Supercritical Carbon Dioxide Extraction of Fatty Acids from Atlantic Sea Cucumber (*Cucumaria frondosa*) Viscera

by

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Dalhousie University is located in Mi'kma'ki,
the ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

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Abstract

Omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), play important roles in human health. Omega-3 PUFAs-enriched nutraceuticals are highly recommended as supplements for daily diets. Currently, EPA and DHA are mainly obtained from fish and its processing by-products through extraction and fractionation. Besides those traditional sources, some marine organisms' by-products, like see cucumber (*Cucumaria frondosa*) viscera, also exhibit potential as natural supplies of Omega-3 PUFAs. Supercritical carbon dioxide ($scCO_2$) extraction technique has attracted increasing interests as a promising green technology for lipids extraction from marine organisms for biofuel production or pharmaceutical development. In order to establish a clean and efficient recovery process, this study applied $scCO_2$ extraction to extract lipids from *Cucumaria frondosa* viscera. Through a response surface design, the extraction process variables, such as temperature (35-75 ˚C), pressure (20-50 MPa), dynamic extraction time (30-70 min), and co-solvents to biomass ratio (0:1-2:1), were evaluated and optimized to obtain the highest yields of EPA and DHA in the extracted product. Temperature of 75 ˚C, pressure of 45 MPa, dynamic extraction time of 30 min, and mass ratio of co-solvent to biomass as 2:1 was determined as the optimal levels, achieving 88% of fatty acids recovery and 80 % of combined EPA and DHA recovery in comparison to maximum contents obtained in *in-situ* transesterification. The effect of pre-treatment methods (hot air drying and freeze drying) on lipid extraction yield was also investigated. It was found the freeze-dried samples yielded significantly more fatty acids but insignificantly different selected Omega-3 fatty acids. However, the cost and energy consumption of freeze drying equipment and the statistically insignificant difference of Omega-3 fatty acids yields still suggested it was not necessary to switch to commercial freeze dryers for fatty acids extraction at the industrial level.

List of Abbreviations Used

12-MTA: 12-methyltetradecanoic acid
ANOVA: analysis of variance
AOAC: Association of Official Agricultural Chemists
BHT: butylated hydroxytoluene
C19:0: nonadecanoate
CCC: central composite circumscribed design
CCF: central composite face-centered design
CCI: central composite inscribed design
DHA: docosahexaenoic acid
DWB: dry weight basis
EPA: eicosapentaenoic acid
FA: fatty acids
FAMEs: fatty acid methyl esters
GC-FID: gas chromatograph-flame ionization detector
GC-MS: gas chromatograph-mass spectrometry
KCl: potassium chloride
NaCl: sodium chloride
$NaHCO_3$: sodium bicarbonate
$NaSO_4$: sodium sulfate
NIST: National Institute of Standards and Technology
PUFAs: polyunsaturated fatty acids
RSM: response surface methodology
$ScCO_2$: supercritical carbon dioxide
SEM: scanning electron microscope
SFT: supercritical fluids technique
WWB: wet weight basis
α: Alpha
β: Beata

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Chapter 1: Introduction

1.1 Background

Sea cucumbers, marine invertebrates under the phylum of Echinoderms, class Holothuroidea, are ideal functional food with high nutritional value and have been exploited and used for hundreds of years as a food delicacy and medicines for a wide variety of disease (Bahrami et al., 2014; Gomes et al., 2014). In the 1950s, the global landing amount of sea cucumbers was 5, 000 tonnes per year, following with a stable increasing reaching to 30, 000 tonnes in 2003 (Conand & Byrne, 1993; Food and Agriculture Organization of the United Stations, 2005; Therkildsen & Petersen, 2006). Furthermore, the year of 2015 witnessed approximately 70,000 metric tonnes of sea cucumbers produced globally, growing 38.3% within the past five years before 2015 (Food and Agriculture Organization of the United Stations, 2017). Comparing with others, sea cucumber fishery is rapidly developing and starts taking up a greater portion in the global market although it is still smaller in scale. Initially, the fishery only focused on the Indian and Pacific Ocean (Therkildsen & Petersen, 2006). The main species in the Indo-Pacific region include *Apostichopus japonicus*, *Holothuria scabra*, *Stichopus chloronotus*, *Thelenota ananas*, *Stichopus variegatus*, etc. As the demand for sea cucumbers and the risk of overexploitation of species increase, various new species of sea cucumbers have been exploited in many new regions (Conand & Byrne, 1993; Therkildsen & Petersen, 2006).

Several studies have proven that sea cucumbers are rich sources of essential amino acids, proteins, vitamins (vitamin A, B1, B2, and B3), mineral elements (zinc, iron, magnesium, calcium), and various bioactive molecules with diverse therapeutic properties (Bordbar et al., 2011; J. Chen et al., 2005; Gomes et al., 2014; Janakiram et al., 2015); and those bioactive molecules possessing pharmacological potential, like fatty acids, saponins, triterpene glycosides, steroid glycosides, phenolics, cerebrosides, lectins, sulfated polysaccharides and peptides, exhibit a wide range of biological activities including immunostimulatory, anticancer, antimicrobial, antiviral, anti-angiogenic, anti-inflammatory, antioxidant, anti-obesity, and anti-aging activities (**Figure 1.1**) (Bordbar et al., 2011; Guo et al., 2018a; Janakiram et al., 2015; Zhao et al., 2018). Therefore, sea cucumbers have attracted significant interests in developing techniques to isolate valuable

compounds and understanding their potential as pharmaceuticals and nutraceuticals and functional food.

