feed additive in nutrient supplements in the poultry and aquaculture industries [16,17]. Primary natural sources of ASX include algae and yeast [18], but finfish (salmon, trout and red seabream) and crustaceans (shrimp, lobster, crawfish and crab) have high ASX levels that have accumulated through the food chain [19].

Crustacean species harvested in Atlantic Canada include lobster, crab, shrimp and scallops. Shrimp harvesting and processing play a key role in Canada's employment, food security, and economic growth [20]. Globally, the value of shrimp exports worldwide was US$19.3 billion in 2017 [21]. In 2016, world shrimp capture production was approximately 6 million metric tonnes [22]. In 2020, shrimp landing was 68,580 metric tonnes in Canada (20 % of total shellfish landings). The value of landed shrimp in Canada was $262,697 and or approximately 13 % of total value of shellfish [23].

The value of the shrimp industry could potentially be increased by extracting the high-value compounds left in the processing by-products. These by-products (shells, heads and tails) can make up to 50+ wt% of landed shrimp [24–26], resulting in a loss of valuable compounds (profits) and environmental burden and costs associated with disposal. The

by-products are a source of valuable bioactive materials (proteins, ASX and lipids) and biomaterials (chitin and minerals). Shrimp processing by-products are a potentially lower-cost source of ASX compared to other sources (algae, yeast, bacteria, vegetables, fruits, and flowers), and generate other valuable products (lipids, chitins and proteins). Lipids and ASX content in Atlantic shrimp processing by-products (*Pandalus borealis)* vary from 2.3 wet wt% to 8.12 dry wt% lipids and ASX from 148- 284.48 $\mu g/g_{dry\ waste}$ depending on extraction methods and treatment prior to analysis [17,27]. As ocean resources are under increasing stress due to climate change, we must focus on extracting more from our landed product. The key is that the process must be environmentally and economically feasible and should have the least impact on the environment.

Conventional chemical ASX extraction methods can involve the use of corrosive/toxic solvents, be energy/waste intensive, and can degrade the product and/or by-products [9,28]. Green solvent processes such as edible oil and supercritical CO_2 extraction are potential alternatives [29–32]. Extraction using edible oils can protect thermolabile compounds such as ASX from oxidation/degradation and delay the oxidation time [33,34]. Furthermore, the edible oil extract can supplement aquaculture feed [33]. Factors that impact lipid and ASX extraction using oils include feedstock water content and particle size, and operating conditions (temperature, time and oil:waste ratio) [28]. Extraction of ASX from animal, plant and marine sources have focused on the use of plant-based oils [25,33–37]. Sachindra and Mahendrakar [25] studied sunflower, groundnut, gingelly, mustard, soybean, coconut, and rice oils in the extraction of ASX from wet shrimp by-products. Refined sunflower oil

extracted the highest carotenoid (26.3 μg/g_{waste}) at an oil to waste ratio of 2:1 v/w, temperature of 70 ˚C, and extraction time of 150 min [25].

There have been studies using vegetable oils, however there is no work on marine oils as a green solvent. While plant-based oils show good performance, often shrimp processing occurs either in the same plant as finfish processing or are in close proximity. This provides an accessible source of "waste" fish oil from fish processing by-products and/or fishmeal production [38]. Moreover, using "waste" to treat "waste" results in a more sustainable alternative to value-added compound recovery and "zero" waste. Waste fish oil is rich in TAG/n-3 FAs, thus the use of the oil in ASX extraction provides pigmented oil with higher TAG compared to shrimp oil extracted using Soxhlet that provides higher PL. PL are more bioavailable and provide superior health benefits compared to TAG-rich oils. In this study, the most commonly used traditional solvent extraction process was applied for ASX extraction from shrimp by-products (*Pandalus borealis*) using edible oil (sunflower and waste fish oils). The edible oil ASX extraction process was validated using sunflower oil and compared to the literature. The protocol was then repeated using waste fish oil. Finally, the performance of the waste fish oil in ASX extraction was compared with the Soxhlet extracts and published vegetable oil results.

The main objectives of this study are to broaden the study of the extraction of lipids/ASX from shrimp by-products using waste fish oil extracted from waste fish to include: the impact of varying process conditions on yields and lipid distributions, process conditions that optimize yield and quality of pigmented oil extracts, and comparison of obtained data with published studies using vegetable oil [25,34,35]. The impact of extraction operating

conditions (factors: time, temperature and oil:waste ratio) on ASX extraction was evaluated using response surface methodology (RSM). RSM combines mathematics with statistics to design experiments, build models, and evaluate the effects of factors [39]. The most common types of RSM models include the central composite, the Box–Behnken design (BBD) and the three-level factorial [40]. BBD was used in this study and two feedstocks were studied: wet and freeze-dried via freeze-drying shrimp by-products. Wet and freeze-dried shrimp by-products were studied to determine the impact of water removal on waste fish oil performance.

4.2. Materials and methods

4.2.1. Materials

Atlantic shrimp processing by-products (*Pandalus Borealis*) collected from the St. Anthony Basin Resources Incorporated (SABRI) shrimp processing plants (Northern Peninsula of Newfoundland and Labrador) were transported to the laboratory frozen (- 4 ˚C) and stored at – 30 ˚C until use. Waste fish oil was extracted from a modified Fishmeal process using salmon fish processing offal (supplied by the Newfoundland Aquaculture Industries Association) [38]. Sunflower oil (100 % pure) was bought from a local market (Compliments, Canada). The ASX standard (≥92%, A9335) was purchased from Sigma–Aldrich Co. (CAS registry No. 472-61-7).

4.2.2. Preparation of shrimp by-products

A lab mixer and a mortar/pestle were used for milling and grinding shrimp by-products. Wet residues (67.72-79.9 wt% water) were freeze-dried using a Labconco® Freeze Dry

System (6 L Benchtop Models, US) at - 52 ˚C over 72 h. The water content of the freeze-dried (FD) residues varied from 2.63–11.97 wt%.

4.2.3. ASX extraction using sunflower oil

Sunflower oil has previously been used to extract ASX from shrimp by-products [25,34,36]; thus, we used this protocol to validate our experimental procedure (Fig. 3) before carrying out the edible oil ASX extraction process using waste fish oil. ASX extraction using sunflower oil was carried out for wet and FD residues at 70 ˚C, solvent to waste ratios of 4:1 /w, and 2:1 v/w for 2 h; and the results were compared to the literature [25,36].

4.2.4. Waste fish oil extraction using modified Fishmeal process

The salmon offal was homogenized using a grinder and heated at 70 ˚C and solids removed. The press liquor contains fish oil, water, and fine solids. Waste fish oil was separated from suspended solids and water using a centrifuge and separatory funnel [38].

4.2.5. Extraction of ASX using waste fish oil

The preliminary waste fish oil experiments were carried out at 70 ˚C with wet residues at fish oil to waste ratios of 2:1 v/w and 2 h, and fish oil to waste ratios of 3:1 v/w and 3 h, and with FD residues at 70 ˚C, fish oil to waste ratios of 3:1 v/w and 3 h. 2 g of the shrimp residues were mixed with the specified volume of waste fish oil and the mixture was heated to the 70 ˚C at 150 rpm. The pigmented oil phase was separated from the solid phase by pressing/centrifuging at 5000 rpm for 15 min for wet residues. For the FD residues, the separation was centrifuged (5000 rpm, 15 min) and washed with hot distilled water (70- 80 ˚C) (Fig. 4.1). In the next step, to optimise the process, further study was carried

out at various ratios, times and temperatures. All pigmented oils separated (Fig. 4.2) were stored at 4 ˚C until further analysis. The leftover solids were stored at -30 ˚C for further study.

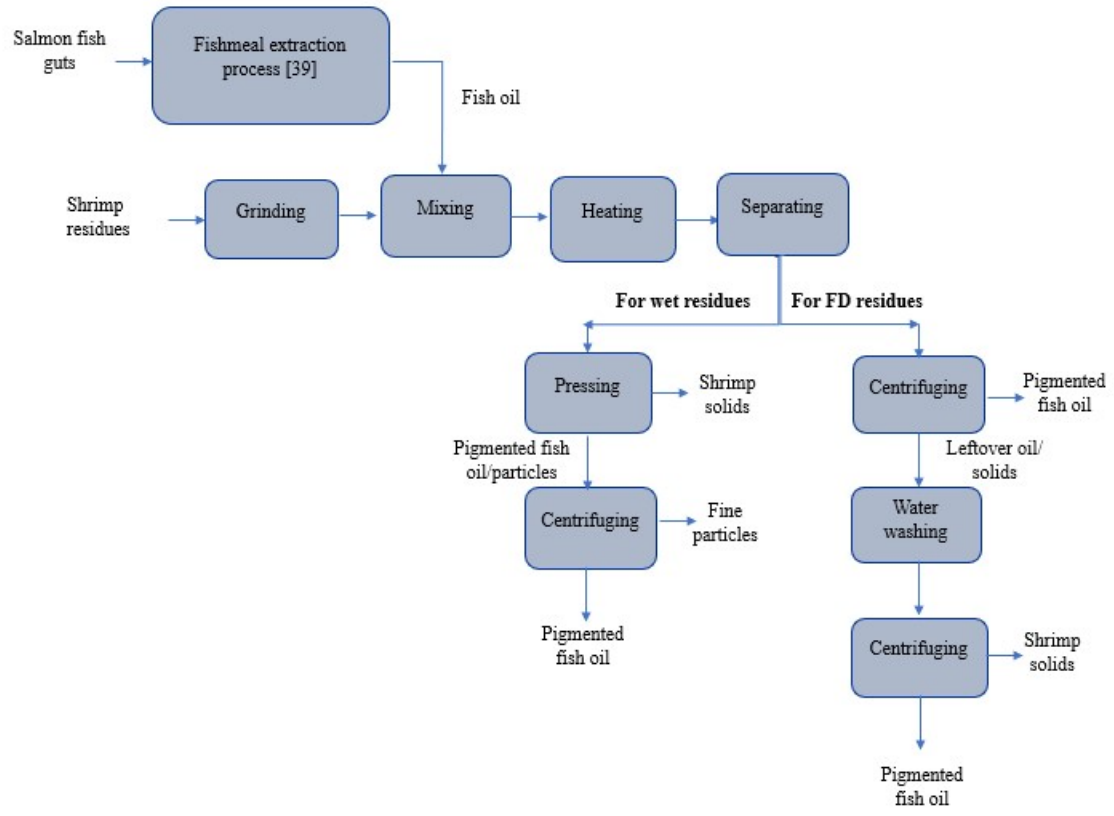

Figure 4.1: Process flow diagram of edible oil extraction of astaxanthin from wet and freeze-dried shrimp by-products.

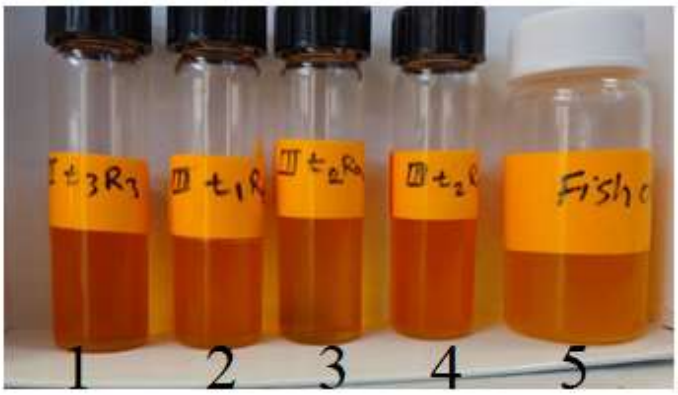

Figure 4.2: Pigmented fish oils (No. 1-4) extracted from wet shrimp by-products at 70 ˚C under various ratios (3-9 v/w) and times (1-3 h) and waste fish oil (No. 5).

4.2.6. ASX quantification

The concentration (mg/L) of ASX in pigmented oils is determined using UV-Vis-NIR spectrophotometer (Agilent Cary 6000i) at CCART-SIRI/MUN Materials Characterization Facility. A calibration curve using standard ASX at different concentrations (0.5-20 mg/L) in waste fish oil/sunflower oils was prepared by measuring the absorbance of the standards at 488 nm for waste fish oil and 475 nm for sunflower oil 475 nm (the λ_{max} of ASX) against the particular solvent as blank. Samples were treated in the same way and concentrations of ASX were determined from the calibration curve. The calibration curves were then used to determine ASX concentration in mg/L. The ASX yield, $\mu g/g_{waste}$ was calculated as follows:

$$ASX\ yield\ (\mu g/g_{waste}) = \frac{C_{ASX} \times V}{m} \qquad 4\text{-}1$$

Where C_{ASX} is concentration (mg/L); V (mL) is volume of the pigmented oil recovered from separation steps (mL); m is mass of wet shrimp by-products (g). All ASX yields in this work are expressed in wet weight basis or the whole mass of the shrimp by-products (~ 2 g). This was done to normalize the ASX yield and compare "wet" to FD experiments.

The lipid profile analysis of waste fish oil and pigmented fish oils was carried out using thin layer chromatography (TLC) equipped with flame ionization detection analysis (FID) (Mark VI Iatroscan with silica coated chromarods). Fatty acid (FA) compositions of waste fish oil and pigmented fish oil extracts were analyzed on a GC-FID (HP 6890) equipped with an autosampler (7683). All results of lipid and FA compositions were expressed in percentage weight (wt%) of total lipid and FA classes. These analyses were carried out at the Ocean Science Centre (Memorial University of Newfoundland and Labrador).

4.2. Percentage recovery of ASX using edible oil extraction

A series of solvent studies in the Soxhlet were carried out and the process yielding the highest lipids and/or ASX was used as the "baseline" and compared to fish oil yields:

$$Recovery\ (\%) = \frac{ASX\ yield\ using\ fish\ oil}{ASX\ yield\ using\ Soxhl} \times 100 \qquad 4\text{-}2$$

4.3. Experimental design and statistical analysis

The BBD with three factors and three levels (-1, 0, +1) was used to compare the extraction conditions on yield of ASX. A total of 15 factor combinations with a center point in triplicate were randomly generated using design-expert software, version 11. The independent variables were extraction temperature (T in ˚C), extraction time (t in h), and oil:waste ratio (R in v/w).

Table 4.1: The independent variables with the levels and codes

Independent variables	**Codes**	**Levels**		
		-1	0	1
Temperature (˚C)	T (X1)	50	60	70
Time (h)	T (X2)	1	2	3
Oil: waste ratio (v/w)	R (X3)	3	6	9

Regression analysis, statistical significance and response surface applications were performed using design-expert 11. The regression model was evaluated with analysis of variance (ANOVA) by the F-test ($P < 0.05$) at a 95% interval of confidence level. A regression model containing 10 coefficients including linear and quadratic effect of factors and the linear effect of interactions was used to describe relationships between response (Y) and the experimental factors (X1, X2, X3) as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{i=i+1}^{3} \beta_{ij} X_i X_j \quad 4\text{-}3$$

4.4. Results and discussion

4.4.1. Preliminary edible oil extraction of ASX

The edible oil extraction process (Fig.4.1) was first validated using the most studied edible oil, sunflower oil (Table 4.2) and then applied with waste fish oil as the solvent. The fish oil results are reported for the first time in this study.

Mezzomo et al. [36] used cooked and dried shrimp by-products with sunflower oil to waste ratio of 4:1 v/w at 70 ˚C for 2 h. The yield in this study is substantially higher than Mezzomo et al. [36]. This follows the same trend as with the Soxhlet results, the pre-treatment (cooking) of the shrimp by-products may have degraded the ASX, thus decreasing yields. When the yields of wet residues were compared with Sachindra and Mahendrakar [25], there is good agreement, again likely due to lack of cooking step. This gave us confidence in our experimental procedure.

Table 4.2: Comparison of astaxanthin (ASX) yields obtained from wet and freeze-dried (FD) residues using sunflower oil in this study with the literature.

ASX yield	Ratio of 4:1 v/w, 70 ˚C for 2 h	
	This work	[36]
	FD residues	Cooked/dried residues
µg/g_{waste}, dry weight basis	127.20 ± 14.29[a]	1.66×10^{-4}
	Ratio of 2:1 v/w, 70 ˚C for 2 h	
	This work	[25]
	Wet residues	
µg/g_{waste}, wet weight basis	20.48 ± 3.54[b]	26.3 ± 2.31

[a]: ASX yield expressed in terms of the dry biomass calculated using the shrimp by-product mass (~2-5 g) and its water content (1.48 ± 0.22 wt %).
[b]: ASX yield expressed in terms of the whole mass of the shrimp by-products (~ 2-5 g).

In the next step, we conducted a preliminary study on the viability of using waste fish oil extracted from waste fish in ASX extraction from the shrimp by-products. The wet extraction of ASX was carried out at 70 ˚C using waste fish oil to waste ratios of 2:1 v/w for 2 h and using waste fish oil to waste ratios of 3:1 v/w for 3 h. ASX extraction from FD residues was run at 70 ˚C, 3:1 v/w and 3 h. The ASX yield for 3:1 v/w and 3 h (16.31 µg/g_{waste}) doubled compared to the yield at 2:1 v/w and 2 h (6.13 µg/g_{waste}) for wet residues. Based on wet residues results at 70 ˚C, 3:1 v/w and 3 h, for the FD residues, ASX yield increased to 123.45±30.82 µg/$g_{dry\ waste}$ for FD residues (2.16 ± 0.08 wt% water content).

This work provided a proof of concept to demonstrate there is potential to increase these yields relative to solvent methods through process optimization.

4.4.2. ASX extraction using waste fish oil

To optimise the process, further study was carried out based on factor combinations randomly defined by BBD. 15 random factor combinations each were for wet and FD residues each. The center point of the experimental design (60 ˚C, 6:1 v/w and 2 h) was run in triplicate to estimate the pure error of the design model. Table 4.3 shows experimental data normalized to a dry weight basis for ASX extraction from wet and FD residues. Table 4.4 and Table 4.5 outline the experimental and model predicted ASX yields in $\mu g/g_{waste}$. The ASX concentration varied from 1.5 to 9.8 mg/L in the lipid extract for wet residues, and 10.8 to 57.45 mg/L for FD residues.

Table 4.3. Experimental values of astaxanthin (ASX) yields in dry weight basis using waste fish oil from wet and freeze-dried (FD) residues

Temperature, ˚C	R, v/w	Time, h	ASX yield, $\mu g/g_{dry\ waste}$ (for wet extraction)	ASX yield, $\mu g/g_{dry\ waste}$ (for FD extraction)
70	9	2	101.81	112
50	3	2	60.54	91.12
50	6	1	45.81	59.67
60	3	1	35.34	97.68
60	3	3	85.44	109.30
70	3	2	67.90	100.50
60	9	1	70.75	117.27
60	9	3	60.54	124.90
50	6	3	100.06	118.94
50	9	2	116.73	131.50
70	6	3	65.93	79.67
70	6	1	82.19	112.76
60	6	2	101.15	122.83
60	6	2	104.26	121.81
60	6	2	131.55	123.48

Table 4.4: Observed and predicted values of astaxanthin (ASX) yields in wet weight basis using waste fish oil from wet residues.