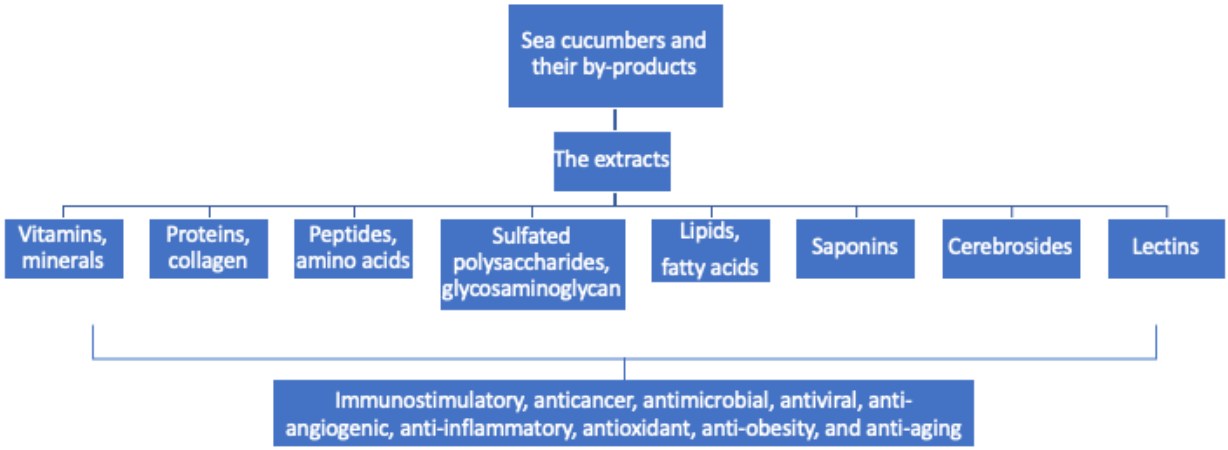


Figure 1.1 Bioactive compounds in the sea cucumber extracts and relevant effects on human.

Sea cucumbers are comprised of three parts, including body walls, tentacles, and viscera, (Hossain et al., 2020). The general practice of preparing sea cucumbers for food market relies on cutting, trimming and removing tentacles and internal organs during processing; as such, considerable amount of sea cucumber by-products containing valuable matter ends up in the landfill. Mamelona et al. (2010) have reported that echinoderm by-products like viscera, representing 50 % of the Atlantic sea cucumber biomass, were discarded in recent years (Mamelona et al., 2010). Currently, sea cucumbers are mainly sold in the form of fresh sea cucumbers and dried sea cucumbers. Also, dried and pulverized sea cucumber tissues are encapsulated as nutraceuticals and sold over the counter (Attoub et al., 2013). Such fresh, dried, or processing products only involve the body wall of sea cucumbers as main ingredients, even though all the constituents are edible, and by-products (i.e., internal organs) also contains various bioactive compounds. For example, Zhong and his coworkers found sea cucumbers with internal organs had higher antioxidant activity due to the orange internal organs containing abundant astaxanthin (Zhong et al., 2007). The growing market for nutraceutical supplements provides an opportunity to enhance the profit margins through generating values from by-products that are typically discarded as wastes (Sánchez-Camargo et al., 2011). Adverse environmental impacts of wastes generated from

marine organisms processing drive research into extraction of value-added compounds, such as lipids and astaxanthin in shrimp residues (Sánchez-Camargo et al., 2011, 2012; Treyvaud Amiguet et al., 2012), astaxanthin in blue crab shell wastes (Félix-Valenzuela et al., 2001), and lipids in fish by-products (Haq et al., 2017; Rubio-Rodríguez et al., 2008, 2012). However, there is limited research into utilization of sea cucumber by-products. Therefore, to increase the market value of sea cucumber and minimize the loss of valuable resources, researchers are required to develop an understanding of the extraction of value-added compounds from sea cucumber by-products.

To the best of our knowledge, current studies on sea cucumber lipids only used conventional methods involving organic solvents such as chloroform and n-hexane. However, chlorinated solvents like chloroform possessing toxic and potentially carcinogenic properties increase concerns on the environment and health; whereas other solvents like n-hexane and n-heptane demonstrate low extraction yield (Sánchez-Camargo et al., 2011). The cost of large amount of solvents required, their health and environmental concerns, and strict requirements for products purity, challenge the use of organic solvents for the extraction of natural products at the industrial levels. Thus, to eliminate/reduce the use of organic solvents, researchers are motivated to seek greener alternatives to conventional extraction methods; among which, the supercritical carbon dioxide ($scCO_2$) extraction method has attracted increasing interests.

1.2. Research Objectives

Even though $scCO_2$ extraction has been extensively studied for its potential to produce lipids and has been used to extract oils from a variety of animal and plant sources, studies associated with the extraction of lipids from sea cucumber by-products (especially *Cucumaria frondosa* viscera) using $scCO_2$ is still blank. Thus, it is critical to gain an understanding of the $scCO_2$ extraction of lipids from *Cucumaria frondosa* viscera by selecting appropriate process variables and pre-treatments, characterizing the products, optimizing the extraction process, and comparing the results to those obtained using conventional methods.