Run no.	Temperature, ˚C	R, v/w	Time, h	ASX yield, $\mu g/g_{waste}$ (experimental value)	ASX yield, $\mu g/g_{waste}$ (predicted value)
1	70	9	2	25.62	25.14
2	50	3	2	9.60	10.10
3	50	6	1	6.55	6.00
4	60	3	1	5.56	5.71
5	60	3	3	14.64	14.61
6	70	3	2	15.24	14.62
7	60	9	1	24.70	24.71
8	60	9	3	12.90	12.74
9	50	6	3	13.73	13.26
10	50	9	2	16.07	16.69
11	70	6	3	10.23	10.87
12	70	6	1	20.83	21.29
13	60	6	2	15.91	15.11
14	60	6	2	16.40	15.11
15	60	6	2	13.02	15.11
13-15				15.11 ± 1.83	

Table 4.5: Observed and predicted values of astaxanthin (ASX) yields in wet weight basis using fish oil from freeze-dried (FD) residues.

Run no.	Temperature, ˚C	R, v/w	Time, h	ASX yield, $\mu g/g_{waste}$ (experimental value)	ASX yield, $\mu g/g_{waste}$ (predicted value)
1	70	9	2	105.96	106.24
2	50	3	2	89.05	88.77
3	50	6	1	54.13	55.55
4	60	3	1	95.40	92.25
5	60	3	3	106.41	108.63
6	70	3	2	97.74	96.88
7	60	9	1	114.13	111.85
8	60	9	3	115.41	116.57
9	50	6	3	116.14	114.14
10	50	9	2	106.05	106.90
11	70	6	3	71.25	69.83
12	70	6	1	105.30	107.30
13	60	6	2	118.95	118.83
14	60	6	2	117.96	118.83
15	60	6	2	119.58	118.83
13-15				118.83± 0.82	

4.4.3. Water content impact on ASX yield

The ASX yields from both wet and FD residues are normalized to a dry weight basis and compared in Fig.4.3. Higher yields of ASX were observed for FD residues compared to wet residues. This is likely due to high water content (up to 67 % in wet waste) which creates mass transfer resistances by sealing pores, limiting contact with waste fish oil (due

to lower mean free pathway) [37,41], and the limited solubility of ASX in water which would limit diffusion of ASX out of the shrimp phase [41]. The impact of freeze-drying and the rate of freezing on lipid integrity could also play a factor as outlined in published studies, however this is a complex mechanism that requires further investigations and is outside the scope of this study [42,43].

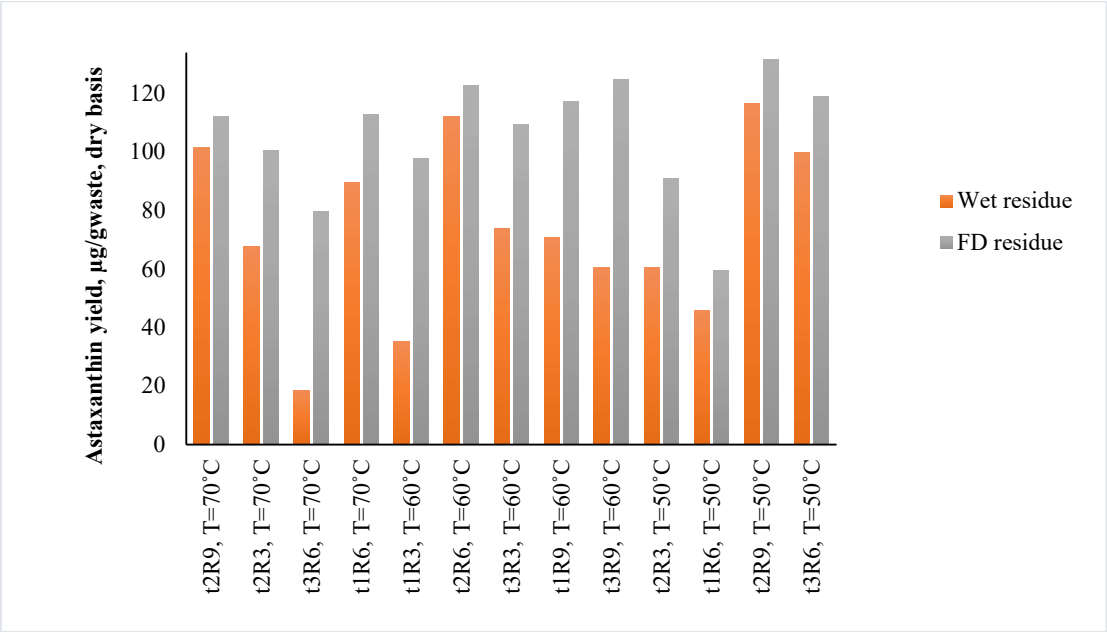

Figure 4.3: Water impact on astaxanthin (ASX) yields recovered using waste fish oil as a function of time (t), temperature (T) and oil:waste ratio (R), e.g., t2R9 represents t in 2 h and R as 9:1 v/w.

4.4.4. Process optimization of the ASX extraction using waste fish oil

4.4.4.1. ANOVA results and model fitting of BBD

ANOVA results were used to estimate the significance and suitability of the yield models (Tables A-1 and A-3). The factor significance was determined using F-test and p-values. The F-values of the regression models for both wet and FD residues indicate that the models were significant and highly predictable. Insignificance ($p > 0.05$) of "lack of fit" for

both models also show the adequacy of the model. Plots of predicted values vs. observed experimental values show a linear with R^2 value exceeding 0.98 for both wet and FD (Fig. 4.4a and Fig. 4.4b) for the response (ASX yield). This represents the accuracy and good fit of the regression models. Adjusted R^2 greater than 0.98 for both models verified the adequacy of the models.

The linear terms of temperature and oil:waste ratio, the quadratic term of time, and interaction of time with temperature and time with oil:waste ratio had significant effects ($p \leq 0.05$) on yield from wet residues. If the interaction term is significant then the effect of one independent depends on the value of the other indicated independent variable. The model equation in actual values of the factors for wet residues can be written as:

$$Y\ (ASX\ yield,\ \mu g/g_{waste}) = -78.01 + 1.07T + 0.86\ R + 45.26t + 0.033T*R - 0.44T*t - 1.74R*t - 0.0004T^2 + 0.17R^2 - 2.23t^2 \quad 4\text{-}4$$

For the FD residue, the oil:waste ratio and time were significant ($p \leq 0.05$) and had positive linear effects on AXS extraction. The quadratic terms ($p \leq 0.05$) of temperature and time, and time/temperature interaction were also significant ($p \leq 0.05$). The negative quadratic term indicates the relationship has a maxima. The model equation (Eq. 4-5) in actual values of the factors for ASX yield for FD residues can be written as:

$$Y\ (ASX\ yield,\ \mu g/g_{wet\ waste}) = -1008.56 + 29.59T + 6.88R + 201.95t - 0.073T*R - 2.40T*t - 0.81R*t - 0.20T^2 + 0.11R^2 - 11.99t^2 \quad 4\text{-}5$$

In the models, an increase in temperature, ratio, or time increases ASX yield due to the positive linear terms, while the negative interaction terms, time/ratio interaction, and time/temperature interaction negatively affect ASX recovery. In practical terms, for a given set of conditions, independently increasing extraction times, or ratio, or temperature will

increase yields. However, the negative interaction of time/temperature means the combined effect is less than the sum of the individual effects. This is discussed in more detail in subsequent sections.

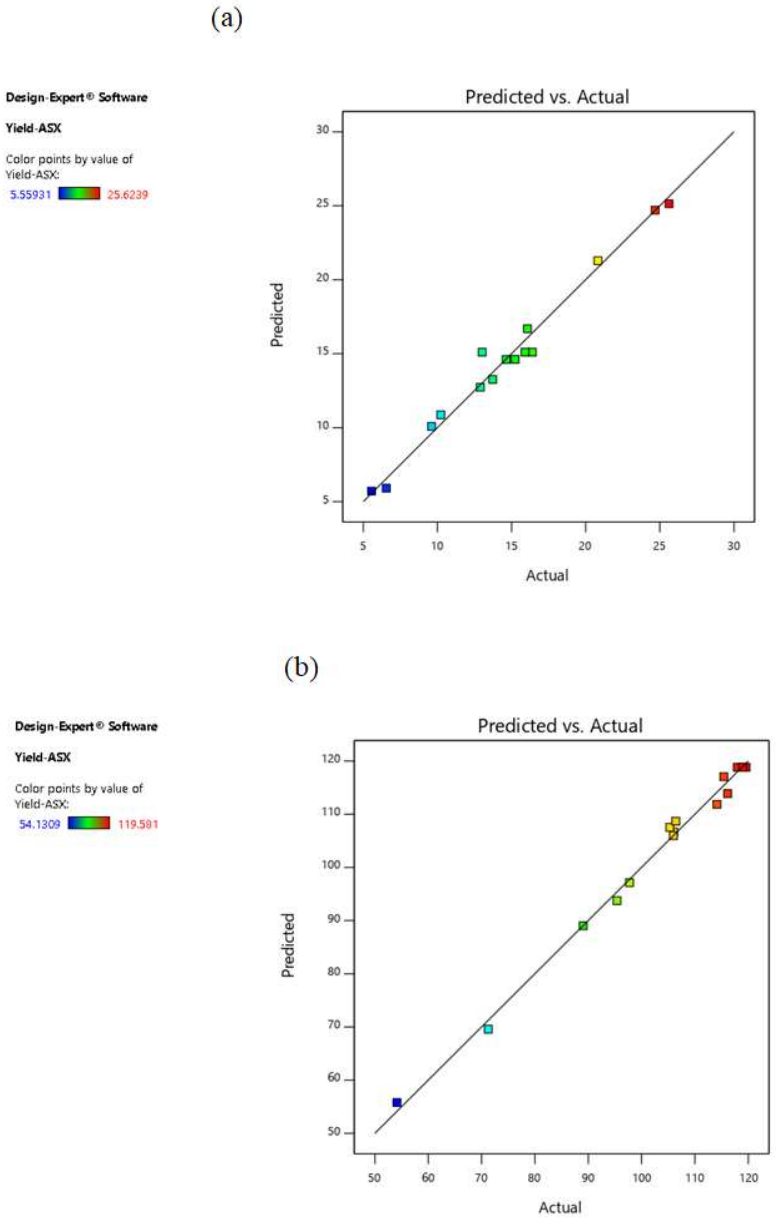

Figure 4.4: Predicted values of astaxanthin (ASX) yields vs. experimental values for wet (a) and freeze-dried (FD) (b) residues.

4.4.4.2. Response surface analysis

The surface plots of the model in two dimensions (2D) and three dimensions (3D) are a convenient way to visualize the effect of the independent variables (temperature, time, and oil:waste ratio) on the yield. The 3D response graphs indicate the effect of two independent variables on a dependent variable, when the third variable remains constant at the center point.

4.4.4.2.1. Effects of temperature, time and their interaction on ASX yield

The response surface graphs (Fig. 4.5a and b) for ASX yield, as a function of temperature and time at a set oil:waste ratio of 6:1 v/w show the impact of temperature and time and interactions. At a fixed oil:waste ratio, the longer the extraction time or the higher the temperature, the higher the yield. However, when temperature is increased at an extraction time of 3 h (highest time studied) or time is increased at the highest temperature studied, the yield decreases (due to a negative impact of temperature/time interaction), which has also been reported previously [35,39,44]. For example, for wet residues, at the shortest extraction time (1 h), the ASX yield increased (6.55-20.83 $\mu g/g_{waste}$) with an increase in temperature to 70 ˚C; however, increasing temperature at 3 h decreased yield (13.73-10.23 $\mu g/g_{waste}$). This is likely a result of the higher temperature combined with longer extraction times which acts to enhance ASX thermal degradation. The same trend was observed in FD residues; increasing the extraction time to 3 h at the lowest temperature studied increased the ASX yield (54.13-116.14 $\mu g/g_{waste}$) while the yield decreased from 105.30 to 71.25 $\mu g/g_{waste}$ at 70 ˚C as extraction time increased. Razi Parjikolaei et al. [34] observed a reduction in ASX yield with sunflower oil as extraction time increased at 70 ˚C and attributed it to thermal degradation. Sachindra and Mahendrakar [25] investigated

temperatures up to 100 ˚C and observed similar behaviour; an increase in temperature above 70 ˚C decreased ASX yield.

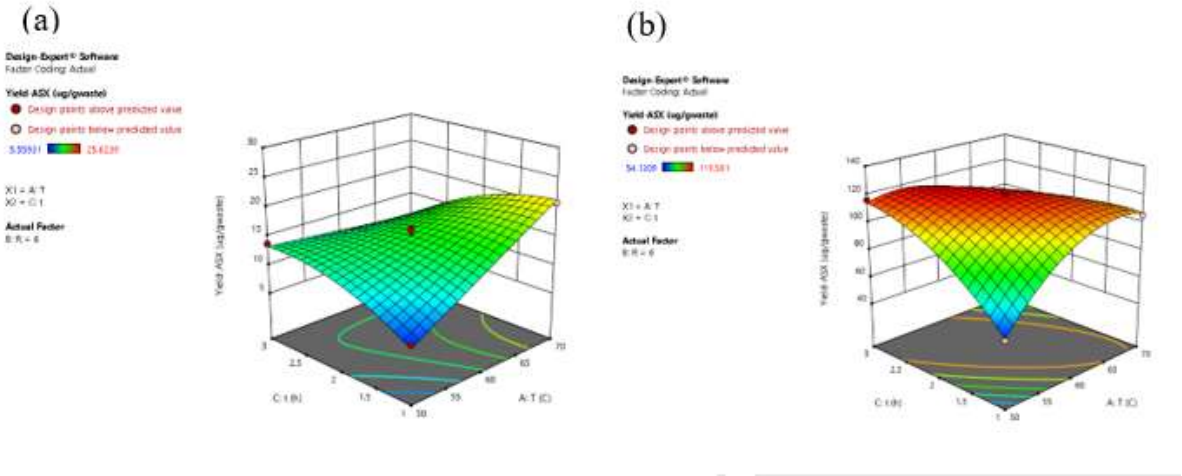

Figure 4.5: Response surface plot of interaction between temperature and extraction time effects on astaxanthin (ASX) yield (a) for wet and (b) for freeze-dried (FD) residues; A: temperature (T, ˚C), C: time (t, h) at oil:waste ratio (R= 6:1 v/w).

As noted above, ASX yield is impacted by mass transfer rate, reactions (ASX hydroxyl groups with other compounds in oil), degradation rate, and solubility [35,44,45]. ASX in crustaceans such as shrimp can be found in free, esterified, or complexed forms (with proteins or lipoproteins) [8,27,34,35,46,47]. One or both hydroxyl groups of free ASX may be esterified (monoester and diester) with fatty acids (such as palmitic, oleic, stearic or linoleic), which easily dissolves in oils [34,35]. The mechanism of extraction of ASX using lipid-based oils, is a combination of mass transfer and potential hydrogen bonding between ASX and oil (reaction of the hydroxyl groups in ASX with fatty acids) [35,44]. Increasing the extraction time (when temperature and oil:waste ratio are constant) favours the reaction or esterification of hydroxyl groups in free ASX with fatty acids in oils, enhancing

extraction [35,44]. As such, at the lowest temperature studied (50 ˚C) ASX yield increased with time (1-3 h).

Similarly, increasing temperature may enhance the esterification reaction as the reaction rate increases with temperature [35]. Further as temperature increases, the viscosity of the oil decreases (enhancing diffusivity of ASX in oil) and solubility of ASX increases, resulting in an overall increase in ASX yield [17,34,35,48]. Again, this in part explains the increase in ASX yield with an increase in temperature at short extraction times (1 h).

Water content, temperature, and contact with atmospheric oxygen [48] affect the stability of ASX, and ASX can degrade as extraction (reaction) time increases [44,49] due to electron-rich conjugated double bonds in its structure [50,51]. High temperatures can also reduce the ASX yield due to the instability of the free form of ASX [44]. As noted above, the low yield of ASX using sunflower oil at 60 and 70 ˚C was a result of the degradation of ASX at these temperatures [44].

In this work, we observed a decrease in the ASX yield using waste fish oil at high temperature (70 ˚C) with an increase in the extraction time (1-3 h), which is likely due to higher rates of ASX degradation and isomerisation at high temperature [34,39,49,52]. This agrees with the literature using sunflower oil for ASX extraction from wet shrimp by-products [25,34] where increasing the temperature and extraction time decreased ASX yield.

4.4.4.2.2. Effects of oil:waste ratio and its interaction with time on the ASX yield

The ASX yield increased with an increase in oil:waste ratio over the range of temperature (50-70 ˚C) for both wet and FD residues at short extraction times. For example, at 2 h, an

increase in the ratio from 3:1 to 9:1 v/w, the ASX yield for wet residues increased by 77 % at 50 ˚C and 66 % at 70 ˚C. In the case of FD residues, the yield increased by 20 % at 50 ˚C and 10 % at 70 ˚C. Razi Parjikolaei et al. [34] reported that increasing the ratio of sunflower oil to waste from 3:1 to 9:1 v/w increased the yield 71 % at 25 ˚C and 17 % at 45 ˚C for wet residues [34]. This is due to an increase in ASX mass transfer between solid and oil phases driven by solubility and concentration gradients [34,53,54].

As shown above, when comparing wet to FD yields, changes in the oil:waste ratio have a larger impact on wet extractions. The limited solubility of ASX in water would limit the diffusion of ASX out of the shrimp solid phase [41]. Thus, wet extraction requires more fish oil to overcome the high-water content compared to FD extraction for a given set of conditions. Given the lower water content, mass transfer is reduced significantly in FD residues. Therefore, increases in the oil:waste ratio have a much more subdued impact. This has an effect on process scale up in that less oil is required for FD extractions.

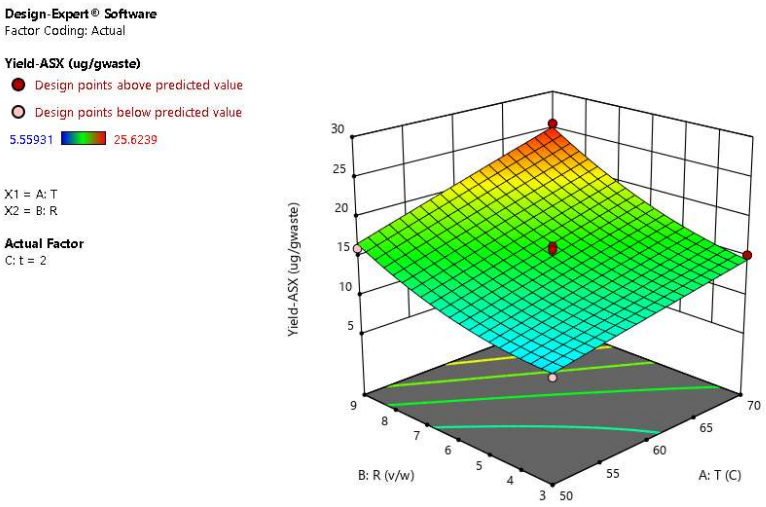

Figure 4.6: Response surface graph for astaxanthin (ASX) yield from wet residues as a function of ratio (B: R, v/w) and temperature (A: T, ˚C) at time (t)= 2 h.

As observed in Fig. 4.7, for wet residues, at a shorter extraction time (1 h), the ASX yield increased from 5.56 to 24.70 µg/g_{waste} with an increase in the ratio to 9:1 v/w at 60 ˚C. This agrees with that reported by Razi Parjikolaei et al. [34], who found that an increase in the ratio over shorter extraction time increased ASX yields obtained using sunflower oil. However, at the longest extraction time (3 h), there was a slight reduction in the yield (14.63- 12.89 µg/g_{waste}) with the increase in the ratio to 9:1 v/w (Fig.4.7). This is likely due to the fact that thermal degradation accelerates with time over extraction of ASX. Sachindra and Mahendrakar [25] also observed a slight reduction in the yield with an increase in a ratio from 2:1-3.5:1 v/w over 2-3 h [25].

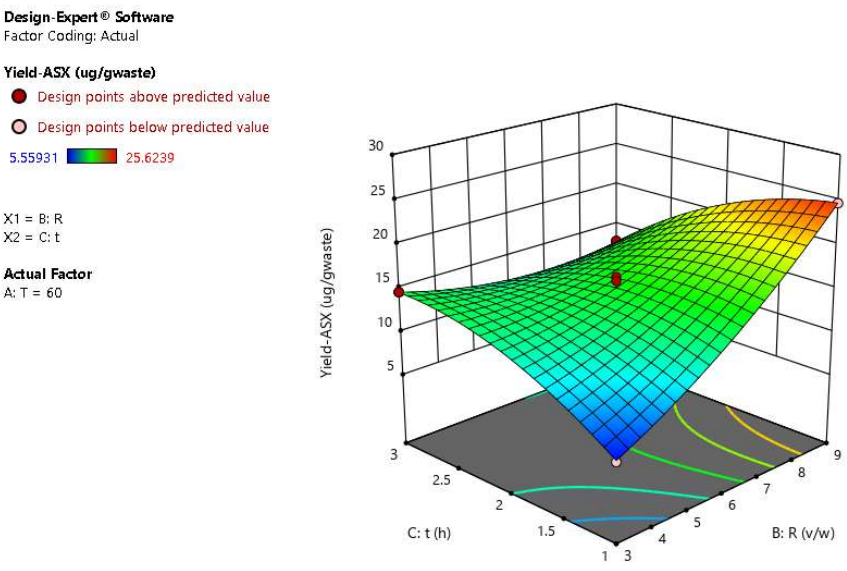

Figure 4.7: Response surface graph for astaxanthin (ASX) yield from wet residues as a function of time (C: t, h) and ratio (B: R, v/w) at temperature (T) = 60 ˚C.

4.4.4.3. Optimal extraction conditions for maximum yield of ASX

The optimum levels of independent factors (temperature, time and the ratio) were predicted using response optimization. It should be noted, this "optimum" is for this set of conditions

and could be used as a tool in scale-up. The numerical optimization showed the ASX yield was optimized (Fig. AI-1) between temperatures 65-70 ˚C, range of 8:1-9:1 v/w and approximately 1.5 h for wet residues, and 55- 65 ˚C, 8:1- 9:1 v/w and less than 2 h for FD residues. Sachindra and Mahendrakar [25] reported the optimum ASX yield from wet shrimp by-products using sunflower oil at 70 ˚C, oil to waste ratio of 2:1 v/w for 2.5 h. Razi Parjikolaei et al. [34] reported an extraction time of 2 h with the temperature of 70 ˚C and the ratio of 9:1 v/w as optimal conditions for maximum ASX yield using sunflower oil.

As ASX is sensitive to temperature, light and oxygen, lower temperature and shorter extraction time are advisable for optimum extraction yield of ASX from shrimp by-products. Therefore, in this study, we suggested 65 ˚C, 9:1 v/w and 1.5 h to obtain the maximum yield of ASX from both wet and FD residues using waste fish oil.

4.4.4.4. Comparison of optimal yields with published work

Razi Parjikolaei et al. [34] extracted ASX from wet shrimp by-products (*Pandalus borealis*) using sunflower oil with ratios of 3:1-9:1 v/w at 25, 45, and 70 ˚C for 24 h. The yield increased with temperature at shorter extraction times, and decreased with time due to thermal degradation. At 9:1 v/w and 6 h, ASX yield increased from 14.7–22.2 $\mu g/g_{waste}$ with an increase in temperature from 25-45 ˚C, while no significant changes in ASX yield were observed with increasing temperature to 70 ˚C. They obtained the same maximum ASX at 45 ˚C and 6 h as at 70 ˚C and 2 h. In their work the optimum operating condition was at 2 h and 70 ˚C with a ratio of 9:1 v/w; these conditions were chosen based on scaling up to the industrial level [34]. Sachindra and Mahendrakar [25] studied ASX extraction from wet shrimp by-products (*Penaeus indicus*) using sunflower oil with ratios of 0.5:1-

3.5:1 v/w at 40- 100 ˚C over 2-3 h. A reduction in the yield was observed with an increase in temperature beyond 70 ˚C and 2.5 h; thus, they chose 70 ˚C and 2.5 h as optimal conditions. The maximum ASX yield (25.6 $\mu g/g_{waste}$) was extracted at 70 ˚C, 2.5 h with the ratio of 2-2.5 v/w [25]. In our study, for wet residues, the maximum ASX was 25.62 $\mu g/g_{waste}$ extracted using waste fish oil at 70 ˚C, 9:1 v/w for 2 h, which is higher than that extracted using sunflower oils in Razi Parjikolaei et al. [34], and the same reported by Sachindra and Mahendrakar [25].

In work using palm oil on FD shrimp at 3 h, 6:1 v/w, 50-70 ˚C for particle sizes of 0.18-0.43 mm, ASX yield increased with temperature regardless of the particle size. The highest yield (132 $\mu g/g_{dry\ waste}$) was obtained for the smallest particle at 70 ˚C. In this study, the maximum yield for FD with the same ratio (6:1 v/w) was 120 $\mu g/g_{dry\ waste}$ at 3 h/50 ˚C to 123 $\mu g/g_{dry\ waste}$ at 2 h/60 ˚C. At the same conditions as the palm oil maximum, the ASX yield for FD fish oil extraction was 80 $\mu g/g_{dry\ waste}$ (particle size of 0.1 mm).

As shown above, performance of oils as a solvent in ASX recovery can vary depending on ranges of operating conditions studied, shrimp species, particle size, water content and heating processes. In this study, the maximum ASX yield (25.62 $\mu g/g_{waste}$) obtained from wet residues at 9:1 v/w and 2 h and 70 ˚C was 56 % of the maximum yield from Soxhlet extraction (40:60 vol% hexane/acetone). With FD residues, an oil:waste ratio of 6:1 v/w over 3 h and 60 ˚C gave the maximum ASX yield (123 $\mu g/g_{dry\ waste}$) which was 45 % of ASX recovered using Soxhlet with 40:60 vol% hexane/acetone.

Table 4.6 shows lipid compositions in waste fish oil and pigmented fish oil extracts compared to shrimp extract using hexane/acetone (40:60 vol%) in Soxhlet for wet and FD residues. Our work showed the pigmented fish oil extract from the FD extraction had higher FFA and lower higher PL compared to wet extraction. This is can due to freeze-drying impact on the lipid compositions. High FFA translates to oil acidity and has a negative impact on the stability of the oil as the higher amount of FFA is an indication of increased oxidation [55]. Moreover, waste fish oil is rich in TAG, thus the use of the oil in ASX extraction provides pigmented oil with higher TAG compared to shrimp oil extracted using Soxhlet that provides higher PL. PL are more bioavailable and provide superior health benefits, compared to TAG rich oils [56]. Thus, the lipid distribution is key in decisions around nutritional.

Table 4.6: Lipid compositions in waste fish oil and pigmented fish oil extracts compared to shrimp oil extracted using hexane/acetone (40:60 vol%) in Soxhlet for wet and freeze-dried (FD) residues.

	Pigmented fish oil extracts from wet residues[b]			Pigmented fish oil extracts from FD residues[b]			Oil solvent	Soxhlet samples	
Lipid compositions[a], wt%	t2R9, 70 ˚C	t1R6, 70 ˚C	t3R6, 50 ˚C	t3R6, 70 ˚C	t1R6, 70˚C	t3R6, 50 ˚C	Waste Fish oil	Shrimp oil from FD residues	Shrimp oil from wet residues
TAGs	61.68	57.31	77.04	66.15	66.35	74.69	59.94	12.65	6.80
FFAs	2.73	2.99	0.00	12.29	11.73	14.03	2.74	10.74	5.93
PLs	16.60	29.68	22.20	0.00	0.00	0.04	28.42	33.93	68.62
STs	7.11	8.64	0.00	3.19	3.03	3.55	0.92	22.16	8.98
AMPLs	7.98	0.00	0.76	18.37	18.58	7.69	7.11	4.02	4.7

[a] triacylglycerols (TAG), free fatty acids (FFA), phospholipids (PL), sterols (ST), and acetone mobile polar lipids (AMPL).
[b] time (t), ratio (R); e.g., t2R9 represents t in 2 h and R as 9:1 v/w.

In addition to knowledge of the lipid classes, the FA distribution (Table 4.7) is also key with respect to downstream processing and applications. The waste fish oil (salmon) used in this work has a FA distribution of 20 wt% SFA and 0.8 PUFA:MUFA ratio with 13 wt% omega (n)-3 and 18 wt% n-6 FAs. Temperature, time and ratio did not have an impact on the FA compositions of the extracts. Only a slight decrease in SFA, and an increase in MUFA was observed for the pigmented fish oil extracts with temperature, time and ratio. The FA distributions with the pigmented fish oil extracts were similar to Soxhlet for SFA, MUFA, and PUFA but the n-3 FA was 0.4 of Soxhlet extracts while n-6 FA in fish oil extracts was 6 times higher.

Table 4.7: FA compositions of waste fish oil and pigmented fish oil extracts compared to Soxhlet for wet residues.

	Pigmented fish oil extracts from wet residues[a]						Oil solvent	Soxhlet for wet residues
FA compositions, wt%	t2R9, 70 ˚C	t2R3, 70 ˚C	t2R9, 50 ˚C	t3R6, 50 ˚C	t3R9, 60 ˚C	t1R9, 60 ˚C	Waste fish oil	Shrimp oil
C14:0	0.06	0.02	0.02	0.04	0.03	0.02	2.38	2.19
C16:0	14.98	15.33	14.85	14.50	15.06	14.54	13.13	14.85
C16:1n7	7.56	7.43	7.48	7.53	7.56	7.45	5.12	7.67
C18:0	3.86	3.95	3.72	3.66	3.75	3.70	3.98	3.00
C18:1n9	41.32	42.18	41.64	41.88	41.30	41.62	35.63	15.95
C18:1n7	0.18	0.15	0.15	0.16	0.16	0.17	3.00	6.00
C18:2n6	16.90	16.69	17.10	17.10	17.02	17.06	14.85	1.39
C18:3n6	0.38	0.33	0.38	0.38	0.39	0.39	0.29	0.01
C18:3n3	3.29	3.18	3.36	3.34	3.36	3.36	2.75	0.42
C20:1n9	0.83	0.85	0.87	0.86	0.82	0.88	1.84	4.02
C20:4n6	0.66	0.57	0.64	0.66	0.64	0.67	0.56	1.11
C20:5n3, EPA	4.30	3.85	4.15	4.20	4.24	4.31	3.57	16.85
C22:5n3	1.84	1.75	1.78	1.81	1.80	1.87	1.63	0.70
C22:6n3, DHA	3.65	3.58	3.69	3.71	3.70	3.79	3.29	12.75
Σ SFA	18.91	19.29	18.59	18.20	18.84	18.27	20.25	21.65
Σ MUFA	49.89	50.60	50.13	50.44	49.84	50.13	47.4	41.96
Σ PUFA	31.20	30.11	31.28	31.35	31.32	31.61	31.96	35.37
n-3	13.08	12.35	12.99	13.07	13.09	13.32	12.93	32.28
n-6	18.12	17.75	18.29	18.29	18.23	18.28	17.79	2.48

[a] time (t), ratio (R); e.g., t2R9 represents t in 2 h and R as 9:1 v/w.

4.4.5. Conclusions

Extraction of ASX from the Atlantic shrimp by-products (*Pandalus borealis)* using waste fish oil was studied as a function of process conditions (temperature, time and oil:waste ratio) and pre-treatment (drying). Lower water content in by-products translated to higher ASX yield in the extract. At the low temperature range of this study (50 ˚C), ASX yield increased with time and yield also increased with temperature at short extraction times (1 h). However, at the highest temperature studied in this work, yield decreased with increasing extraction time and at the longest extraction time yield decreased with increased temperature. Increased oil:waste ratio increased ASX yield. Thermal degradation at high temperatures and longer reaction times combine to decrease the yield at high temperatures and extraction times. This work determined 65 ˚C, 9:1 v/w and 1.5 h using BBD to maximize yield of ASX from both wet and FD residues using waste fish oil. The maximum yields of ASX extracted for wet and FD residues were the same or higher compared to published work using sunflower/palm oils. Although ASX extractions using waste fish oil were lower compared to Soxhlet, the pigmented fish oil extracts were higher in TAG and n-6 FAs. Further, freeze drying impacts lipid compositions as higher FFA and lower PL were observed in pigmented fish oil extract from the FD extraction compared to wet extraction. This is the first report to evaluate and optimize ASX recovery using waste fish oil from waste fish in extraction of ASX from shrimp residues. This study suggests that fish oil from waste fish can be a sustainable alternative to vegetable oil/organic solvents for ASX recovery. These results can be used as a reference to evaluate other shrimp species

by-product utilization and the quality of ASX in a fish oil (lipid) medium in food/medical applications.

Conflict of interest statement

We know of no conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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Chapter 5 Experimental experiments and mathematical modeling of supercritical CO_2 extraction

Supercritical CO_2 Extraction of Lipids and Astaxanthin from the Atlantic Shrimp By-products with Static Co-solvents:

Optimization, Kinetics Experiment and Mathematical Modeling Studies

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Abstract

Shrimp processing by-products from harvesting and processing are a source of valuable biomaterials/bioactive materials (such as lipids and astaxanthin). Extraction of these compounds could not only decrease environmental impacts associated with shrimp harvesting but also result in economic benefits. This work provides the first comprehensive analysis of co-solvent effectiveness and pressure/temperature in lipid compositions of extract from the Atlantic Northern shrimp by-products (*Pandalus borealis*). In this study, extraction conditions (pressure and temperature) were studied to determine optimal operating conditions to maximize lipid and astaxanthin (ASX) yields and significant impacts on yields using the central composite design (CCD). This work also provides a

study on the evaluation of the impact of static co-solvents on lipid/ASX recovery using supercritical CO_2 extraction (SC-CO_2) at the "optimum" pressure and temperature from the response surface methodology (RSM) analysis. Furthermore, the SC-CO_2 extraction rate of lipids/ASX at the "optimum" pressure/temperature was experimentally studied and then validated using the Goto et al. model.

The highest lipid yield was extracted at 50 ˚C and 30 MPa and at 60 ˚C and 32 MPa the highest ASX yield, and total carotenoid content (TCC) were obtained. The conditions that maximize lipid and ASX yields, and TCC were 50 ˚C and 30 MPa. Lipid/ASX recovery increased with an increase in pressure; however, the impact of temperature was more complex. Overall extraction rates of lipid/ASX were controlled by the strong solid–solute interaction. Pure SC-CO_2 extract had high percentages of neutral lipids but low phospholipids. Over the range of temperature and pressure studied, there was no impact on FA compositions. Static co-solvent processing resulted in the same lipid yield and a higher ASX yield compared to studies using a continuous co-solvent/SC-CO_2 process. The addition of a polar co-solvent increased lipid/ASX recovery and increased extract phospholipids (PLs) and saturated fatty acids (SFA) but decreased monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFAs). Sunflower oil as a co-solvent recovered higher ASX compared to waste fish oil. The lipid profile of the extract using sunflower oil as a co-solvent reflected the composition of the sunflower oil while the extract using waste fish oil had higher sterols (STs) and free fatty acids (FFAs) compared to the lipid profile of waste fish oil.

A version of this paper has been published in the *Journal of CO_2 Utilization.* The lead author is Sara Ahmadkelayeh and co-authors are Dr. Kelly Hawboldt and Dr. Sukhinder Kaur Cheema. Miss Ahmadkelayeh's contributions to this paper include the following:

- writing the paper
- performing all the laboratory testing and analyses (except where noted)
- conducting all data processing and interpretation of results
- optimizing all data using Design-Expert software
- mathematical modeling using coding in MATLAB
- performing all literature searches required for background information
- writing the paper and performed all literature searches required for background information

Dr. Hawboldt and Dr. Cheema provided technical guidance and editing of the manuscript.

5.1. Introduction

Shrimp processing by-products include the head, tail and shell, and accounts for over 50% of the landed product [1–3]. Shrimp by-products are a source of value-added compounds, particularly carotenoids (mainly ASX) and lipids. ASX has health benefits due to its high antioxidant properties and is used as a source of pigmentation/feed additive in the aquaculture and food industries. Marine sourced lipids are rich in PLs and omega (n)-3 FAs which have important applications in medicine [1,4–6]. Recovery of lipids and ASX from shrimp processing by-products could provide economic benefits and reduce environmental impacts associated with processing [4,5,7,8].

Lipids and ASX are traditionally extracted using organic solvents at room or elevated temperatures and atmospheric pressure [9]. Organic-solvent based processes have disadvantages including high costs and potential product degradation (for higher temperature processes and prolonged extraction times), and toxicity of solvents [1,8]. One promising alternative for heat-labile and compounds prone to oxidation is SC-CO_2. SC-CO_2 can extract soluble compounds at relatively low temperatures (40-50 ˚C) and high pressures (30-50 MPa) over a short extraction time without any subsequent separation steps. The solubility of the target extract and the selectivity power of solvent can be improved by manipulating temperature and pressure [5,6]. CO_2 is inert, non-toxic, non-flammable, and ideal for use in the food industry, and deemed as GRAS (generally regarded as safe) [4,6]. Despite high capital investment costs (initial costs), this technology can be profitable on a large scale, especially for value-added compound extraction from industrial waste [8]. SC-CO_2 is a non-polar solvent and can extract non-polar and

low/medium molecular weight compounds with slight polarity [10]. However, polar lipids and ASX have relatively low solubility in supercritical CO_2 and therefore must operate at the higher end of the pressure range (>40 MPa), or a polar co-solvent must be added [1,9]. Adding polar solvents (e.g., ethanol) to SC-CO_2 increases the density and polarity of SC-CO_2 and consequently increases the target compound solubility. A few studies have investigated the impact of co-solvents (organic solvents and vegetable oils) on lipid/ASX recovery using SC-CO_2 from shrimp by-products [8,11,12]; Radzali et al. [11] showed that adding pure ethanol or pure methanol to SC-CO_2 increased the ASX yield and gave the higher yield compared to when aqueous ethanol (50-70 vol% in water) or aqueous methanol (50-70 vol% in water) was added [11]. The most common organic co-solvent used is ethanol [1,5,11,13–15]. Ethanol increases lipid yield due to the increased solubility of polar compounds such as phospholipids and more polar carotenoids such as ASX in SC-CO_2 [6,16–18]. The presence of ethanol in SC-CO_2 enables the formation of hydrogen bonds with ASX and swells the pores of the solids matrix which eases ASX extraction [1,11,15].

Sunflower oil and its methyl ester have been used as alternative co-solvents in SC-CO_2 for ASX recovery from shrimp by-products [8,15]; with sunflower oil/SC-CO_2 ratio of 5 wt% ASX yield ranged from 4.8×10^{-4} (for cooked/dried residues at 50 ˚C, 30 MPa, 3 h, 13.3 g/min) to 25 $\mu g/g_{dry\ waste}$ (for dried residues at 55 ˚C, 40 MPa, 3 h, 20 g/min), and with 5 wt% methyl ester of sunflower oil/SC-CO_2 ratio, ASX yield was 38 $\mu g/g_{dry\ waste}$ (for dried residues at 55 ˚C, 40 MPa, 3 h, 20 g/min). There is no publication on marine oils as a co-solvent in SC-CO_2.