The overall objective of this study is to extract value-added compounds Omega-3 fatty acids from *Cucumaria frondosa* viscera using $scCO_2$. Specific research objectives include:

1. To extract lipids from Cucumaria frondosa viscera using $scCO_2$ and compare product yields and lipidic profile with those obtained using conventional and modified traditional methods.
2. To examine the impacts of parameters (temperature, pressure, extraction time, and co-solvents) on fatty acids yield.
3. To optimize the $scCO_2$ extraction processing conditions for maximizing yields of target products fatty acids.
4. To investigate the effects of pre-treatments on fatty acids production and their composition.

1.3. Thesis Organization

Chapter 2 provides a literature review on a general lipidomic profile of *Cucumaria frondosa* viscera, conventional extraction methods applied for lipids recovery and $scCO_2$ extraction and their efficiency comparison, as well as effects of pre-treatments on extraction yields. Chapter 3 presents materials and methods used in this project, including the procedures of the Bligh & Dyer method and transesterification (*in-situ* and after extraction), the setup of $scCO_2$ extraction, the methods of product characterization, the operations of pre-treatments, and the design of experiments. Chapter 4 explores the performance of conventional methods and improved methods assisted with ultrasonic pre-treatment on the extraction of fatty acids from *Cucumaria frondosa* viscera. Chapter 5 offers preliminary experiments to determine the range of process variables that influence $scCO_2$ extraction of fatty acids from *Cucumaria frondosa* viscera. Chapter 6 explores optimization of $scCO_2$ extraction of fatty acids from *Cucumaria frondosa* viscera using response surface methodology. The effects of process variables on the target compounds extraction are discussed. Chapter 7 compares the conventional and $scCO_2$ methods on the extraction of fatty acids from *Cucumaria frondosa* viscera. In Chapter 8, the effects of pre-treatments (hot air drying and freeze drying) on fatty acids recovery are studied. Chapter 9 concludes the key points of the whole study and gives recommendations for the future work.

Chapter 2: Literature Review

2.1. *Cucumaria frondosa*

Cucumaria frondosa (**Figure 2.1**) is a dendrochirotic sea cucumber species with equally developed dendritic tentacles, which is well known as the orange-footed sea cucumber or the northern sea cucumber. This species can reach a maximum length of 50 cm, a maximum width of 10 cm, and a maximum weight of 2-5 kg. Although it is normally light to dark brown in color, some have been seen to be a pale shade of orange or cream (Abuzaytoun, 2017). The body shape is long and cylindrical, and the body surface grows five rows of tube feet. Its viscera can be divided into three different systems: digestive system, reproductive system (gonad), and respiratory system (Gudimova et al., 2004); among which, gonad morphology is highly related to its reproductive cycle. The reproductive cycle of *Cucumaria frondosa* in eastern Canada shows gametogenesis occurs firstly in the beginning of winter in which day length increases, while simultaneously energy transfer from the body wall to the gonad happens. In the early spring, the increase of food supply and temperature initiates the following gamete synthesis. Finally, the size, index, and calorific value of the tubules decrease in around early summer, which means sea cucumbers start spawning (J. Hamel & Mercier, 1996).

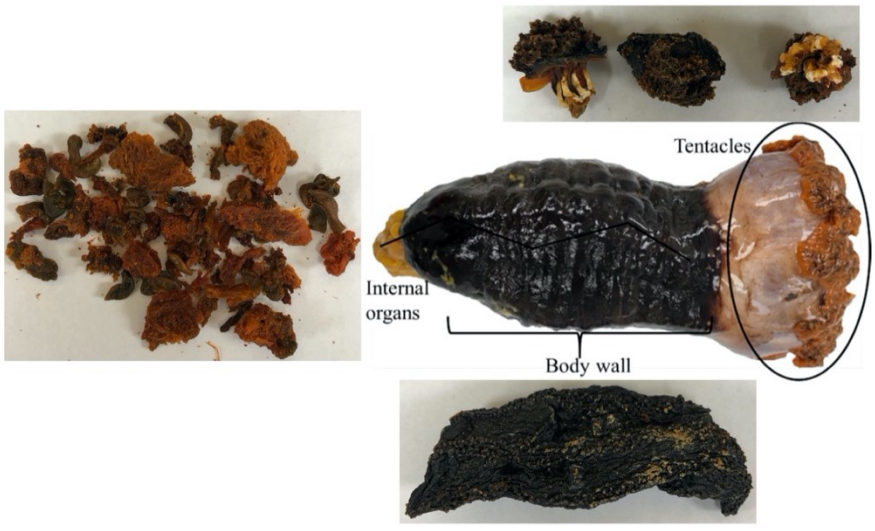


Figure 2.1 The structure of sea cucumber, *Cucumaria frondosa* (fresh sea cucumber entirety and dehydrated parts)

The sea cucumber is a widespread temperate species in the North Atlantic and Arctic Ocean. Thus, it is regarded as pan-Atlantic (Christian et al., 2010; Nelson et al., 2012; Singh et al., 1998). Its distribution ranges from the east coast of Canada, down to the southwest coast of New England, and along the way also include southern Iceland, the coast of Greenland and the northern Europe and Scandinavia, as well as the Faroe Islands (**Figure 2.2**) (Nelson et al., 2012; Singh et al., 1998). They are usually found in the 30-meter-deep sea or shallower waters. Also, they can be harvested in either shallow tide pools or ocean at the depths of 300 to 400 m, even some small ones can be detected in 800 m (Hamel & Mercier, 2008; Nelson et al., 2012; Singh et al., 1998). As recorded, *Cucumaria frondosa* in Atlantic Canada is harvested in between May and November using dragging equipment that is modified based on drags used in scallop and urchin fisheries (Abuzaytoun, 2017; Nelson et al., 2012).