While SC-CO_2 has advantages over solvent-based extraction processes [9], there are limited studies on lipid/ASX extraction using SC-CO_2 from shrimp by-products [1,5,6,8,11,15,19,20]. Four of these studies investigated the impacts of temperature/pressure and co-solvents on lipid/ASX recovery: two on the quality, lipid/ASX yields and FA distributions [1,5], one on the quality, lipid/ASX yields and carotenoid distributions [8] and the other on only lipid/ASX yields [15]. There is one study on the effect of temperatures/pressures on lipid/ASX recovery combined with FA compositions [6]. Another evaluated the impact of operating conditions (time, temperature, pressure, flow rate and co-solvent) on only lipid yield and FA distributions [20]. The impact of co-solvents on ASX yield and carotenoid distributions has been studied [11]. Optimization of operating conditions for ASX yield and quality using SC-CO_2/ethanol was studied in [19]. Although these studies are useful, there is no comprehensive study that encompasses all of the above and no work in the impact of operating conditions (temperature, pressure and co-solvents) on lipid compositions (lipid profiles) and distribution of lipids/ASX in the extract. This data is important as the distribution (i.e., quality) has a vital role in decisions around the final application of the extract.

The mechanism of bioactive extraction under supercritical conditions from a solid matrix can be simplified into three steps: (1) local desorption of the solute and solubilisation in solvent; (2) internal solute/solvent diffusion through the pores to the surface; and (3) external solute diffusion into the bulk medium [21]. The initial extraction rate (step 1) is controlled by solute-fluid phase equilibrium. To assess the feasibility of SC-CO_2 on a larger scale, models which describe fundamental mass transfer phenomena are required; however,

there has been only one study on the modeling of SC-CO_2 for recovery of lipids from shrimp by-products [8]. Mezzomo [8] used Sovová model [22] to describe SC-CO_2 extraction of lipids from shrimp by-products. The Sovová model [22] assumes plug flow for the fluid phase with no accumulation in the fluid phase [22]. In this model [22], solute-solid interaction is not considered in the mass balance equations, and therefore, the initial extraction process is controlled by the solubility equilibrium between the solute and the fluid phase where the external mass transfer process was dominant. However, depending on the initial solute concentration in the sources, the phase equilibrium can be controlled by the solute-fluid interaction (solubility) or the solute-solid interaction (adsorption) [23,24]. In the Goto et al. model, the differential mass balance equations were written for three phases; the solid matrix, the pores within the solid (with solute-solid interaction), and the fluid phases (assuming well-mixed flow) [25]. Goto et al. [25] showed that due to the high solute-interaction, the SC-CO_2 extraction of essential oil from peppermint leaves was slow and the fluid-phase equilibrium concentration was lower than that predicted by oil solubility. This means the local equilibrium was controlled by solute–solid interaction. In a separate study [26], the extraction rate of cuticular waxes was controlled by mass transfer process and the solute concentration in fluid leaving the extractor was close to solubility limit (due to the low solute-solid interaction) [26].

The first objective of this study is to investigate SC-CO_2 optimization of lipids/ASX from processing by-products of Atlantic shrimp (*Pandalus borealis*). Extraction conditions (pressure and temperature) are studied to determine significant impacts on lipid/ASX yields using CCD. CCD is RSM used in optimization studies. The second objective is to evaluate

the impact of co-solvents on lipid/ASX recovery using SC-CO_2 at the "optimum" pressure and temperature from the RSM analysis. In all published SC-CO_2/co-solvent studies except one [27], co-solvents are continuously pumped to an extraction system and mixed with SC-CO_2 flow at a constant ratio over a fixed extraction time. The disadvantages of working with continuous co-solvent input in lab-scale studies are considerable amounts of solvents and extra equipment such as extra co-solvent pumps and tanks, etc., are required. In this study, we used a static co-solvent system (outlined in [27]) and then compared results with continuous approaches in the literature to validate the approach [1,8]. Co-solvents used in this study are; ethanol, 40:60 vol% hexane/acetone, sunflower oil, and waste fish oil. These solvents were chosen based on an extensive literature review and work in our lab [9]. Waste fish oil is of particular interest as it is associated with finfish processing by-products and often represents a disposal cost. Waste fish oil was extracted from salmonid aquaculture processing by-products using a method outlined in previous work [28]. The use of waste finfish oil as a solvent in ASX extraction is potentially more feasible than vegetable oils as "waste" fish oil from fish processing and/or fishmeal production are easily accessible due to co-locations of processing plants. Lipid/FA compositions of SC-CO_2 extracts extracted either with or without co-solvents are compared. The third objective is to study the SC-CO_2 extraction rate of lipids/ASX at the "optimum" pressure and temperature from the RSM analysis. In addition, the rate equation describing the extraction of lipids and ASX was determined using the Goto et al. model [25]. As noted above, this model is made of up mass balances for fluid, pores and the solid phase, and the linear local adsorption equilibrium relation is used to model solute-solid interaction.

This study provides the first comprehensive analysis of lipid compositions of extract as a function of pressure/temperature and co-solvents. Furthermore, SC-CO_2 performance was compared to solvent-based extraction processes.

5.2. Materials and methods

5.2.1. Materials and chemicals

St. Anthony Basin Resources Incorporated (SABRI) shrimp processing plant (NL, Canada) supplied the Atlantic shrimp processing by-products (*Pandalus Borealis*). Shrimp by-products were stored at -30 ˚C until use. Waste fish oil was provided from salmonid aquaculture offal using the modified fishmeal process developed in our previous work [28]. Sunflower oil (100% pure) was bought from a local market (Compliments). The standard ASX (≥92%, A9335) used for UV-vis analysis to obtain calibration curve was purchased from Sigma–Aldrich Co. (CAS registry No. 472-61-7). Hexane (CAS registry No. 110-54-3), acetone (CAS registry No. 67-64-1) and ethanol, anhydrous (100%) were of ACS grade and purchased from Fisher Scientific and ACP Chemicals Inc.

5.2.2. Preparation of shrimp by-products

Shrimp by-products were ground and freeze-dried using a freeze drier (Labconco® Freeze Dry Systems, 6 L Benchtop Models, US) operated at -52 ˚C over 72 h and then kept in a desiccator at - 20 ˚C to keep water content stable. The freeze-dried shrimp by-products, refer to FD residues, contain 12.85 wt% water.

5.2.3. Dynamic SC-CO_2 without co-solvents

Approximately 3 g of FD residues was loaded into a 13.6 cm^3 extraction vessel (Penn Manufacturing Inc.,10,000 Psig @194°F, USA) per experimental run. The extraction

vessel and inlet/outlet tubing were heated with heating tapes (Omega Engineering, Inc., USA; model HTWC101-010) to keep the system at a certain temperature (46-74 ˚C). The liquid CO_2 was compressed to the desired pressure (18-32 MPa) and then continuously pumped to the extractor at a constant flow rate (2.76 g/min which is equivalent to 1.5 L/min of CO_2 measured at atmospheric and room temperature) over 3 h. SC-CO_2 continuously flowed through the system while the outlet needle valve was open over 3 h, and SC-CO_2 with the extract was depressurized to gaseous CO_2 in a collection bottle. At the end of every extraction, the outlet tubing was washed with hexane to recover the extract retained in the system. To quantity the mass of CO_2 used in extraction, the gaseous CO_2 leaving the collection bottle flowed through a gas flow meter and totalizer – XFM series (Aalborg Instruments & Controls, Inc. USA). The collected extracts after removing solvent were weighed at the end of each experiment, and stored at 4 ˚C.

5.2.4. Kinetic SC-CO_2 extraction without co-solvents

Kinetic SC-CO_2 without co-solvents was run the same as outlined above but run at 1.5 L/min, 50 ˚C and 30 MPa over 3 h. The extract was collected each 30 min over 3 h to measure total lipid and ASX amounts versus time or consumed CO_2 amount per 30 min. The collected extract representing the total lipid mass was weighed and stored at 4 ˚C till UV analysis to measure ASX concentration (µg/mL) in the extract. ASX amount (µg) in each extract was calculated by multiplying ASX concentration (µg/mL) by the volume (mL) of the extract diluted in the solvent, 80:20 vol% hexane/acetone.

5.2.5. Dynamic SC-CO_2 with static co-solvents

FD residues (1-2 g) was mixed with a co-solvent at a ratio of 5 v/w and loaded into the extractor. The liquid CO_2 was compressed and heated to 50 ˚C and 30 MPa (determined from CCD optimization). After reaching the specified temperature and pressure, SC-CO_2 was loaded into the extractor and left for 1 h. This static time is to provide enough contact time between the residues, co-solvent and SC-CO_2, and avoid loss of co-solvent. After the static extraction period, CO_2 continuously flowed through the system at a constant flow rate of 1.5 L/min for 1 h. During the 1-h dynamic extraction, the flow with the extract was collected in an atmospheric separation vessel. As with the experiments without co-solvent, at the end of extraction, the outlet tubing was washed with hexane to recover the extract and CO_2 was quantified using the gas flow meter and totalizer. The collected/dried extracts after removing hexane were weighed at the end of each experiment, and stored at 4 ˚C.

5.3. Extract quantity and quality

The extract quantity was measured as total lipid yield (dry wt%) and ASX yield ($\mu g/g_{waste}$) and quality by TCC in the extract ($mg/g_{extract}$) UV-Vis-NIR spectrophotometer (Agilent Cary 6000i) was used for ASX concentration measurement. Lipids were analyzed using an Iatroscan Mark VI TLC-FID for various lipid classes, and FA profiles were analyzed with an Agilent GC-FID equipped with an autosampler at the Ocean Science Centre (Memorial University of Newfoundland and Labrador).

5.3.1. Total lipid yield calculation

The collected extract from the extraction process and the tubing washing step represents the total lipid mass, g. The total lipid yield was calculated using Eq.5-1 on a dry wt%.

$$\textit{Total lipid yield, dry wt\%} = \frac{m_{extract}}{mass\ of\ \ dry\ biomass} \times 100 \qquad \textit{5-1}$$

Where m is mass of extract (g); mass of dry biomass (g) is calculated using shrimp by-product mass (g) and its water content.

5.3.2. ASX yield and TCC calculations

To measure ASX in the extract, the concentrated extract was re-dissolved in a mixture of hexane and acetone (80:20 vol%) before UV-analysis. The concentration of ASX (mg/L) is determined by measuring absorbance between 475-488 nm using UV-Vis-NIR spectrophotometer at CCART-SIRI/MUN Materials Characterization Facility. A calibration curve using standard ASX at different concentrations (0.5-40 mg/L) in each solvent was determined by measuring the absorbance of the standards at the λ_{max} of ASX (475 nm for 80:20 vol% hexane/acetone, 475 nm for sunflower oil, and 488 nm for waste fish oil) against the particular solvent as blank. The measured absorbances at the maximum wavelength and the obtained calibration curves were used to calculate ASX concentration in mg/L. TCC represents the extract quality and was calculated via the following formula:

$$\textit{TCC (mg/g}_{extract}\textit{)} = \frac{C_{ASX} \times V}{m_{extract}} \qquad \textit{5-2}$$

Where C_{ASX} is concentration (mg/L); V (L) is volume of the extract diluted in solvent for analysis (solvent extract solution volume); m is mass of extract (g). The ASX content in the shrimp by-products reported as ASX yield, $\mu g/g_{dry\ waste}$, dry weight basis was calculated as follows:

$$\textit{ASX yield (}\mu\textit{g/g}_{dry\ waste}\textit{)} = \frac{C_{ASX} \times V}{mass\ of\ \ dry\ biomass} \qquad \textit{5-3}$$

Where C_{ASX} is concentration (mg/L); V (mL) is volume of extract diluted in solvent or of the pigmented oil (mL); mass of dry biomass (g) is calculated using shrimp by-product mass (g) and its water content.

5.3.3. Lipid/FA profile analysis

The lipid classes and FA compositional analysis of extracts were carried out at the Ocean Science Centre (Memorial University of Newfoundland and Labrador). The lipid compositions were analyzed using thin layer chromatography (TLC) equipped with flame ionization detection analysis (FID) (Mark VI Iatroscan with silica coated chromarods). The lipid classes include straight chain hydrocarbons (HCs), steryl esters (SEs), ethyl esters (EEs), methyl esters (MEs), ethyl ketones (EKs), methyl ketones (MKs), glycerol ethers (GEs), triacylglycerols (TAGs), FFAs, alcohols (ALCs), STs, diacylglycerols (DAGs), acetone mobile polar lipids (AMPLs), and PLs.

FA compositions of the extract were analyzed on a GC-FID (HP 6890) equipped with an autosampler (7683). All results of lipid and FA compositions were expressed in percentage weight (wt%) of total lipid and FA classes.

5.3.4. Recovery of lipid/ASX using SC-CO_2

The Soxhlet process was used as the "baseline" to compare with SC-CO_2 in terms of lipid and ASX recovery:

$$Recovery = \frac{Yield\ using\ SC-CO}{Yield\ using\ Soxhl \quad or\ fish\ oil} \qquad 5\text{-}4$$

5.4. Experimental design and statistical analysis

The CCD with two factors and 5 levels (-1.41, -1, 0, +1, +1.41) was used to compare the extraction conditions for three responses, Y1, total lipid yield (dry wt%), Y2, ASX yield ($\mu g/g_{dry\ waste}$) and Y3, TCC ($mg/g_{extract}$). A total of 12 factor combinations with a center point in quadruplicate were randomly generated using design-expert software, version 11. The independent variables were extraction temperature (T in ˚C) and pressure (P in MPa).

Table 5.1: Independent variables with the levels and codes

Independent variables	Codes	Levels				
		-1.41	-1	0	1	1.41
Temperature (˚C)	T (X1)	46	50	60	70	74
Pressure (MPa)	P (X2)	18	20	25	30	32

Regression analysis, statistical significance and response surface applications were performed using design-expert 11. The terms in the regression model were evaluated with analysis of variance (ANOVA) by the F-test ($P < 0.05$) at a 95% interval of confidence level. The regression model containing 6 coefficients, including the linear and quadratic effect of factors and linear effect of interactions, was assumed to describe relationships between each response (Y1, Y2 and Y3) and the experimental factors (X1, T and X2, P) as follows:

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1}^{1} \sum_{j=i+1}^{2} \beta_{ij} X_i X_j \qquad 5\text{-}5$$

5.5. Mathematical modeling

5.5.1. Extraction mechanism and general assumptions

According to Goto et al. model [25], the solid particles have a porous structure, and the extraction mechanism includes desorption of the solute from the solid matrix, dissolution

of the solute into the solvent present in the solid pores and diffusion of the dissolved solute into the bulk solvent through intra-particle and external mass transfer [25].

In this study, the fixed-bed extraction process contains FD shrimp particles as the stationary phase and SC-CO_2 flow as the mobile phase with the following assumptions: (1) porous shrimp particles (2) two single extractable components, ASX (free/esterified ASX) and lipids consisting of free fatty acids, triacylglycerols, and phospholipids (3) no interaction between the solutes in the fluid phase or solid phase (4) isobaric and isothermal process (5) negligible radial/axial dispersion (6) fast adsorption/desorption equilibrium between the solute in pores and the solid (7) gradiantless differential bed (8) constant physical properties of SC-CO_2 (9) a parabolic concentration profile of the solute within the solid (10) spherical geometry for shrimp particles with initial concentrations of the solute, lipids and ASX which were obtained from the best Soxhlet method (11) pure solvent input, free of the solute, and no initial solute in the bed and the pores of the solid.

5.5.2. Fundamental mass balance equations

The governing models contain two differential solute mass balances in fluid and solid phases combined with local equilibrium adsorption that describes the relationship between solute in the pores and the solid. A well-mixed reactor model was assumed in this study, meaning changes of extracted solute through the bed are negligible. According to the above assumptions, the general mass balance equations for the solute in the solid and solvent phases were simplified and the differential mass balance equation for the solute in the SC-CO_2 (the bulk solvent) in the packed bed is written as:

$$\alpha \frac{\partial C}{\partial t} + \frac{C}{\tau} = -(1-\alpha)\, k_f a_p \left[C - \left(C_p\right)_{r=r_p} \right] \qquad 5\text{-}6$$

where τ is a residence time (the total bed volume divided by the volumetric flow rate of SC-CO_2 and r_p is the half of the particle diameter (d_p); a_p is specific surface area given by $a_p = 6/d_p$ for spherical particles. C_p is solute concentration in pore of particles and C is solute concentration in SC-CO_2. k_f is the external mass transfer coefficient and α is void fraction in the bed.

The differential mass balance for the solute in the pores is given as:

$$\beta \frac{\partial C_p}{\partial t} = D_e \frac{\partial^2 C_p}{\partial r^2} - (1-\beta) \frac{\partial C_s}{\partial t} \qquad 5\text{-}7$$

where C_s is solute concentration in the solid and β is solid porosity. D_e is effective intraparticle diffusion coefficient.

Desorption-dissolution-diffusion model is used to define the term $\partial C_s/\partial t$ in Eq. (5-7). In this model, the desorption rate, which is equivalent to the local extraction rate, is assumed to be reversible and linear.

$$\frac{\partial C_s}{\partial t} = k_a \left(C_p - \frac{C_s}{K} \right) \qquad 5\text{-}8$$

where $K(= ka/kd)$, the adsorption equilibrium constant, k_a and k_d represent first-order kinetic adsorption and desorption constant, respectively. When values of K are large ($K \rightarrow \infty$) the solute-solid interactions are strong, and when values of K are small ($K \rightarrow 0$) the solute-solid interactions become weaker. Instantaneous equilibrium in the pores is assumed due to a relatively fast adsorption–desorption rate assumption and Eq. (5-8) is re-written as:

$$C_s = K\, C_p \qquad 5\text{-}9$$

Internal and external mass transfer processes are combined using linear driving-force approximation defined by:

$$(1-\alpha)k_f a_p\left[C-\left(C_p\right)_{r=r_p}\right]=\frac{15\ e}{R^2}(1-\alpha)\left[\left(C_p\right)_{r=r_p}-C_p\right] \quad 5\text{-}10$$

Initial and boundary conditions are:

$$D_e\left(\frac{\partial C_p}{\partial t}\right)_{r=r_p}=k_f\left[C-\left(C_p\right)_{r=r_p}\right] \quad 5\text{-}11$$

$$C\ (r,t=0)=0 \quad 5\text{-}12$$

$$C_p(r,t=0)=0 \quad 5\text{-}13$$

$$C_s(r,t=0)=C_{s,0} \quad 5\text{-}14$$

$C_{s,0}$ is an initial concentration of solute in solid phase and C_p concentration of solute in the pores. The total initial concentration of solute present both in solid phase and within the pore at equilibrium is given by:

$$C_0=\left[\frac{\beta}{K}+(1-\beta)\right]C_{s,0} \quad 5\text{-}15$$

To further simplify the model equations, some dimensionless variables are defined as: x = C/C0, x_s= Cs/C0, $\theta=\frac{t}{\tau}$, and $\emptyset=k_p a_p \tau$.

where t, time and k_p, overall mass transfer coefficient, accounts for both the internal and external mass transfer resistances, for spherical geometry is given by:

$$k_p=\frac{k_f}{1+Bi/5} \quad 5\text{-}16$$

where *Bi* is dimensionless Biot number and is given as:

$$Bi=\frac{k_f\,d_p}{D_e} \quad 5\text{-}17$$

The model Eqs.(5-1)–(5-17) can be further transformed into dimensionless forms as described by Goto et al.[25]:

$$\frac{dx}{d\theta} + \frac{x}{\alpha} = -\frac{\emptyset(1-\alpha)}{\alpha}\left(x - \frac{x_s}{K}\right) \quad 5\text{-}18$$

$$\frac{dx_s}{d\theta} = \frac{\emptyset}{[\beta+(1-\beta)]}\left(x - \frac{x_s}{K}\right) \quad 5\text{-}19$$

Initial conditions are:

$$x = 0 \text{ at } \theta = 0 \quad 5\text{-}20$$

$$x_s = \frac{K}{[\beta+(1-\beta)K]} \text{ at } \theta = 0 \quad 5\text{-}21$$

These equations are simplified using Laplace transform to obtain an analytical solution of the model in terms of the dimensionless solute concentration in the bulk fluid phase given by Eq. (5-22), as:

$$x(\theta) = A[exp(a_1\theta) - exp(a_2\theta)] \quad 5\text{-}22$$

where,

$$a_1 = \frac{1}{2}\left(-b + \sqrt{b^2 - 4c}\right),\ a_2 = \frac{1}{2}\left(-b - \sqrt{b^2 - 4c}\right) \quad 5\text{-}23$$

$$A = \frac{(1-\alpha)\emptyset}{[\beta+(1-\beta)K]\alpha(a_1 - a_2)} \quad 5\text{-}24$$

$$b = \frac{\emptyset}{[\beta+(1-\beta)K]} + \frac{1}{\alpha} + \frac{(1-\alpha)\emptyset}{\alpha} \quad 5\text{-}25$$

$$c = \frac{\emptyset}{[\beta+(1-\beta)K]\alpha} \quad 5\text{-}26$$

The cumulative fraction of solute extracted up to dimensionless time θ is given by:

$$F(\theta) = \frac{1}{1-\alpha}\int_0^\theta x d\theta = \frac{A}{1-\alpha}\left[\frac{exp(a_1\theta)-1}{a_1} - \frac{exp(a_2\theta)-1}{a_2}\right] \quad 5\text{-}27$$

Therefore, the solute mass (m) at the bed outlet as a function of time can be calculated from Eq. (5-27) and Eq. (5-15):

$$m(t) = m_{s0}F(\theta)\left[\frac{\beta}{K} + (1-\beta)\right] \quad 5\text{-}28$$

where, m_{s0} is initial mass of the solutes in the solid obtained using the Soxhlet in our previous research.

5.5.3. Model parameter estimation

The initial amounts of lipids and ASX in the shrimp by-products were determined using Soxhlet extraction. The shrimp particle porosity β, was determined using a mercury porosimeter (Micromeritics Instrument Co., Auto-pore IV). The bed void fraction (α) was estimated from the volume of shrimp particles and the bulk volume of bed.

The physical properties of SC-CO_2, such as density and viscosity under the operating condition, are determined using the NIST database [29]. The binary diffusion coefficients of lipid-CO_2 and ASX-CO_2 were estimated as a function of reduced temperature, pressure, solvent density (kg/m^3) and critical molar volume of solvent, and the solvent/solute molecular weight (M_{lipids} = 239.72 g/mol, M_{ASX}= 596.8 g/mol and M_{CO2}=44 g/mol) using He et al., [30] as:

$$D_{21} = \left[0.61614 + 3.0902\, exp(-0.87756\sqrt{M_1 V_{c_1}}/P_{c_1})\right]10^{-1}\ \left(V_1^k - 23\right)\sqrt{T/M_2} \quad 5\text{-}29$$

where M_1 is molecular weight of CO_2, V_{c_1}, critical molar volume of CO_2, V_1, moral volume of CO_2, P_{c_1}, critical pressure of CO_2, T (K), temperature, M_2, the solute molecular weight and k, a correlation parameter is given by:

$$k=1 \quad \rho_r \geq 1.2 \qquad 5\text{-}30$$

$$k=1+(\rho_r - 1.2)/\sqrt{M_1} \quad \rho_r < 1.2 \qquad 5\text{-}31$$

where ρ_r is reduced solvent density.

Given the binary coefficient obtained using Eqs. (29)-(31), the mass transfer coefficient, k_f and the effective intra-particle diffusion coefficient, D_e are required. The Wakao-Funazkri correlation [31] is used to estimate the external mass transfer coefficient:

$$Sh = 2 + 1.1\, Re^{0.6} Sc^{0.33} \qquad Re \leq 3000 \text{ and } Sc \leq 10000 \qquad 5\text{-}32$$

The dimensionless parameters (Re, Sc and Sh) equations are as follows:

$$Re = \frac{\rho_1 u d_p}{\mu_1} \quad 5\text{-}33$$

$$Sc = \frac{\mu_1}{\rho_1 D_{21}} \quad 5\text{-}34$$

$$Sh = \frac{d_p k_f}{D_{21}} \quad 5\text{-}35$$

The superficial velocity, u, was estimated from the ratio of solvent volumetric flow rate to the bed cross sectional area, ρ_1 solvent density and μ_1 solvent viscosity.

$$D_e = \beta^2 D_{21} \quad 5\text{-}36$$

The experimental data related to extraction of lipid and ASX from shrimp by-products was used to predict the estimated cumulative extraction yields using the model. We determined the tuning parameter of the model, K by minimizing of the error equation, average absolute relative deviation, % (AARD) in MATLAB environment as defined below:

$$AARD\% = \frac{1}{N} \sum_{i=1}^{N} \left| \frac{F_{i,exP} - F(\theta)_{i,cal}}{F(\theta)_{i,exp}} \right| \times 100 \quad 5\text{-}37$$

where N is the number of data point, $F_{i,exp}$ and $F_{i,cal}$ are the experimental cumulative yields for lipids and ASX extraction and the estimated data using the model equation (Eq. 5-27), respectively.

5.6. Results and discussion

5.6.1. Dynamic SC-CO_2 process without co-solvents

5.6.1.1.Total lipid yield, TCC and ASX yield

To compare with the literature, results were normalized to CO_2 consumed. In this study, at 1.5 L/min, 50 ˚C, 30 MPa and 3 h the total lipid yield was 0.12 mg/g_{CO2} and ASX was 0.43 µg/g_{CO2}. Published work from FD shrimp by-products at the same conditions (plus a 20 min static time at the start) showed a lipid yield of 0.26 mg/g_{CO2} and ASX yield of

0.29 μg/g_{CO2} [5]. Our ASX yield was 1.5 times more than [5] but lipid yield was half of [5]. In a separate study with cooked/dried shrimp by-products at 60 ˚C, 30 MPa and 3 h, the lipid yield was 0.19 mg/g_{CO2} and ASX yield was 0.22 μg/g_{CO2} [8]. In this study the same lipid yield as [8] obtained at 1.5 L/min, 60 ˚C, 32 MPa and 3 h. However, our ASX yield (0.71 μg/g_{CO2}) was more than doubled [8]. The initial tests show extraction time plays a role in the balance of lipids/ASX in the extract.

Preliminary tests were carried out at different flow rates,1.5 and 3 L/min at 50 ˚C, 30 MPa and 2 h to determine the range of conditions to be studied. At 1.5 L/min, total lipid yield was 1.20 ± 0.05 dry wt%, ASX yield, 81.15 ± 5.97 μg/$g_{dry\ waste}$, and TCC, 6.76 ± 0.80 mg/$g_{extract}$. At 3 L/min, total lipid yield was 1.51 ± 0.25 dry wt%, ASX yield, 78.83 ± 9.75 μg/$g_{dry\ waste}$, and TCC, 5.11 ± 0.50 mg/$g_{extract}$. As such, a range of 50-70 ˚C and 15-30 MPa, and a CO_2 flow rate of 1.5 L/min and 3 h were selected for extraction experiments without co-solvent.

Table 5.2 summarizes lipid/ASX yields at 46-74 ˚C, 18-32 MPa (generated ranges using CCD based on the selected temperature/pressure ranges) and 1.5 L/min over 3 h with FD residues. Total lipid yield varied from 1.12-2.16 dry wt %, ASX yield from 4.75-86.30 μg ASX/$g_{dry\ waste}$, and TCC from 0.29-5.07 mg ASX/$g_{extract}$. These yields were the same or higher compared to a FD redspotted shrimp (*Farfantepenaeus paulensis*) by-products study at similar conditions, where total lipid yield varied from 1.74 to 2.26 dry wt%, ASX yield from 5.9-20.7 μg ASX/$g_{dry\ waste}$ and TCC from 0.34-1.07 mg ASX/$g_{extract}$ (40-60 ˚C and 20-40 MPa over 3.3 h) [5].

In this study, the highest lipid yield (2.16 dry wt %) was extracted at 50 ˚C and 30 MPa, however the highest ASX yield (86.30 µg ASX/$g_{dry\ waste}$) and TCC (5.07 mg ASX/$g_{extract}$) were obtained at 60 ˚C and 32 MPa. A study using FD shrimp by-products of *Farfantepenaeus paulensis* showed the same maximum for lipid yield and ASX yield and TCC was maximized at a lower temperature and higher pressure (43 ˚C and 37 MPa for ASX and 50 ˚C and 40 MPa for TCC) [5].

Table 5.2: Central composite design combination runs, total lipid yield, astaxanthin (ASX) yield and total carotenoid content (TCC) value obtained using freeze-dried (FD) shrimp by-products.

Run	X1=T, ˚C	X2=P, MPa	Y1=total lipid yield, dry wt%	Y2=ASX yield, µg/$g_{dry\ waste}$	Y3=TCC, mg/$g_{extract}$
1	46	25	1.72	58.32	3.38
2	50	30	2.16	81.53	3.59
3	50	20	1.24	10.32	0.83
4	60	32	1.70	86.30	5.07
5	60	18	1.12	11.10	0.98
6	70	30	1.30	56.61	4.35
7	70	20	1.65	4.75	0.29
8	74	25	1.29	55.54	4.30
9	60	25	1.43	64.31	4.50
10	60	25	1.50	58.84	3.93
11	60	25	1.41	64.31	2.91
12	60	25	1.58	56.09	3.54
9-12			1.48±0.07	60.89±3.56	3.72±0.58

5.6.1.2.SC-CO_2 performance compared with Soxhlet process

Different solvents studies were used in Soxhlet experiments to determine the maximum extractable lipid, ASX and TCC values. These were used as a basis to compare the SC-CO_2 results (using Eq. 5-4). The lipid yield recovered using SC-CO_2 was between 0.5-1.67 times of lipids extracted using Soxhlet (depending on solvent used). For instance, Soxhlet using ethanol recovered twice that of SC-CO_2, however with hexane as solvent the yield was 0.4 times. The ASX yields in SC-CO_2 vs Soxhlet had a much narrower range of difference, from 0.3-0.7 of ASX yield using Soxhlet, (maximum achieved at 40:60 vol% hexane/acetone). The highest TCC extracted using SC-CO_2 was approximately 0.4 of the Soxhlet (60:40 vol% hexane/isopropanol).

5.6.1.3. ANOVA results and model fitting of CCD

ANOVA results were used to estimate the significance and suitability of the proposed models (Tables AI-4, AI-5, and AI-6). The factor significance was determined using F-test and p-values ($p \leq 0.05$). The F-values of the regression models indicate that the models were significant and highly predictable. Insignificance ($p > 0.05$) of "lack of fit" for the models also show the adequacy of the model.

As observed in Tables A4-5, temperature, pressure, and temperature/pressure interactions had significant effects on lipid recovery while the most significant effect on ASX recovery was pressure. Plots of predicted values vs. observed experimental values show a linear with R^2 values ranging 0.85-0.96 for the responses, total lipid yield (Y1), ASX yield (Y2), and TCC (Y3) (Fig.5.1a, b and c). Adjusted R^2 ranged from 0.72-0.92 for all the models verified the adequacy of the models.

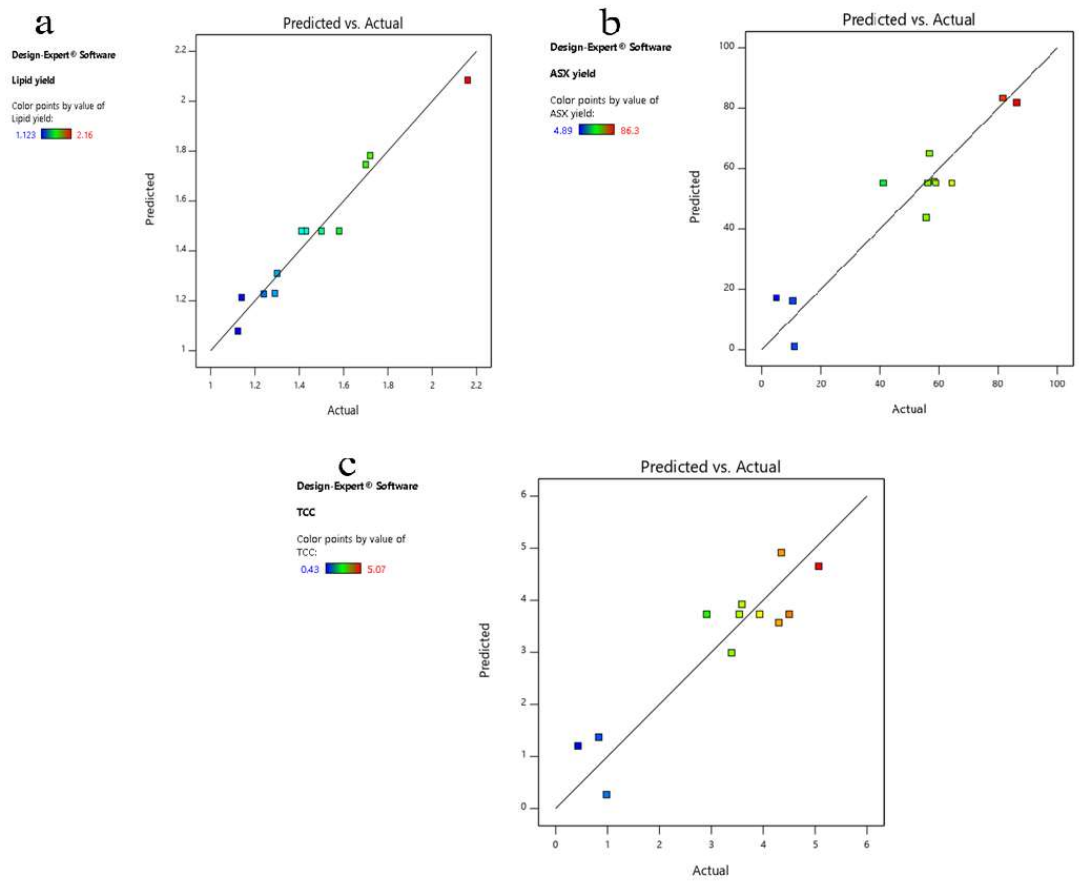

Figure 5.1:Predicted values vs experimental values for (a) total lipid yield, (b) astaxanthin (ASX) yield and (c) total carotenoid content (TCC).

The model equations in actual values of the factors for total lipid yield (Y1), ASX yield (Y2) and TCC (Y3) can be written as:

$$Y1 \text{ (total lipid yield, dry wt\%)} = -4.608 + 0.060\,T + 0.35\,P - 0.004\,T*P + 0.00013\,T^2 - 0.0014\,P^2 \quad 5\text{-}38$$

$$Y2 \text{ (ASX yield, } \mu g/g_{dry\ waste}) = -485.96 + 5.365\,T + 25.687\,P - 0.097\,T*P - 0.028\,T^2 - 0.281\,P^2 \quad 5\text{-}39$$

$$Y3 \text{ (TCC, } mg/g_{extract}) = -21.12 + 0.15\,T + 1.26\,P + 0.0058\,T*P - 0.0023\,T^2 - 0.026\,P^2 \quad 5\text{-}40$$

5.6.1.3.1. Pressure and temperature effects on lipid/ASX recovery

The response surface graphs (Fig. 5.2a, b and c) for total lipid/ASX yields and TCC are function of temperature and pressure.

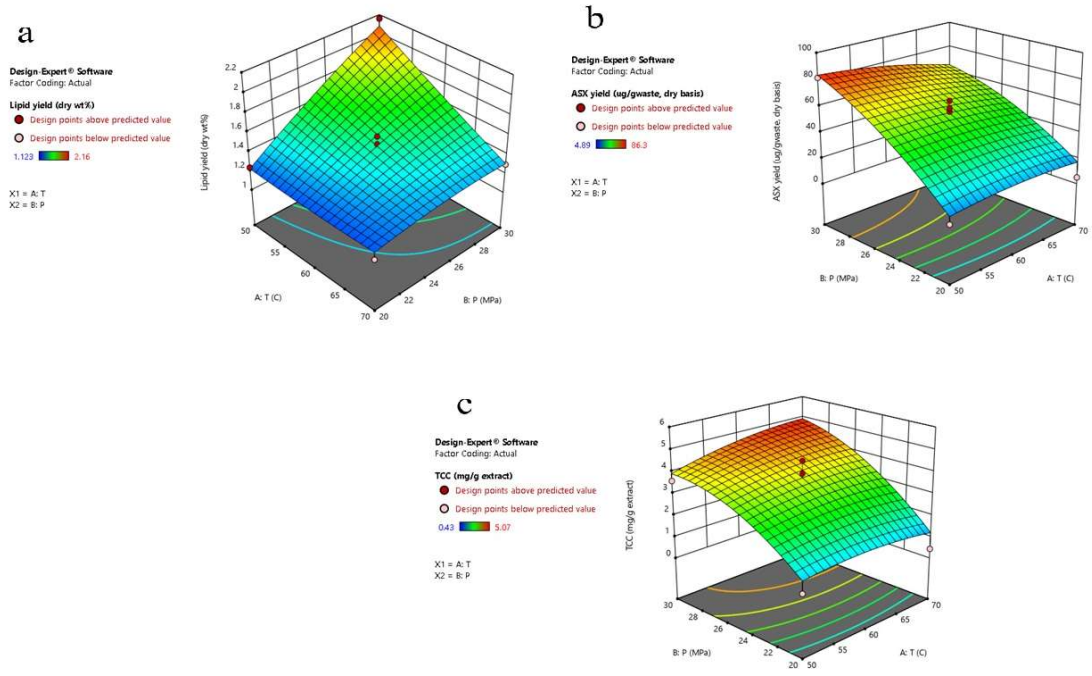

Figure 5.2: Response surface plots of interaction between temperature and pressure effects on (a) total lipid yield, (b) astaxanthin (ASX) yield and (c) total carotenoid content (TCC).

As pressure increases from 20 to 30 MPa at a constant temperature, lipid/ASX recovery increases due to increases in lipid/ASX solubility as CO_2 solvating power (density) increases with pressure [5,15,32]. However, the impact of temperature at constant pressure is more complex as temperature impacts both solvent density and solute vapor pressure. At constant pressure, an increase in temperature decreases the solvent density, and therefore the solute solubility decreases. However, an increase in temperature increases the solute vapor pressure, thereby increasing the solute solubility [5,15,32,33]. As such, there is a balance between the solvent density and the solute vapor pressure in solute solubility [34]. Further,

at high temperatures, the rate of mass transfer increases [32] but there is a corresponding increase in the degradation of key compounds [35–37].

There is an overall decrease in lipid and ASX yields with an increase in temperature from 50-60 ˚C. The decrease in lipid was more pronounced at pressures above 30 MPa; however, at pressures below 25 MPa there was no significant change in lipid yield with temperature. The same behaviour was observed for ASX yield and TCC (Fig.5.2 b and c). At 20-25 MPa, changes to temperature had little influence on lipid/ASX recovery. This pressure range is close to the cross-over pressure of lipid/ASX solubility in SC-CO_2. The exact range of cross-over pressure varies in different studies, from as low as 18-20 MPa [8] to 20-35 MPa [5] and 30-35 MPa [15].