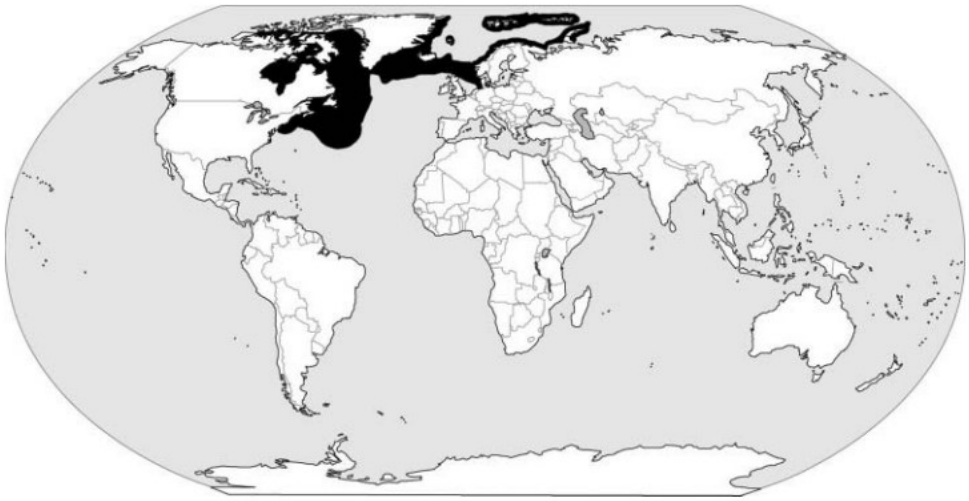


Figure 2.2 Distribution of *Cucumaria frondosa*. The black areas represent the distributing regions. Retrieved from Nelson et al., 2012.

Cucumaria frondosa is a benthic suspension-feeding sea cucumber species that lives by food particles suspending in the water. It normally gathers in areas with adequate water flows in which food can suspended reaching the feeding tentacles. The water column delivers the suspended particles to their adhesive ten tentacles, and then food (e.g., phytoplanktonic cells, small crustaceans and various eggs and larvae) is captured and fed into its oral cavity one at a time (J.-F. Hamel & Mercier, 1998; Holtz & MacDonald, 2009;

Singh et al., 1998). As such, they are different from other deposit-feeding sea cucumbers (Hamel & Mercier, 1998; Singh et al., 1998, 1999).

Before 1990s, *Cucumaria frondosa* aquaculture and capture did not develop. However, as the demand for sea cucumbers is higher than their production, a fishery for such an emerging species was established in the east coast of North America. The expansion of the *Cucumaria frondosa* fishery was rapid, which made the USA and Canada the second and fourth largest producer of wild-caught sea cucumbers in 2003, respectively (Therkildsen & Petersen, 2006).

The market demand for foods that are high in polyunsaturated fatty acids (PUFAs) particularly Omega-3 fatty acids including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) or relevant nutraceuticals from natural sources, has led to an increase in sea cucumber consumption in recent years. EPA and DHA are long chain Omega-3 polyunsaturated fatty acids with more than 18 carbons. They promote anti-inflammatory activities and reduce inflammatory processes in the body, and then further support cardiovascular health, brain function, metabolism and immune function, and anti-cancer. (Calder, 2015).

Zhong et al. (2007) reported that the proximate composition of fresh *Cucumaria frondosa* with viscera (Newfoundland, Canada) contained 90.1% of moisture, 5.11% of protein, 0.70% of lipid, 1.19% of carbohydrates, and 3.03% of ash; meanwhile, a high portion of PUFAs were detected in its extracts with a predominant EPA accounting for 43.2% of the total fatty acids content (Zhong et al., 2007). Liu et al. (2021) found 82.07% of moisture, 2.14% of ash, 4,68% of lipids, and 8.65% of protein in fresh *Cucumaria frondosa* viscera (Newfoundland, Canada); and the compositions of PUFAs and EPA were 33.01% and 27.76% of the total fatty acids (Liu et al., 2021). In comparison with fresh *Apostichopus japonicus* viscera that was reported to contain 0.66-2.12% lipids with EPA level at 15.12-17.76% of the total fatty acids (Lee et al., 2012), *Cucumaria frondosa* viscera with relatively abundant EPA content is regarded as a good material for the development of Omega-3-enriched product.

Furthermore, researchers experimentally confirmed the total lipid content of frozen *Cumaria frondosa* viscera (Nova Scotia, Canada) on the dry weight basis was varying from 20.81 to 28.87% with approximately 29-30% of PUFAs and 24-28% of EPA in the total

fatty acids (Abuzaytoun, 2017). In the same vein, air-dried and freeze-dried *Cumaria frondosa* viscera (Newfoundland, Canada) were found to contain 25.69% and 23.19% of lipids on the dry weight basis with a similar composition of PUFAs and EPA at 30.58% and 28.71% as well as 31.65% and 27.97% of the total, respectively (Liu et al., 2021). However, *Cucumaria frondosa* viscera separated from living bodies are decomposed very quickly without proper preservation because of the presence of digestive enzymes. At industrial level, the convective drying using a continuous flow of hot air is the most common method due to the low costs and easy operation.

2.2. Conventional Methods

The most commonly used conventional methods for lipids extraction include the Folch method and modified Folch method (Christie, 1982; Folch et al., 1957; Iverson et al., 2001; Parrish, 1999; Ramalhosa et al., 2012), the Bligh and Dyer method and its modified version (Abuzaytoun, 2017; Bligh & Dyer, 1959; Breil et al., 2017; Christie, 1982; Iverson et al., 2001; Manirakiza et al., 2001; Ramalhosa et al., 2012; Smedes, 1999), Soxhlet extraction (Ewald et al., 1998; Manirakiza et al., 2001; Ramalhosa et al., 2012), the Roese–Gottlieb method (Manirakiza et al., 2001; Ramalhosa et al., 2012), the Hara & Radin method (Hara & Radin, 1978; Ramalhosa et al., 2012), the Randall method (Fiori et al., 2012). **Table 2.1** summarizes representative examples of each method.

Ramalhosa et al. (2012) contrasted the performance of several conventional extraction methods (Soxhlet using hexane, the Bligh & Dyer, modified Bligh & Dyer using 2-propanol/cyclohexane/water, Folch, modified Folch using ethyl acetate/ethyl alcohol, Hara & Radin, Roese-Gottlieb) on fish lipids extraction. They found the Bligh & Dyer, Folch, modified Folch, and Hara & Radin showed highest extraction efficiencies (Ramalhosa et al., 2012). A comparative study, on Bligh & Dyer, Modified Bligh & Dyer, Soxhlet, and Roese-Gottlieb extraction methods, was conducted to evaluate their performance using animal source materials like eggs and dairy products, and animal feeds. The outcomes indicated the Bligh & Dyer method, modified Bligh & Dyer method, and Soxhlet extraction using acetone/hexane or dichloromethane/hexane were suitable for all chosen samples and exhibited relatively high extraction efficiencies compared to the reference values provided by producers; but Soxhlet method required longer extraction time and was convenient only for solid samples (Manirakiza et al., 2001). The view that Soxhlet

method is time consuming, was also supported by Ewald et al. (1998), who investigated the differences between the Bligh & dyer and Soxhlet extraction of lipids from four different fish species. Moreover, their data revealed the Bligh & Dyer method worked better in fish lipid extraction (Ewald et al., 1998). The modified Soxhlet method – the Randell method – also requires long extraction time; and lipid yields could be effectively improved through extending the time spending on the washing step, but the fatty acids composition did not change (Fiori et al., 2012).

The fact that the lipid yields given by the Folch method and the Bligh & Dyer method were not significantly different – was supported by Bligh & Dyer (1959) and Ramalhosa et al. (2012). However, Iverson et al. (2001) noticed some limitations of the Bligh & Dyer method – the extraction yields of low-fat samples (<2%) were similar to those obtained from the Folch extraction process, while the yields for samples with high lipid contents were relatively lower. Ewald et al. (1998) discovered a comparable result that the Bligh & Dyer worked better for lipids extraction from lean fish (Ewald et al., 1998). As a method based on the Folch, the Bligh & Dyer was developed for the economical purpose to minimize the volume of solvents used and maximize the yield of lipids. Additionally, the Bligh & Dyer method is a rapid solvents extraction system that only requires approximately ten minutes to complete the entire procedure, and the method allows the extraction of all lipids, including polar and non-polar lipids.

Breil et al. (2017) compared the performances of the solvents system of the Bligh & Dyer method and found water extracts about 13% of lipids, 43% of proteins, and 44% of glucose (Breil et al., 2017). As such, the addition of distilled water helps in not only separating proteins and sugars but extracting more lipids. The modified Bligh & Dyer added aqueous 0.88% potassium chloride instead of distilled water, to the solvent mixture (Christie, 1982). The addition of aqueous 0.88% potassium chloride solution yielded the target ratio of chloroform/methanol/water at 2:2:1.8 and at the same time induced the monophasic system to layer (Breil et al., 2017; Ren et al., 2017). After adding the salt solution, the cations from the salts decreases the dissociation of lipids by a mass action effect – assist the exchange of lipids between the aqueous phase and the organic phase (Folch et al., 1957). Therefore, the addition of an aqueous salt solution effectively reduces the loss of lipids.

Based on the assessment of conventional extraction methods above, it is evident the modified Bligh & Dyer method gives relatively higher lipid yield in a shorter extraction period and performs well in animal lipids extraction. Recently, it has been commonly used for sea cucumber lipids extraction (**Table 2.2**) (Abuzaytoun, 2017; Anisuzzaman et al., 2019; Barzkar et al., 2017; Budzinski et al., 2020; Nishanthan et al., 2018; Yahyavi et al., 2012).

Table 2.1 A list of commonly used conventional extraction methods.

Method	Material	Procedure	Lipid yield (%, gravimetrical method)	Ref.
The Folch	Salmon viscera	Mixing chloroform and methanol (v/v=2:1) with samples, followed by centrifugation. Then, adding 0.2 equivalents (of the total volume of the chloroform/methanol mixture) of water or aqueous salt solution to the suspension to induce phase separation. The biphasic solvent system consists of chloroform/methanol/water in a volumetric ratio of 8:4:3 (v/v/v). Collecting and drying the lower chloroform layer.	44.3% wwb.	Rincón-Cervera et al., 2017
Modified Folch	Fish (horse mackerel, chub mackerel, and sardine)	Replacing chloroform and methanol with ethyl acetate and ethyl alcohol at the same proportion. The rest operations were the same as the above.	3.381% wwb.	Ramalhosa et al., 2012
The Bligh & Dyer	Cod muscle	Samples containing 80% water were homogenized with a mixture of chloroform and methanol (v/v=1:2) for	0.666-0.76% wwb.	Bligh & Dyer, 1959