At pressures lower than cross-over pressure, an increase in temperature decreases the solubility of the solute, and at pressures above the cross-over pressure, increasing temperature increases the solubility. At the cross-over pressure, the solubility does not change with temperature [5,15,32,33]. Given the range of cross-over pressures, the increase in ASX/lipid yield as temperature increased at pressures greater than 20-25 MPa can be in part explained by this behaviour.

5.6.1.3.2. Optimal extraction conditions for maximum lipid/ASX recovery

The optimum levels of independent factors (temperature, time and the ratio) to achieve the "best" responses (total lipid yield, dry wt%, ASX yield, $\mu g/g_{dry\ waste}$ and TCC, $mg/g_{extract}$) were determined using response optimization. The conditions that maximize lipid and ASX yields, and TCC was 50 ˚C and 30 MPa (2.09 dry wt% for total lipid yield, 83.42 $\mu g/g_{dry\ waste}$ for ASX yield and 3.92 $mg/g_{extract}$ for TCC). These values are in good

agreement with experimental values (lipid yield of 2.16 dry wt%, ASX yield of 81.53 $\mu g/g_{dry\ waste}$, and TCC of 3.59 $mg/g_{extract}$).

5.6.1.4. Lipid/FA compositions of SC-CO_2 extract and pressure/temperature effects

The lipid profile plays a key role in the final application and informs storage and stability of extract. There is no available literature on the lipid profile of SC-CO_2 extract from shrimp by-products, thus we compared our results with the literature related to other marine feedstocks used in SC-CO_2 studies.

Lipid classification in terms of polarity includes neutral lipids and polar lipids. Neutral lipids are such as FFAs, STs, TAGs, and polar lipids are such as PLs [9]. As shown in Table 5-3, at a given pressure and temperature, SC-CO_2 extract fractions had high percentages of neutral lipids (FFAs, STs and TAGs) but a low concentration of PLs. Lipid extraction using SC-CO_2 (e.g., algal, yeast, seeds and scallop by-products) favors neutral lipids (FFA, ST and TAG), and is less effective in PL extraction [16–18,38] due to the non-polar nature of CO_2. In addition, PL has a strong interaction with proteins/polysaccharides, binding it more tightly to the solid matrix [17]. Thus, PL extraction using SC-CO_2 requires very high pressure (>50 MPa) [16] or the addition of polar co-solvents such as ethanol [1,37].

Compared to the other neutral lipids, TAG yield was lower at a given temperature and pressure. The higher the FFA in extracts, the more soluble the extract in SC-CO_2 [39,40]. A study on the solubility of two vegetable oils (blackcurrant and grape seed oils) in SC-CO_2 showed the FFA, mono- and diglycerides are more soluble in SC-CO_2 compared to TAG [39,41–43]. Furthermore, any pre-processing (e.g., water removal) can impact the biomass cell

structure and water/biomass interaction, and consequently, lipid compositions [44]. The type of water removal process can also impact the degree of degradation of TAG to FFA which would shift ratios of TAG:FFA [18,44–46]. Freeze drying was used in this study, which can impact lipid composition as we observed in previous work where FFA almost doubled in the extract from Soxhlet extraction when the by-products were freeze-dried. In a study of neutral lipid extraction from algae biomass using SC-CO_2, forced air drying was compared to ring drying. The FFA in the extract from forced air drying (13 wt% water content) was higher compared to ring drying (3.4 wt% water content). This was attributed to the degradation/hydrolysis of TAG to FFA due to the intensity/duration of the forced airflow as well as the high water content in biomass [44]. In general, the solubility of FFA in SC-CO_2 is higher than TAG, and drying processes and water content can intensify the degradation/hydrolysis of TAG to FFA, increasing FFA in the extract which can explain the high level of FFA in this present work.

Table 5.3: Lipid compositions (wt%) of the SC-CO_2 extracts at various temperature/pressure compared to different solvents/solvent mixtures in Soxhlet for freeze-dried (FD) shrimp by-products.

% Lipid composition[a]	50 ˚C, 30 MPa	60 ˚C, 32 MPa	46 ˚C, 25 MPa	50 ˚C, 20 MPa	70 ˚C, 20 MPa	Ethanol	Hexane/acetone (40:60 vol%)	Hexane
TAG	3.37	4.68	4.81	7.09	6.43	2.39	12.65	31.36 ± 0.07
FFA	46.17	49.05	46.35	40.16	41.12	19.03	10.74	Not detected
ST	46.40	41.17	43.07	48.90	45.08	37.85	22.16	40.38 ± 3.56
AMPL	3.31	2.50	3.62	3.19	3.49	4.02	13.89	4.93 ± 0.02
PL	Not detected	Not detected	Not detected	0.28	1.15	35.92	33.93	31.41 ± 6.93

[a] triacylglycerols (TAG), free fatty acids (FFA), phospholipids (PL), sterols (ST), and acetone mobile polar lipids (AMPL).

Increases in pressure and/or temperature increase FFA in the extract. This was observed in other studies [40,44,47]. The highest FFA was at 60 ˚C/32 MPa used in this study. FFA increased from 40.16-46.17 wt%, and TAG decreased from 7.09-3.37 wt% with an increase in pressure from 20-30 MPa at 50 ˚C. At a 20 MPa increasing temperature from 50-70 ˚C increased FFA by 2 % and decreased TAG by 9 %. The increase/decrease changes in FFA and TAG are potentially due to thermal degradation at the higher temperature. The trends in ASX concentrations and neutral lipids, particularly FFA, indicate a link between ASX recovery and FFA content, which has also been observed in other work [44,47]. The higher the FFA in vegetable oils and lipid fraction of extract, the higher the ASX yield and the higher solubility of vegetable oils or extract in SC-CO_2 [39,47].

Table 5.4 shows FA compositions of SC-CO_2 extracts compared to Soxhlet extracts using different solvents (FD residues). Over the range of temperature and pressure studied in SC-CO_2 extracts, SFA ranged from 18-19 wt%, MUFA from 43-45 wt% and PUFA from 36-37 wt% which represents there is no impact on FA compositions. The Soxhlet extracts had higher MUFA (49-50 wt%) but less PUFA (30-31 wt%) compared to the SC-CO_2 extracts. The percentage of n-3 FAs (31-33 wt%) in SC-CO_2 extracts was higher than the Soxhlet extracts using 40:60 vol% of hexane/acetone (28 wt%). Compared to a SC-CO_2 study of lipid extraction from Brazilian redspotted shrimp (*Farfantepenaeus paulensis*) [5], in our work, MUFA (1.7 times) and PUFA (1.5 times) were higher but less SFA was extracted (0.5 times). Key FAs include C18:1n9 (15-15.44 wt% in SC-CO_2 extract), docosahexaenoic acid (DHA) (12-14 wt%), eicosapentaenoic acid (EPA) (17.40-18 wt%) which were higher

compared to the Soxhlet extracts. C16:0 (13-14 wt%) was approximately the same as the Soxhlet extracts.

Table 5.4: FA compositions of SC-CO_2 extracts at various temperatures/pressures compared to Soxhlet extracts using different solvents for freeze-dried (FD) residues.

	SC-CO_2 extracts					Soxhlet extracts	
FAs, wt %	50 ˚C/30 MPa	60 ˚C/32 MPa	46 ˚C/25 MPa	50 ˚C/20 MPa	70 ˚C/ 20 MPa	Hexane	Hexane/acetone (40:60 vol%)
C14:0	2.00	2.05	1.95	2.15	2.16	3.35± 0.53	2.81± 0.23
C16:0	13.59	13.39	13.05	12.97	13.34	12.12± 1.25	12.42± 1.63
C16:1n7	6.56	6.85	6.87	7.48	7.20	9.91± 1.05	8.74± 0.90
C18:0	2.71	2.69	2.61	2.48	2.72	2.16± 0.66	2.48± 0.58
C18:1n9	14.85	14.94	15.05	15.44	15.20	15.26± 0.60	14.12± 1.17
C18:1n7	9.62	9.41	9.45	9.35	9.36	4.70± 0.28	5.16± 0.63
C18:2n6	1.27	1.27	1.29	1.32	1.29	1.29± 0.09	1.45± 0.48
C18:3n6	0.06	0.06	0.06	0.07	0.06	0.05± 0.04	0.01± 0.02
C18:3n3	0.26	0.26	0.26	0.27	0.26	0.53± 0.02	0.51± 0.09
C20:1n9	4.34	4.44	4.45	4.55	4.48	5.98± 0.98	5.24± 0.48
C20:4n6	1.66	1.63	1.65	1.65	1.68	1.10± 0.18	0.76± 0.69
C20:5n3, EPA	17.47	17.48	17.59	17.39	17.54	13.33± 2.36	14.74± 3.44

	SC-CO_2 extracts					Soxhlet extracts	
FAs, wt %	50 ˚C/30 MPa	60 ˚C/32 MPa	46 ˚C/25 MPa	50 ˚C/20 MPa	70 ˚C/ 20 MPa	Hexane	Hexane/acetone (40:60 vol%)
C22:5n3	0.50	0.50	0.52	0.50	0.49	0.77± 0.42	0.78± 0.35
C22:6n3, DHA	13.72	13.39	13.33	12.25	12.50	8.89± 0.69	10.38± 1.86
Σ SFA	19.09	18.84	18.53	18.42	19.05	18.66± 1.47	18.94± 2.20
Σ MUFA	43.17	43.73	43.87	45.18	44.09	51.27± 2.75	49.16± 4.87
Σ PUFA	36.90	36.61	36.75	35.55	36.03	28.98± 3.96	31.04± 6.71
n-3 FA	32.60	32.25	32.48	31.18	31.51	25.24± 3.52	27.95± 5.99
n-6 FA	3.72	3.75	3.67	3.74	3.91	2.82± 0.21	2.73± 0.41

5.6.2. Dynamic SC-CO_2 with static co-solvents

Solubility of compounds with higher polarity and molecular weight such as ASX and PLs is low in SC-CO_2 and extraction requires high pressures (P > 30-50 MPa) or adding a polar co-solvent such as ethanol for higher recovery. High pressures or extending extraction time increases processing costs [37]. Polar co-solvents increase the polarity of CO_2 and therefore increase more polar compound extraction at lower pressures [1,16,17].

In this work, different co-solvents were added to the SC-CO_2 using a static method to determine if the enhanced recovery would justify costs. The optimal pressure and temperature (50 ˚C and 30 MPa) were used with a co-solvent/waste ratio of 5:1 v/w. In the experiments, the residue was premixed with the co-solvent in the extraction unit and loaded with SC-CO_2 at 50 ˚C and left 30 MPa for a static extraction time of 1 h. This provides additional contact time between co-solvent, the residues, and SC-CO_2. After 1-h static time, the CO_2 continuously flowed at 1.5 L/min for another 1 h. The 1-h static time was selected based a SC-CO_2/static co-solvent study on chlorophyll A extraction from algae biomass [27]. The results were compared to an experiment without co-solvent (50 ˚C, 30 MPa over 1-h static time and then 1-h dynamic time).

The experiment without co-solvent recovered 1.94 dry wt% lipids, 32.14 $\mu g/g_{dry\ waste}$ ASX and 1.66 $mg/g_{extract}$ TCC. The addition of co-solvent to the SC-CO_2 system improved lipid/ASX recovery. The lipid yield increased to 2.48 dry wt% or 0.22 mg/g_{CO2}, ASX yield to 51.79 $\mu g/g_{dry\ waste}$ or 0.42 $\mu g/g_{CO2}$ and TCC to 2.09 $mg/g_{extract}$ using ethanol. Hexane/acetone (40:60 vol%) as a co-solvent increased these values to a lesser extent, with

lipid yield at 2 dry wt% (0.18 mg/g_{CO2}), 43.31 µg/$g_{dry\ waste}$ (0.48 µg/g_{CO2}) for ASX yield, and 2.17 for TCC mg/$g_{extract}$.

In published work of dynamic or continuous co-solvent/SC-CO_2 extraction (3 L/min for CO_2 and 0.33-1.12 mL/min for ethanol) using FD redspotted shrimp by-products (*Farfantepenaeus paulensis*) at 50 ˚C and 30 MPa over 100 min (including a 20 min static time at the start), lipid yield ranged from 1.96-4.94 dry wt% (0.13-0.33 mg/g_{CO2}), ASX yield varied from 26-35 µg/$g_{dry\ waste}$ (or 0.35-0.47 µg/g_{CO2}), and TCC was 0.75-1.33 mg/$g_{extract}$ [1]. The 20 min static time was used in [1] to enhance contact time between residues and SC-CO_2 before flowing ethanol to the system. Compared to [1], the lipid yield in this present work is within the same range and the ASX and TCC is higher. The lower ASX and TCC can be attributed to the lower contact time between ethanol/SC-CO_2 and residues in [1].

While ethanol showed good results, a potentially more feasible approach is edible oils as a co-solvent. Sunflower oil recovered higher ASX (18.91 µg/$g_{dry\ waste}$, 13 µg/$g_{sunflower\ oil}$ or 0.24 µg/g_{CO2}) compared to waste fish oil (4.57 µg/$g_{dry\ waste}$, 2 µg/$g_{fish\ oil}$ or 0.04 µg/g_{CO2}). This can be attributed to the higher solubility of sunflower in SC-CO_2 compared to waste fish oil due to the higher FFA in sunflower oil (as shown in Table 5.7). FFA can potentially act as a co-solvent in ASX extraction. As indicated earlier, studies have shown that FFA had higher solubility in SC-CO_2 compared to the other lipid compounds, and increased solubility of oil/extracts in SC-CO_2 [39,41–43,47]. Studies also observed a direct connection between ASX recovery and FFA content [44,47].

A SC-CO_2 study of cooked/dried shrimp (*P. brasiliensis and P. paulensis*) processing residue using dynamic SC-CO_2/sunflower oil with a CO_2 flow rate of 7.22 L/min at 60 ˚C, 30 MPa and 3-h dynamic time showed a maximum recovery of 4.80×10^{-4} $\mu g/g_{dry\ waste}$ ASX for 5 wt% sunflower/SC-CO_2 [8]. The very low value compared to this present work may be due to the cooking step where boiling water and drying at a high temperature can cause thermal degradation of ASX.

Waste fish oil has not been used in published work as a solvent or/and co-solvent; however, one paper optimized dynamic SC-CO_2 of ASX from FD shrimp waste mixed with fish skin. At a shrimp waste:fish waste ratio of 60:40 w/w at 50 ˚C, 30 MPa and 2 h the ASX yield was 15.7 $\mu g/g_{extract}$ [37], approximately 3 times the ASX recovered in this study. As the process conditions of SC-CO_2/fish oil (static/dynamic time, temperature/pressure, fish oil:waste ratio and flow rate) were not optimized in this work, further study is required to enhance yield.

5.6.2.1. Co-solvent effect on lipid/FA profiles of SC-CO_2 extracts and compared with Soxhlet

Before evaluating co-solvent effect on lipid/FA compositions of the extracts, we first compared lipid compositions of 3 h dynamic SC-CO_2 extract with the extract obtained at 1-h static time and then 1-h dynamic time without co-solvent at the same conditions (50 ˚C and 30 MPa). This comparison shows the impact of extraction time on lipid distributions. Compared to the SC-CO_2 extraction at 50 ˚C and 30 MPa for 1 h static followed by 1 h dynamic time, the 3 h dynamic extraction had lower TAG and ST. Longer extraction time may cause further degradation of TAG to FFA, and also degradation of ST [40].

Adding polar co-solvents (ethanol or 40:60 vol% of hexane/acetone) to the SC-CO_2 extraction (at 50 ˚C and 30 MPa for 1 h static followed by 1 h dynamic time) increased PL but decreased TAG and ST. Sunflower oil had higher FFA/ST and lower PL compared to waste fish oil. There was little difference in the lipid profile of the virgin sunflower oil and sunflower oil extract. Fish oil extracts had higher ST and FFA compared to waste fish oil. Adding sunflower or waste fish oil to the system provided extracts with less FFA/ST but higher TAG. This represents lower possible degradation of lipid compounds when the edible oil was used.

Adding polar solvents to the system increased SFA by 10-11 % but decreased MUFA by 0.5-2 % and PUFA by 4-5 %. N-3 FAs decreased by 3-6 % and n-6 FAs decreased by 1-8 % when polar co-solvents were added to SC-CO_2. These FA compositions/groups except MUFA were higher compared to Soxhlet with hexane/acetone (40:60 vol%). However, ethanol in Soxhlet extracted higher DHA, EPA and n-3 FA but lower SFA compared to the extracts using SC-CO_2 with or without polar co-solvents (Table 5.6).

Table 5.5: lipid compositions of edible oils (as co-solvents), and SC-CO_2 extracts using co-solvents at 50 ˚C and 30 MPa for 1h static/1 h dynamic time compared with SC-CO_2 extracts without co-solvents.

	SC-CO_2 extracts			
Lipid compositions [a], wt%	SC-CO_2/ethanol	SC-CO_2/ hexane: acetone (40:60 vol%)	SC-CO_2, 1 h static, 1 h dynamic	SC-CO_2, 3 h dynamic
TAG	4.48	4.70	6.41	3.37
FFA	41.19	38.48	37.90	46.17
ST	46.20	43.70	50.26	46.40
AMPL	2.86	3.18	1.43	3.31
PL	4.11	3.54	0.88	Not detected
	SC-CO_2 extracts		Edible oils compositions	
Lipid compositions[a], wt%	SC-CO_2/sunflower oil	SC-CO_2/fish oil	Sunflower oil	Waste fish oil
TAG	65.45	71.31	68.77	59.94
FFA	13.63	11.19	12.86	2.74
ST	16.00	17.50	17.65	0.92
AMPL	4.18	Not detected	Not detected	7.11
PL	0.44	Not detected	0.72	28.42

[a] triacylglycerols (TAG), free fatty acids (FFA), phospholipids (PL), sterols (ST), and acetone mobile polar lipids (AMPL).

Table 5.6: FA compositions of extracts recovered from freeze-dried (FD) residues using SC-CO_2/organic solvents, SC-CO_2 at 50 ˚C/30 MPa for 1h static/1 h dynamic time and Soxhlet with ethanol, and hexane/acetone (40:60 vol%).