		~2 min. Then adding methanol and distilled water (v/v=1:1) respectively and blending for ~30s each time. The resultant ratio of chloroform/methanol/ water is 2:2:1.8 (v/v/v). Filtrating the mixture through Buchner funnel and washing the residues and the filter paper. Leaving the filtrate to set until break into two layers. Collecting and drying the lower chloroform layer.		
Modified Bligh & Dyer	*Cucumaria frondosa* viscera	Replacing the distilled water with 0.88% potassium chloride. Other steps were the same as the above.	4.9-7.4 % wwb.	Abuzayto un, 2017
	Mussel	Mixing diisopropyl ether and methanol or cyclohexane and propan-2-ol at the same ratio as chloroform and methanol in the Bligh and Dyer method.	24.5% wwb.	Smedes, 1999
Soxhlet extraction	Fish (horse mackerel, chub mackerel, and sardine)	Placing samples and sodium sulfate in a Soxhlet extractor. Passing hexane through in hot extraction mode at 140 °C for 4 h or more. Drying the extract in an oven at 104 °C until obtaining constant weight.	2.917% wwb.	Ramalho sa et al., 2012
Roese–Gottlieb	Chicken feed	Lipids extraction with petroleum ether after hydrolysis of the bounded lipids. Adding boiling water to samples and swirling for ~1 min. After cooling down, adding 25% ammonia solution and swirling for ~2 min. Then, adding methanol to the mixture and swirling ~2 min. Adding diethyl ether and	7.35% wwb.	Maniraki za et al., 2001

		petroleum ether and shaking vigorously. Collecting the upper layer, followed by concentration.		
Hara & Radin	Mouse brain	Adding the hexane and isopropanol mixture (3:2) into samples, then homogenizing for ~30 s. Separating organic solvents from the suspension and washing the homogenizer, funnel, and residue three times using the same solvents mixture (resuspending residues each time and letting solvents soak for about 2 min). Mixing 66.7% aqueous sodium sulfate with the extract to remove nonlipids. Collecting the upper, lipid-rich layer.	17.73% wwb.	Hara & Radin, 1978
Randall	Trout (*Oncorhynchus mykiss*) heads, spines, and viscera	Immersing a porous sample container in the Randall extractor directly into the boiling solvent, followed by washing. The immersion step helped a fast extraction of soluble components, and the extraction was carried out under 69 °C (the boiling point of n-hexane at atmospheric pressure) for 2-5 h (1 h for immersion, 1 or 4 h for washing).	Viscera: 57-70% dwb. Spines: 33-42% dwb. Head: 33-41% dwb.	Fiori et al., 2012

Note: dwb: dry weight basis, wwb: wet weight basis.

Table 2.2 Representative studies about sea cucumber lipids extraction using the Bligh & Dyer method.

Materials	Lipid yield (%)	Reference
Cucumaria frondosa viscera	20.81-28.87% dwb.	Abuzaytoun, 2017
Apostichopus japonicus	4.00% dwb.	Anisuzzaman et al., 2019
Holothuria nobilis	2.06% dwb.	Budzinski et al., 2020
Actinopyga miliaris	0.49% dwb.	
Stichopus horrens body wall	0.41% wwb.	
Holothuria Arenicola body wall	0.60% wwb.	Barzkar et al., 2017

Note: dwb: dry weight basis, wwb: wet weight basis. The lipids here represent the global extracts.

2.3. $ScCO_2$ Extraction of Lipids

2.3.1. Overview

As a promising green alternative to the conventional extraction process, supercritical fluids extraction (SFE) using $scCO_2$ has attracted considerable attention as an extraction technique for the development of functional foods, pharmaceuticals, and nutraceutical supplements. SFE is an extraction technique based on supercritical fluid properties, which can be achieved through regulating pressure and temperature above the critical point of the solvent used; for example, the critical point of $scCO_2$ is obtained under 31.1 ˚C and 7.4 MPa (**Figure 2.3**) (Sharif et al., 2014). There are several parameters that influence SFE extraction performance including temperature, pressure, the nature of sample matrix, extraction time (static and dynamic status) and presence of modifier (co-solvents) (Caudell, 1999).

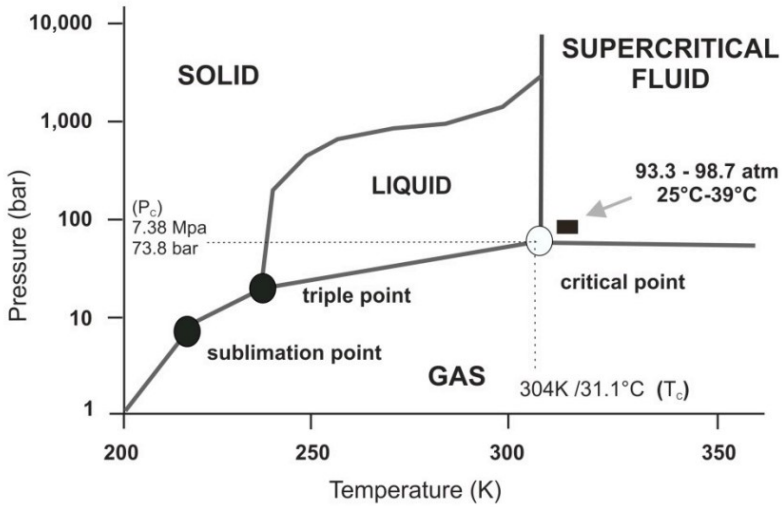


Figure 2.3 Schematic p-T phase diagram of CO_2. Four phases of CO_2 – solid, liquid, gas, and supercritical fluid – are demonstrated. CO_2 will transition to supercritical status at the critical point of 31.1 ˚C and 7.38 MPa. Retrieved from Budisa & Schulze-Makuch, 2014.