FAs, wt %	SC-CO_2/ethanol	SC-CO_2/ hexane: acetone (40:60 vol%)	SC-CO_2 for 1 h static, 1 h dynamic	Ethanol	Hexane: acetone (40:60 vol%)
C14:0	2.14	2.10	1.96	2.02	2.81± 0.23
C16:0	14.66	14.27	13.46	12.51	12.42± 1.63
C16:1n7	6.55	6.69	6.83	6.82	8.74± 0.90
C18:0	3.43	3.75	2.85	2.25	2.48± 0.58
C18:1n9	15.34	15.21	15.61	15.38	14.12± 1.17
C18:1n7	8.91	8.90	9.32	7.8	5.16± 0.63
C18:2n6	1.66	1.63	1.75	1.21	1.45± 0.48
C18:3n6	0.07	0.06	0.06	0.06	0.01± 0.02
C18:3n3	0.27	0.27	0.29	0.24	0.51± 0.09
C20:1n9	4.37	4.41	4.28	4.75	5.24± 0.48
C20:4n6	1.50	1.52	1.58	1.55	0.76± 0.69
C20:5n3, EPA	16.18	15.99	17.16	17.76	14.74± 3.44
C22:5n3	0.42	0.48	0.51	0.6	0.78± 0.35

FAs, wt %	SC-CO_2/ethanol	SC-CO_2/ hexane: acetone (40:60 vol%)	SC-CO_2 for 1 h static, 1 h dynamic	Ethanol	Hexane: acetone (40:60 vol%)
C22:6n3, DHA	12.90	12.28	12.73	13.43	10.38± 1.86
Σ SFA	21.19	21.02	19.03	18.03	18.94± 2.20
Σ MUFA	43.29	43.89	44.13	44.05	49.16± 4.87
Σ PUFA	34.68	34.23	36.01	37.03	31.04± 6.71
n-3	30.39	29.65	31.38	32.9	27.95± 5.99
n-6	3.73	4.00	4.06	3.54	2.73± 0.41

Sánchez-Camargo et al. [1] extracted lipids/ASX from FD redspotted shrimp by-products using continuous ethanol/SC-CO_2 with 5-15 wt% ratios at 50 ˚C and 30 MPa, 100 min. They observed increasing ethanol percentage in SC-CO_2 increased n-3 FAs, especially EPA (6-11 wt%) and DHA (4-10 wt%) but decreased SFA (38-34 wt%); this indicates more polar compounds were linked with these n-3 FAs. Again, compared to the literature, we extracted higher n-3 FAs (approximately 2 times) using static co-solvents in SC-CO_2 at the same temperature/pressure but at a shorter time.

As shown in Table 5.7, sunflower oil is rich in MUFA and PUFA (mainly n-6 FAs but no n-3 FAs, e.g., EPA and DHA). Waste fish oil has higher SFA and MUFA, and less PUFA (including lower n-6 FAs and higher n-3 FAs) compared to sunflower oil.

Adding sunflower oil or waste fish oil to SC-CO_2 did not increase n-3 FAs but increased n-6 FAs in the extract. This means SC-CO_2 extracts without any co-solvents or with polar co-solvents had higher n-3 FAs ranging from 30-33 wt% (3 times). While the edible oils provide the extract with higher n-6 FAs (6 times with waste fish oil and 14 times with sunflower oil) compared to SC-CO_2 extracts without any co-solvents or with polar co-solvents.

Table 5.7: FA compositions of extracts recovered using SC-CO_2/edible oils compared to SC-CO_2 without co-solvents at 50 ˚C/30 MPa for 1h static/1 h dynamic time and the edible oils.

FAs, wt %	SC-CO_2 for 1 h static, 1 h dynamic	SC-CO_2/sunflower oil	sunflower oil	SC-CO_2/fish oil	Waste fish oil
C14:0	1.96	0.06	0.06	2.16	2.38
C16:0	13.46	5.88	5.90	12.09	13.13
C16:1n7	6.83	0.12	0.11	4.60	5.12
C18:0	2.85	3.55	3.65	3.84	3.98
C18:1n9	15.61	34.15	34.10	35.67	35.63
C18:1n7	9.32	0.70	0.69	2.75	3.00
C18:2n6	1.75	53.65	53.55	19.63	14.85
C18:3n6	0.06	Not detected	Not detected	0.24	0.29
C18:3n3	0.29	0.07	0.07	2.42	2.75
C20:1n9	4.28	0.17	0.18	1.63	1.84
C20:4n6	1.58	Not detected	Not detected	0.48	0.56
C20:5n3, EPA	17.16	0.01	Not detected	3.11	3.57
C22:5n3	0.51	Not detected	Not detected	1.38	1.63
C22:6n3, DHA	12.73	Not detected	Not detected	2.88	3.29

FAs, wt %	SC-CO_2 for 1 h static, 1 h dynamic	SC-CO_2/sunflower oil	sunflower oil	SC-CO_2/fish oil	Waste fish oil
Σ SFA	19.03	10.75	10.95	18.88	20.25
Σ MUFA	44.13	35.45	35.34	46.29	47.40
Σ PUFA	36.01	53.78	53.69	34.50	31.96
n-3	31.38	0.08	0.07	11.24	12.93
n-6	4.06	53.65	53.55	22.18	17.79

5.6.3. Kinetic study of lipid/ASX extraction using SC-CO_2

We assumed the extractable compounds from shrimp by-products were lipids and total ASX which were treated as two separate single (pseudosolute) components. Overall extraction curves are shown in Fig.5.3a (lipid mass), and Fig.5.3b (ASX mass) versus the amount of CO_2 consumed at 50 ˚C and 30 MPa and flow rate of 1.5 L/min. The maximum mass of lipids (0.1139 g) and ASX (764 μg) extracted using the Soxhlet was used as an initial mass of the solutes (m_{s0}) in the model.

For both lipid/ASX extraction, the overall extraction curves are linear initially. In this stage, more free solute is available on the external surface of the solid particles [21]. Thus, the extraction rate at the beginning is faster than other stages. The initial extraction period is controlled by the phase equilibrium which depends on solute/solvent compositions, pressure and temperature. At high initial solute concentration, the solute solubility controls the equilibrium and fluid-phase equilibrium concentration is equal to the solubility while at low initial solute concentration, solute–solid interaction controls the equilibrium resulting in lower fluid-phase concentration than the solute solubility [24].

In the next step, the rate starts to decrease. This is likely due to less free solute available on the solid surface and the solutes inside the particles start to diffuse to the surface and then into solvent which is controlled by combined mass transfer (external and internal mass transfer). The third stage which is controlled by mainly internal diffusion of the solute with the slow extraction rate involves the extraction of solutes inside of the particles [21].

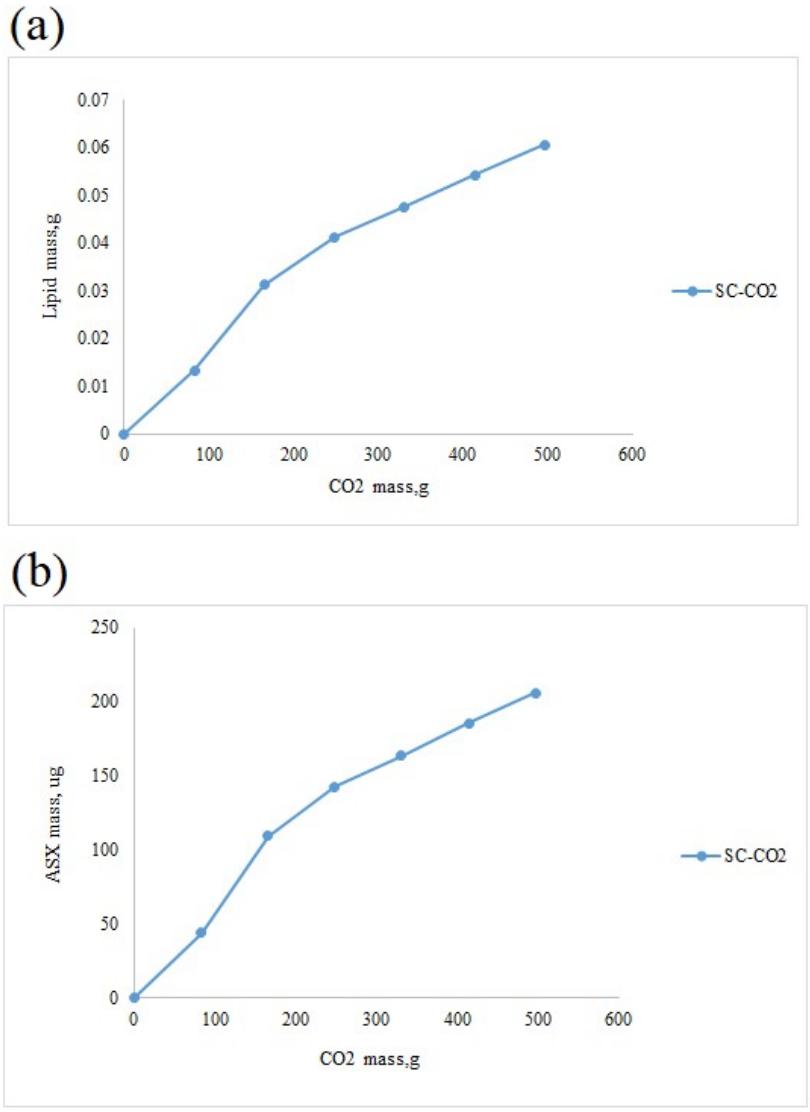

Figure 5.3: Overall extraction curve of lipids (a) and astaxanthin (ASX) (b) at 50 ◦C and 30 MPa and flow rate of 1.5 L/min.

5.6.3.1.Validation of mathematical modeling and the estimated parameter evaluation

As indicated above, the Goto et al. model [25] has a single fitting parameter, K, optimized by minimizing the AARD between experimental data and model values. The AARD values were 6% for lipid extraction and 8.8% for ASX extraction which indicated the mathematical modeling gives good representation of the SC-CO_2 extraction of both

lipids/ASX from the shrimp by-products (Fig.5.4). Fig.5.4 (a) shows the plot of the cumulative fractions of lipids calculated using Eq. (5-27) compared to experimental data as a function of the time (min) and Fig.5.4 (b) compares the cumulative fractions of ASX calculated using Eq. (5-27) with the experimental data versus time.

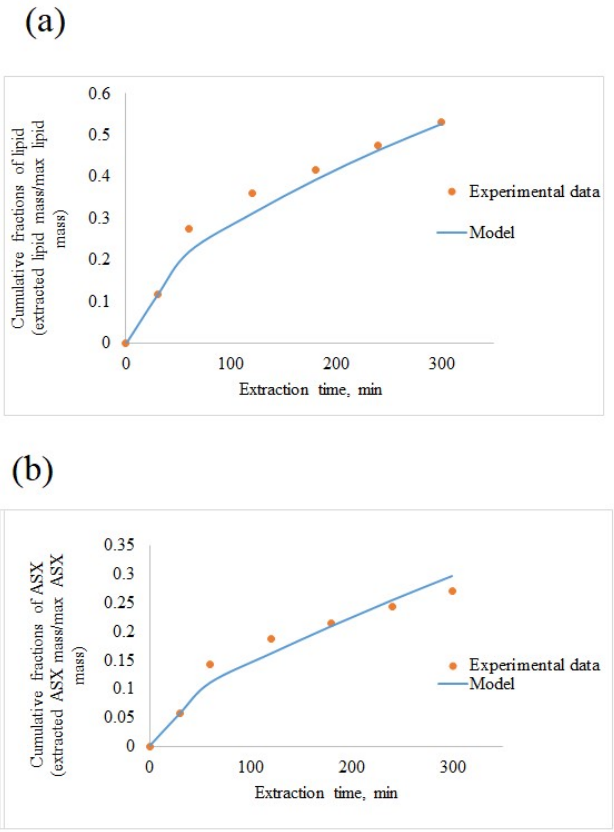

Figure 5.4: Comparison of cumulative fractions of lipid (a) and astaxanthin (ASX) (b) predicted from the Goto model with experimental data at 50 ◦C and 30 MPa and flow rate of 1.5 L/min for dp=0.104 mm, β= 0.76 and α= .0.41.

The low value of Bi (~ 3) in this studied condition confirms the linear driving force approximation. The kinetic parameters estimated using the correlations indicate the external mass transfer coefficient (2-5 $\times 10^{-5}$ m/s) in the fluid phase was higher than effective internal diffusion coefficients (3-5 $\times 10^{-10}$ m^2/s) which mean that the diffusion rate

of solute inside the particles into the solid–fluid interface is slower than the diffusion rate of solute on the particle surface to the solvent bulk. The equilibrium adsorption coefficient (K) was high (>1) for both lipids and ASX, which represents the strong interaction of lipid-the shrimp particles and of ASX-the shrimp. Thus, the solute concentration leaving the extractor is less than its solubility in fluid phase [25,26]. The K value of ASX (824) was higher than that of lipid (372) which means the interaction between ASX and the shrimp particles is stronger than the interaction between lipids and the shrimp. Therefore, the difference of ASX concentration in the fluid leaving the extractor (obtained from the linear curve slope in Fig.5.3) and its solubility is much larger compared to the difference of exit lipid concertation and lipid solubility.

Solubility of pure ASX in SC-CO_2 at 50 ˚C and 30-31.5 MPa measured in the published studies ranged from 4.2×10^{-7}- 4.62×10^{-5} mole fraction [48,49]. In this study, the exit ASX concentration obtained from linear part of Fig.5.3 was 4.8×10^{-8} which was lower than the solubility range obtained in the published studies. This is likely due to the strong ASX-solid interaction, and interaction of other compounds in the solid which is not considered in this model. In fact, shrimp by-products are a mixture of multicomponent and the solubility of ASX present in the shrimp by-products in SC-CO_2 can be affected by other compounds.

5.7. Conclusions

Extraction of lipids/ASX from the Atlantic shrimp by-products (*Pandalus borealis)* using SC-CO_2 was studied as a function of process conditions (temperature and pressure) and

static co-solvents. This study provides the first comprehensive analysis of lipid compositions of extract as a function of pressure/temperature and co-solvents.

In this study, the highest lipid yield extracted at 50 ˚C and 30 MPa was 0.5 times of lipids extracted using ethanol in Soxhlet. At 60 ˚C and 32 MPa the highest ASX yield was 0.3 of ASX yield using a mixture of 40:60 vol% hexane/acetone in Soxhlet and the highest TCC was 0.4 of the highest TCC obtained using Soxhlet (60:40 vol% hexane/isopropanol).

Lipid/ASX recovery increased with an increase in pressure; however, temperature at a constant pressure below the cross-over pressures (20-25 MPa) decreased ASX/lipid yield and above the cross-over pressure increased the yields. The best conditions to maximize lipid and ASX yields, and TCC using CCD were 50 ˚C and 30 MPa.

SC-CO_2 extract fractions had high percentages of neutral lipids (FFAs, STs and TAGs) but low PLs. This study showed that FFA in the extracts was higher compared to TAG which represents degradation/hydrolysis of TAG to FFA due to freeze-drying and water content in biomass. Increases in pressure and/or temperature increased FFA and decreased TAG in the extract. However, over the range of temperature and pressure studied in SC-CO_2 extracts, there was no impact on FA compositions. Key FAs, C18:1n9, DHA, EPA in SC-CO_2 extracts were higher compared to the Soxhlet extracts.

Polar co-solvent increased lipid/ASX recovery using SC-CO_2. Sunflower oil recovered higher ASX compared to waste fish oil. This can be attributed to the higher solubility of sunflower in SC-CO_2 compared to waste fish oil, due to the higher FFA in sunflower oil. This work showed that using static co-solvent in SC-CO_2 without any extra cost of pump

for co-solvent provided the same lipid yield as and the higher ASX yield than the published papers using continuous co-solvent/ SC-CO_2. Polar co-solvents increased PL and SFA but decreased MUFA and PUFA in the SC-CO_2 extract. Compared to Soxhlet with hexane/acetone (40:60 vol%), FA compositions except for MUFA in the SC-CO_2 extract were higher. Sunflower oil used in this study had higher FFA, lower PL, SFA and MUFA, PUFA (with higher n-6 FAs and no n-3 FAs, EPA and DHA) compared to waste fish oil. Lipid compositions of sunflower oil and sunflower oil extract did not differ but the extract using waste fish oil in SC-CO_2 had higher ST and FFA compared to the lipid profile of waste fish oil. Adding sunflower or waste fish oil to the SC-CO_2 system provided extracts with less FFA/ST but higher TAG. Compared to SC-CO_2 extracts without any co-solvents or with polar co-solvents adding sunflower oil or waste fish oil to SC-CO_2 did not increase n-3 FAs but provided extracts with higher n-6 FAs. Although edible oils had lower efficiency in ASX recovery using SC-CO_2 compared to polar co-solvents, the process efficiency can be improved by optimization of the process conditions as a function of static/dynamic time, temperature/pressure, fish oil:waste ratio and flow rate.

There was good agreement of the experimental data with the fitted data from the mathematical model for both lipid/ASX extraction. Overall extraction rates of lipid/ASX were controlled by the strong solid–solute interaction, leading to a reduction in the solubility of lipids/ASX in SC-CO_2. Compared to the literature, the solubility of ASX extracted from shrimp by-products in this study was lower; This is likely due to the strong ASX-shrimp interaction, and interaction of other compounds in the shrimp which is not considered in this model.

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Chapter 6 Conclusion and Recommendations

6.1. Summary and Conclusion

The overall objective of this research was to investigate sustainable and "green" extraction of lipids/astaxanthin from the Atlantic shrimp by-products (*Pandalus borealis*) to improve the yield/quality of extract in terms of lipids/astaxanthin. The shrimp by-products are a source of lipids (phospholipids (PLs), omega (n)-3 fatty acids (FAs)), carotenoids, mainly astaxanthin (ASX), chitin and proteins. Lipids and ASX are valuable feedstocks for the food, pharmaceutical and aquaculture industries. Given the decreasing feedstock of many fish species and impacts on the ocean under increasing stress due to climate change, it is obvious we must extract more from less. The value of the shrimp industry could potentially be increased by extracting the high-value compounds left in the processing by-products. Further, recovery of these compounds decreases environmental impacts associated with by-product disposal. The key is the process must be environmentally and economically feasible.

This thesis includes four main sections:(1) Literature review (Chapter 2); (2) Evaluation of conventional solvent processes of lipid/ASX extraction (Chapter 3); (3) Study of process optimization of ASX extraction using waste fish oil with operational parameter effect studies (Chapter 4); (4) study of supercritical CO_2 extraction of lipids and ASX including optimization, static co-solvent impact, kinetic experiment and mathematical modeling studies on lipid/ASX yields and the quality (Chapter 5).

6.1.1. Literature review

This review focuses on the extraction processes of lipids/ASX from crustacean by-products. A comprehensive review on solvent extraction, edible oil extraction and SC-CO_2 process used in processing shrimp by-products for lipid/ASX recovery was presented in Chapter 2.

Lipids and ASX are traditionally extracted using organic solvents at room or elevated temperatures at atmospheric pressure. The conventional chemical lipid/ASX extraction methods can involve the use of corrosive/toxic solvents and be energy/waste intensive although it results in an overall high quantity and quality lipid and ASX. Green solvent processes such as edible oil and supercritical CO_2 extraction are potential alternatives [1–4]. Extraction using edible oils can protect thermolabile compounds such as ASX from oxidation/degradation and delay the oxidation time [5,6]. Furthermore, the edible oil extract can supplement aquaculture feed [5]. Factors that impact lipid/ASX extraction using oils include feedstock water content and particle size, and operating conditions (temperature, time and oil:waste ratio) [7]. Vegetable oils have been studied for ASX recovery from various sources [5,6,8–11]. No marine oils have been used in ASX extraction.

One promising alternative to organic solvents for heat-labile and compounds prone to oxidation is supercritical CO_2 extraction (SC-CO_2). SC-CO_2 can extract soluble compounds at relatively low temperatures (40-50 ˚C) and high pressures (30-50 MPa) over a short extraction time without any subsequent separation steps. The solubility of the target extract and the selectivity power of solvent can be improved by manipulating temperature and pressure [12,13]. SC-CO_2 is a non-polar solvent and can extract non-polar, low/medium

molecular weight compounds with slight polarity [14]. However, polar lipids and ASX have relatively low solubility in supercritical CO_2 and therefore must operate at the higher end of the pressure range (>40 MPa) or a polar co-solvent must be added [7,15]. Adding polar solvents to SC-CO_2 increases the density and polarity of SC-CO_2, consequently increases the target compound solubility. The most common organic co-solvent used is ethanol [2,12,15–18]. Sunflower oil and its methyl ester have been used as green alternative co-solvents in SC-CO_2 for ASX recovery from shrimp by-products [18,19], and there is no work on marine oils as a co-solvent adding to SC-CO_2.

6.1.1.1. Evaluation of the yields/quality of extract as a function of water content/solvent in Soxhlet

In the chapter 3, the performance of the Soxhlet process was compared with published solvent processes. The impact of drying on lipid/ASX yields and quality from shrimp by-products was studied in this work by using "wet" and freeze-dried (FD) shrimp by-products.

This study shows that a mixture of polar/non-polar solvents in Soxhlet extraction maximized lipid/ASX yield. ASX yields varied from 57-88 $\mu g/g_{waste}$ depending on Soxhlet solvent(s) for wet by-products to 118-218 $\mu g/g_{waste}$ for the FD. Lipid extracts are rich in n-3 FAs and Pls. The composition of lipid classes varied with solvent(s) used and pre-treatment. Freeze-drying decreased lipid yield while was positive in ASX yield and total carotenoid content (TCC). This is likely due to the fact that the high polar lipid percentage in lipid fraction facilitate lipid extraction in the water medium, but ASX are not miscible in water.

The polarity of organic solvents and water content play a vital role in lipid and ASX extraction. This study validates solvent and/or solvent mixtures with higher polarity were effective in lipid extraction, but did not impact the extract quality (TCC, ASX content in the extract). The highest recovery of ASX was achieved with hexane/acetone (40:60 vol%) from FD residues and the highest lipid recovery with the same solvent mixture but with wet residues. The highest extract quality (the highest TCC value) was obtained using hexane/isopropanol (60:40 vol%) with FD residues. The highest recovery for both lipids and ASX was ethanol, followed by 40:60 vol% hexane/acetone with FD residues.

6.1.1.2. "Green" extraction process performance on the yields/quality

The next objective of this work is the evaluation of "green" approaches which would improve the safety and environmental sustainability of the extraction. In this study, sunflower and waste fish oils were used to extract ASX from shrimp by-products. The edible oil ASX extraction process was first validated using sunflower oil and compared with the literature to validate the experimental protocol (Chapter 4). The protocol was then repeated using waste fish oil. These results showed waste fish oil was a viable alternative to organic solvents and had the added benefit as using a "waste" to valorize a "waste". Chapter 4 provides the impact of varying process conditions on yields and lipid distributions, process conditions that optimize yield and quality of extract, and comparison with studies using vegetable oil [6,9,20]. The impact of extraction operating conditions (factors: time, temperature and oil:waste ratio) on ASX extraction was evaluated using Box–Behnken Design (BBD). ASX results using waste fish oil were compared with

traditional Soxhlet extraction and studies using sunflower/palm oils. The impact of water content on process efficiency was also studied.

Similarly observed in the Soxhlet extraction, the lower ASX recovered using waste fish oil from wet residues compared to FD residues. At lower extraction times with an increase in temperature or at lower temperatures with an increase in extraction time, the ASX yield increased. However, at longer extraction times/higher temperatures, ASX yield decreased with increasing time/temperature. This trend is largely due to thermal degradation at high temperatures and long extraction times. Increasing the oil:waste ratio increased ASX yield. The maximum ASX extracted was 25.62 $\mu g/g_{waste}$ for wet residues and 123 $\mu g/g_{dry\ waste}$ for FD residues. This is 93-113 % of yields using sunflower/palm oils in the published studies. Compared to the Soxhlet, this is 45-56 % of the yields using 40:60 vol% hexane/acetone. According to BBD optimization results, the optimal conditions were 65 ˚C, 9:1 v/w and 1.5 h to maximize yield of ASX from both wet and freeze-dried shrimp by-products using waste fish oil. This work showed that the freeze-drying (water removal) prior to extraction had a positive impact on the quantity of ASX but decreased lipid yield and lipid compositions of the extract.

Chapter 5 studies SC-CO_2 (with and without co-solvents) extraction of lipids/ASX from shrimp processing by-products. Extraction conditions (pressure and temperature) were studied to determine the impacts on lipid/ASX yields using the central composite design (CCD). Using pure SC-CO_2 study, the highest lipid yield extracted at 50 ˚C and 30 MPa was 0.5 times of lipids extracted using ethanol in Soxhlet. At 60 ˚C and 32 MPa the highest ASX yield was 0.3 of ASX yield using a mixture of 40:60 vol% hexane/acetone in Soxhlet

and the highest TCC was 0.4 of the highest TCC obtained using Soxhlet (60:40 vol% hexane/isopropanol).

Lipid/ASX recovery increased with an increase in pressure of SC-CO_2. At temperatures below the cross-over pressures (20-25 MPa), the ASX/lipid yield decreased with temperature increase and above the cross-over pressure, an increase in temperature increased yields. The conditions to maximize both lipids and ASX, and TCC were 50 ˚C and 30 MPa. SC-CO_2 extract fractions had high percentages of neutral lipids (FFAs, STs and TAGs) but low PLs. The higher FFA in the extracts compared to TAG represents degradation/hydrolysis of TAG to FFA due to freeze-drying and water content in biomass.

The impact of co-solvents was studied at the "optimum" pressure and temperature from the RSM analysis. In this study, we used a static co-solvent system and then compared results with continuous approaches in the literature to validate the approach [15,19][21]Ethanol and 40:60 .vol% hexane/acetone as a co-solvent increased lipid/ASX recovery using SC-CO_2. Sunflower oil recovered higher ASX compared to waste fish oil. This work showed that using static co-solvent in SC-CO_2 without any extra cost of pump for co-solvent provided the same lipid yield as and the higher ASX yield than the published papers using continuous co-solvent/SC-CO_2.

The SC-CO_2 study showed that polar co-solvents increased PL and SFA but decreased MUFA and PUFA in the SC-CO_2 extract. Compared to Soxhlet with hexane/acetone (40:60 vol%), FA compositions except MUFA in the SC-CO_2 extract were higher. Adding sunflower or waste fish oil to the SC-CO_2 system provided extracts with less FFA/ST but

higher TAG. Compared to SC-CO_2 extracts without any co-solvents or with polar co-solvents adding sunflower oil or waste fish oil to SC-CO_2 did not increase n-3 FAs but provided extracts with higher n-6 FAs.

6.1.1.3. Kinetic and Mathematical modeling of SC-CO_2 for lipid/ASX recovery

In chapter 5 a mass transfer model of the lipid/ASX extraction process was developed. In this study, we used the Goto et al. model [22] which has a single fitting parameter, equilibrium adsorption coefficient (K). The model showed good agreement with the experimental data for both lipid/ASX extraction with AARD values of 6-8%. Overall extraction rates of lipid/ASX were controlled by the strong solid–solute interaction (K=372 for lipids, K=824 for ASX), leading to a reduction in the solubility of lipids/ASX in SC-CO_2. Compared to the literature, the solubility of ASX extracted from shrimp by-products in this study was lower; This is likely due to the strong ASX-shrimp interaction (K=824), and interaction of other compounds in the shrimp which is not considered in this model.

To assess the feasibility of SC-CO_2 on a larger scale, it is necessary to understand the SC-CO_2 extraction mechanism and discover the optimum extraction condition and controlling parameters in extraction rates of lipids/ASX. Either Solute solubility or the solute-solid interaction can control the phase equilibrium depending on the initial solute concentration in the sources. This leads to the study of the mass transfer model. The results of this model would be applicable to other shrimp species which has a low initial amount of lipids/ASX and play a vital role in industrial SC-CO_2 operations of shrimp processing by-products. Thus, to design/operate optimally scale-up equipment based on this model result, it is required to keep optimal inputs constant such as temperature, pressure and solvent/solid

ratio. However, in scale-up production, the mass transfer can impact the overall extraction rate which was not observed in this lab-scale extraction process (solid-solute interaction limited the overall extraction rate). Furthermore, scaling up all factors may be constrained by major parameters such as flow rate, solid moisture, etc. which limit the extraction process. As such, it is critical to determine effective scale-up factors for larger-scale production.

Overall, this study showed due to the polar nature of lipids in Atlantic shrimp by-products, solvents with high polarity are preferred. Moreover, freeze-drying used to remove water impacted the lipid compositions, consequently decreased lipid yield but improved ASX yield. ASX is not miscible in water, and esterified forms of ASX with low polarity present in the shrimp by-products dominate the free form of ASX (which has more polar nature). Therefore, to maximize total ASX extraction using solvents, a low-medium polarity solvent is required when the solid is in the dried medium but high polarity solvent is required in the wet medium. ASX extraction using waste fish oil requires either long extraction times at lower temperatures or high temperatures at lower extraction times to reduce possible degradation/isomerisation during extraction. Since waste fish oil is a source of TAGs, the extract is high in TAGs, whereas for Soxhlet extract PLs from shrimp by-products dominate, while SC-CO_2 extract includes more TAGs relative to Soxhlet. Polar co-solvents in SC-CO_2 improved PL recovery in addition to ASX.

6.2. Recommendations

- This research provided information on a laboratory scale basis. Design parameters should be validated at larger scales for design purposes.

- All focus of this study was on lipid/ASX extraction, but further work can focus on assessing any other potential value-added products (protein, chitin, etc.) of the residual leftover from all extraction processes. This can be used for animal feed and materials and drive towards a zero effluent process.
- The total carotenoid content referred to as total ASX was measured in this study. As various carotenoids, mainly forms of ASX (with different degrees of polarity) present in the marine sources, characterization/quantification of carotenoid profile as a further work can facilitate optimization of the solvent-based extraction processes for high yield/quality which reduces extraction runs and costs.
- This study showed pre-treatment, freeze-drying can impact the quality of extract in terms of lipids/ASX. Thus, further work can be optimization study of various pre-treatment such as pre-washing, freeze-drying (focusing on various drying times) or/and combination of pre-washing/freeze-drying on lipid distributions and ASX yield.
- The optimization of the SC-CO_2 extraction process in this research focused on only temperature and pressure. Future work should focus on the study of other factor impacts such as flow rate, extraction time, and particle size. Also, a broad range of temperature/pressure should be studied as future work for deep evaluation of their impacts on lipid/ASX extraction.
- In this study, FD sample was processed for lipid/ASX extraction using SC-CO_2. Water can act as a co-solvent in bioactive recovery as shown in some published studies. Thus, further work should be the study of water impact on lipid distributions and ASX yield using SC-CO_2 at optimal conditions.

- We evaluated static co-solvent impacts on lipid/ASX extraction using SC-CO_2 at the optimal condition as a preliminary study. To maximize the efficiency of waste fish oil in ASX recovery, further work on optimization of SC-CO_2/fish oil system with an impact study of co-solvent/waste ratio, static time, dynamic time, solvent flow rate, temperature and pressure on ASX extraction should be considered.
- Future work on the SC-CO_2 system can be the study of a mixture of fish waste and shrimp waste as a feedstock in SC-CO_2 for ASX recovery in fish oil medium instead of adding waste fish oil as a co-solvent to the system. This reduces extra extraction processes for waste fish oil production and saves time/money.
- The current work does not include cost evaluation of all the processes studied. In future studies, the evaluation of the costs associated with each of the extraction process is highly recommended in order to determine their economic feasibility.

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Appendix

Table A 1: ANOVA for the astaxanthin (ASX) yield (Y) obtained from wet residues as a function of temperature (T), oil-to-waste ratio (R) and time extraction (t) and their interactions.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	456.88	9	50.76	27.57	0.0010	significant
T-Temperature, ˚C	84.27	1	84.27	45.77	0.0011	
R-Ratio of oil to waste, v/w	146.51	1	146.51	79.57	0.0003	
t-time, h	4.71	1	4.71	2.56	0.1705	
T*R	3.83	1	3.83	2.08	0.2087	
T*t	78.98	1	78.98	42.89	0.0012	

Source	Sum of Squares	df	Mean Square	F-value	p-value	
R*t	108.92	1	108.92	59.15	0.0006	
T^2	0.0065	1	0.0065	0.0035	0.9548	
R^2	9.07	1	9.07	4.93	0.0772	
t^2	18.42	1	18.42	10.00	0.0250	
Residual	9.21	5	1.84			
Lack of Fit	2.52	3	0.8416	0.2519	0.8564	ıot significant
Pure Error	6.68	2	3.34			
Cor Total	466.08	14				

Table A 2:ANOVA for the astaxanthin (ASX) yield (Y) obtained from freeze-dried (FD) residues as a function of temperature (T), oil-to-waste ratio (R) and time extraction (t) and their interactions.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	4872.54	9	541.39	80.80	< 0.0001	significant
T-Temperature, ˚C	27.68	1	27.68	4.13	0.0978	
R-Ratio of oil to waste, v/w	350.61	1	350.61	52.33	0.0008	
t-time, h	202.72	1	202.72	30.26	0.0027	
T*R	19.25	1	19.25	2.87	0.1508	
T*t	2306.13	1	2306.13	344.19	< 0.0001	
R*t	23.68	1	23.68	3.53	0.1189	
T^2	1496.52	1	1496.52	223.36	< 0.0001	

Source	Sum of Squares	df	Mean Square	F-value	p-value	
R^2	3.71	1	3.71	0.5532	0.4905	
t^2	531.30	1	531.30	79.30	0.0003	
Residual	33.50	5	6.70			
Lack of Fit	32.16	3	10.72	15.99	0.0594	not significant
Pure Error	1.34	2	0.6703			
Cor Total	4906.04	14				

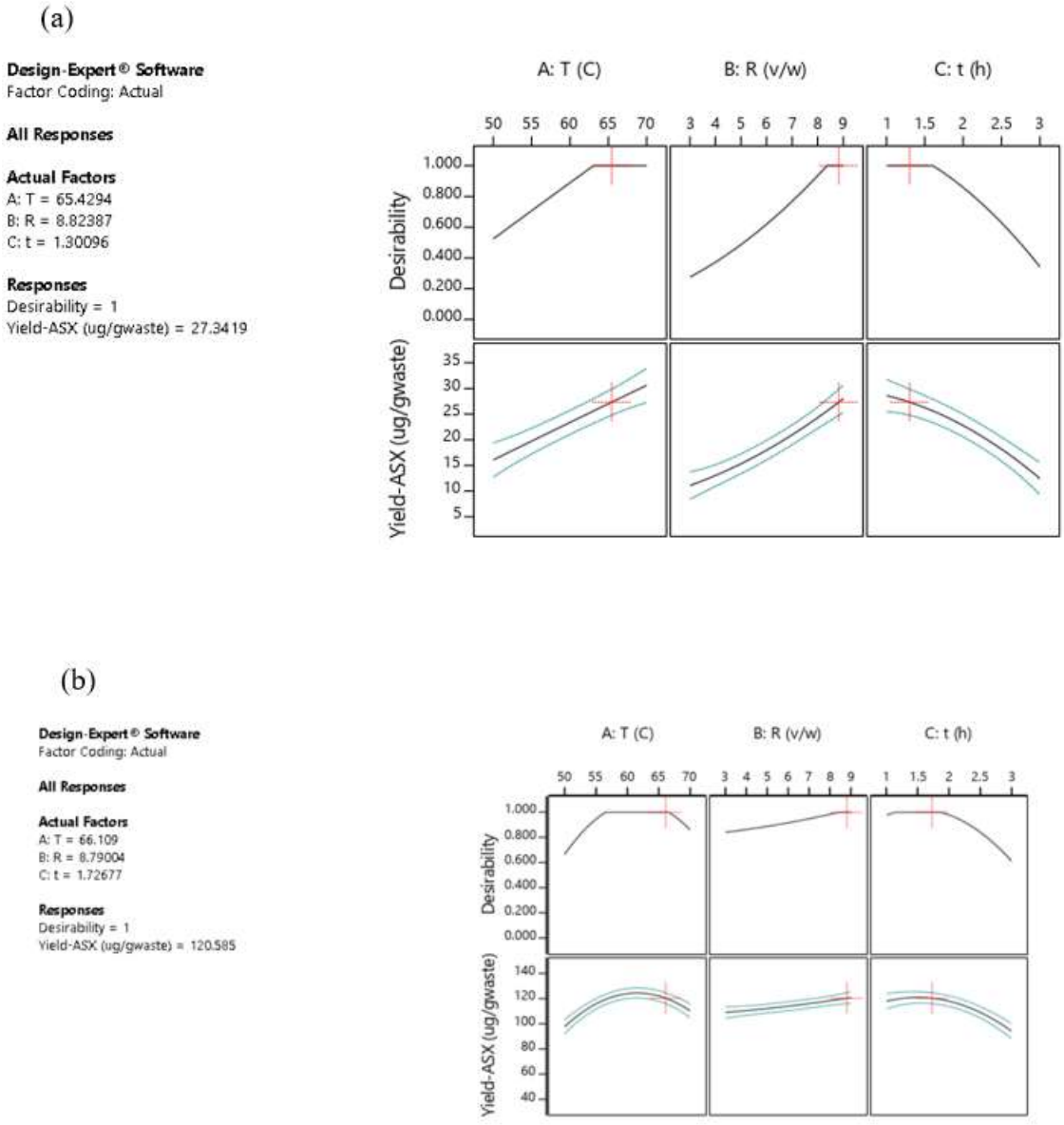

Figure A 1: *Profiles for predicted values of astaxanthin (ASX) yield and the desirability level for different factors for optimum ASX yield from (a) wet residues and (b) freeze-dried (FD) residues.*

Table A 3: *ANOVA results for the total lipid yield (Y1) obtained using SC-CO_2 without co-solvents as a function of temperature (T), pressure (P) and their interactions.*

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.9123	5	0.1825	26.91	0.0005	significant
X1-T	0.3081	1	0.3081	45.43	0.0005	
X2-P	0.4500	1	0.4500	66.35	0.0002	
X1*X2	0.1444	1	0.1444	21.29	0.0036	
$X1^2$	0.0011	1	0.0011	0.1629	0.7005	
$X2^2$	0.0074	1	0.0074	1.08	0.3378	
Residual	0.0407	6	0.0068			
Lack of Fit	0.0229	3	0.0076	1.29	0.4206	not significant
Pure Error	0.0178	3	0.0059			
Cor Total	0.9530	11				

Table A 4: ANOVA results for ASX yield (Y2) obtained using SC-CO_2 without co-solvents as a function of temperature (T), pressure (P) and their interactions.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	7151.98	5	1430.40	10.50	0.0063	significant
X1-T	148.06	1	148.06	1.09	0.3373	
X2-P	6587.03	1	6587.03	48.37	0.0004	
X1*X2	94.97	1	94.97	0.6973	0.4357	
$X1^2$	48.84	1	48.84	0.3586	0.5712	
$X2^2$	308.09	1	308.09	2.26	0.1833	
Residual	817.15	6	136.19			
Lack of Fit	521.08	3	173.69	1.76	0.3269	not significant

Pure Error	296.07	3	98.69			
Cor Total	7969.14	11				

Table A 5:ANOVA results for TCC (Y3) obtained using SC-CO_2 without co-solvents as a function of temperature (T), pressure (P) and their interactions.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	22.80	5	4.56	6.78	0.0187	significant
X1-T	0.3371	1	0.3371	0.5013	0.5055	
X2-P	19.43	1	19.43	28.90	0.0017	
X1*X2	0.3364	1	0.3364	0.5002	0.5059	
$X1^2$	0.3290	1	0.3290	0.4891	0.5105	
$X2^2$	2.62	1	2.62	3.89	0.0961	
Residual	4.04	6	0.6725			
Lack of Fit	2.69	3	0.8980	2.01	0.2906	not significant
Pure Error	1.34	3	0.4470			
Cor Total	26.83	11				