$ScCO_2$ is generally recognized as safe – non-toxic and non-flammable – and cheap, and also easy to remove due to its gas form at room temperature and pressure (Jiao & Kermanshahi pour, 2018; Sánchez-Camargo et al., 2011). Comparing with traditional processes, SFE shows high selectivity of bioactive compounds and avoids the use of toxic organic solvents; meanwhile, thanks to the characteristics of supercritical fluids – low viscosity and high diffusivity – this technique improves the extraction efficiency through enhancing mass transfer (Abbas et al., 2008; Bonilla-Méndez et al., 2018; Jiao & Kermanshahi pour, 2018). The findings in Cheung et al.'s study (1998) exhibited the highest yields of total fatty acids and Omega-3 fatty acids (33.8 mg/g & 16.2 mg/g) extracted from algae (*Sargassu hemiphyllum*) were obtained by $scCO_2$ extraction under 50 ˚C and 37.9 MPa, which were significantly higher than those observed in the Soxhlet method using chloroform/methanol (28.2 mg/g & 13.1 mg/g), and crude oils yield (55.8 mg/g) was comparable to that obtained from that conventional method (53.9 mg/g) (Cheung et al., 1998). Cheng and his coworkers used microalgae (*Pavlova sp.*) to evaluate the efficiency of $scCO_2$ and organic solvent extraction and concluded the $scCO_2$ extraction method was more effective with higher fatty acid methyl esters (FAMEs) recovery efficiency (98.7%) compared with the Soxhlet (58.5%) and mixed solvent extraction

(98.1%) (Cheng et al., 2011). The high selectivity of $scCO_2$ for bioactive compounds/nonpolar compounds was further examined by Rubio-Rodríguez et al. (2012) and Hajeb et al. (2015). They assessed different extraction methods (including enzymatic extraction, Soxhlet extraction, wet reduction, cold extraction, and SFE), and found $scCO_2$ extraction allowed significant reduction of pollutants like arsenic, mercury, cadmium, and lead, whereas other methods gave higher levels of toxic elements that exceeded the acceptable limit of 0.1 mg/g (Hajeb et al., 2015; Rubio-Rodríguez et al., 2012). Biological properties of the compounds of interest can be preserved well in the SFE process, because the target compounds are not diluted by organic solvents then becoming susceptible to oxidation (Sánchez-Camargo et al., 2011). Hao et al. (2015), Haq et al. (2017), and Rubio-Rodríguez et al. (2012) observed similar phenomenon – less lipid oxidation and longer oxidative stability period – when they employed $scCO_2$ to extract fish oils (Hao et al., 2015; Haq et al., 2017; Rubio-Rodríguez et al., 2012). Moreover, due to the high volatility of $scCO_2$, $scCO_2$ extraction normally does not require subsequent separation, which reduces the degradation of target compounds (Sánchez-Camargo et al., 2011). The biggest limitation that SFE suffers is this technique is not well-suited for the extraction of polar compounds, but adding co-solvents (an enhancer, like ethanol and water) can modify the non-polar nature of $scCO_2$ enhancing the extraction efficiency (Grosso et al., 2015). However, to the best of our knowledge, few published scientific papers employed $scCO_2$ extraction to isolate lipids from sea cucumbers and their by-products; only some patents in China reported the application of this technique in sea cucumber oils extraction (Li et al., 2016; Shao & Jiao, 2009; Sun et al., 2017; Sun et al., 2017).

2.3.2. $ScCO_2$ Extraction of Lipids from Marine Organisms

Lipid extraction from marine organisms using $scCO_2$ has been reported in several studies. This literature review offers at least three threads – the range of extraction conditions and the effects of different process variables, the potential impacts of co-solvents and pre-treatments, and the evaluation of SFE versus conventional extraction approaches – from which theory, methods, and insights might be woven. A selected studies on $scCO_2$ extraction of lipids from marine organisms is presented in **Table 2.2,** showing the process condition along with the corresponding yield.

The efficiency of $scCO_2$ extraction of nonpolar compounds is highly associated with $scCO_2$ density which depends on the temperature and pressure. Also, extraction time, co-solvents addition, and pre-treatments have been reported to play important roles in the $scCO_2$ extraction. The relationship between marine organism-based lipids yields and those process variables was well-established. $ScCO_2$ extraction of lipids from air-dried, ground northern shrimp (*Pandalus borealis Kreyer*) processing by-products under 15 MPa and 50 °C and 35 MPa and 40 °C at a certain range of flow rates (3-5 L/min) without co-solvent added was investigated by Treyvaud Amiguet et al. (2012). The extraction efficiencies at different extraction time (10-90 min) were also examined as well. The selection of the pressures was because a high selectivity of DHA and EPA and an improved extraction efficiency were observed under 15 MPa and 35 MPa, respectively. The relatively low temperature used was because of the thermal instability of the polyene. As a result, the oil yield increased with time of extraction, and the highest oil yield was achieved under 35 MPa and 40 °C after 90 min extraction. (Treyvaud Amiguet et al., 2012). Sánchez-Camargo et al. (2011) employed $scCO_2$ extraction technique to isolate lipids from freeze-dried, ground Brazilian redspotted shrimp waste (*Farfantepenaeus paulensis*) and explored the effect of pressure (200-400 bar) and temperature (40-60 °C) on the global extraction yield. In the $scCO_2$ extraction process, static phase of 20 min and dynamic phase of 200 min allowed for sufficient contact between samples and $scCO_2$; and the flow rate of $scCO_2$ was maintained at 1.5 L/min. It was reported pressure and temperature exhibited very insignificant effect on the lipid extraction yield, and the central point of 300 bar and 50 °C resulted in a maximum lipid extraction yield (2.21%) (Sánchez-Camargo et al., 2011). Similar conclusions were drawn by Yamaguchi et al. (1986) and Hardardottir and Kinsella (1988). Yamaguchi et al. (1986) applied $scCO_2$ to extract lipids from freeze-dried Antarctic krill (*Euphausia superba*) through fixing all experimental variables except one – varying the temperature from 40 to 80 °C under a fixed pressure (245 bar) and changing the pressure from 245 to 392 bar at a fixed temperature of 40 °C – the result highlighted the oil yield was almost constant (Yamaguchi et al., 1986). Hardardottir and Kinsella (1988) found increasing the pressure from 2000 to 5000 psig and the temperature from 40 to 50 °C had little effect on the $scCO_2$ extraction of lipids and cholesterol from freeze-dried, ground rainbow trout (Hardardottir & Kinsella, 1988). Létisse et al. (2006) designed a more

comprehensive study to optimize the extraction of EPA and DHA from lyophilized sardine heads using $scCO_2$. The parameters involve in the experimental design include pressure (100-350 bars), temperature (40-80 °C), time extraction (9-55 min), and $scCO_2$ flow rate (0.5-3.5 mL/mi). At temperature of 75 °C, pressure of 300 bars, scCO2 flow rate of 2.5 ml/min, and extraction time of 45 min, 10.78% of optimum lipids yields, and 12.65% of EPA and 11.59% of DHA in the extract (12.65 g of EPA and 11.59 g of DHA presented in 100 g of the extract) were obtained (Létisse et al., 2006).

Except for those common variables, researchers also attempted to examine the effects of co-solvents and pre-treatments on the $scCO_2$ extraction process. Sánchez-Camargo and her co-workers introduced ethanol as enhancer to the $scCO_2$ extraction process to investigate the effect of co-solvent on the extraction of lipids from freeze-dried, ground shrimp by-products after they accomplished the preliminary tests. The pressure (300 bar), temperature (50 °C), static time (20 min), dynamic time (100 min), and $scCO_2$ flow rate (3.0 L/min) were maintained but the ratio of $scCO_2$ to ethanol (95:5, 90:10, and 85:15 (m/m)) and the ethanol flow rates (0.334, 0.705, and 1.12 mL/min) changed. Remarkably, the lipid extraction yields increased dramatically as the ratio of absolute ethanol in $scCO_2$ increased, and an increase of 136% on lipids extraction yield was observed when the proportion of ethanol increased from 5% to 15%, resulting in maximum recovery of 93.8% of lipids (Sánchez-Camargo et al., 2012). Comparing with their previous work, the proportion of EPA and DHA in the lipid extract increased (Sánchez-Camargo et al., 2011, 2012). The addition of co-solvents (non-toxic) has been proven to be effective in modifying $scCO_2$ polarity, achieving enhanced lipid extraction recovery in a shorter extraction time. Patil et al. (2018) used a mixed-polarity co-solvent (hexane/ethanol = 1/1) to enhance the $scCO_2$ affinity towards non-polar and polar lipids. They found maintaining a fixed extraction condition but increasing co-solvent to solid ratio enhanced the total microalgae lipids yields (Patil et al., 2018). Hardardottir and Kinsella (1988) got comparable observations for the $scCO_2$ extraction of lipids from freeze-dried, ground rainbow trout with or without ethanol (Hardardottir & Kinsella, 1988). Those investigations indicated some fatty acids in polar lipids, like phospholipids, preferred to dissolve in more polar solvents. The addition of co-solvents modifies the solubility of extraction solvents, turning them to less selective; as such, both nonpolar and polar substances are extracted

(Sánchez-Camargo et al., 2012). Additionally, prior to $scCO_2$ extraction, Patil and his coworkers tested different co-solvents (dichloromethane, ethanol, hexane, methanol, and hexane and ethanol mixture), and identified $scCO_2$ coupled with those co-solvents under the same reaction conditions resulted in 10–12%, 12–15%, 13–15%, 18–20%, and 25-30% of total algal lipid on dry basis, respectively (Patil et al., 2018); as such, the mixture of hexane and ethanol may be a good candidate for the co-solvent. The use of co-solvent needs to be minimized, given that these solvents are petroleum-based and non-renewable. Additionally, presence of residual organic solvents in the final extract is a source of concern for products intended for pharmaceutical and nutraceutical applications (Chemat et al., 2019).

In order to improve the extraction efficiency, pre-treatments for cell disruption can be employed to enhance mass transfer and facilitate solvent penetration. The view that cell disruption before extraction would improve oils extraction yield was supported by Cheng et al. (2011) and Taher et al. (2014), who drew on the comparison of the amount of lipids extracted from ground and unground samples. Combining both process variables (the temperature at 35, 50, and 65 °C, the pressure at 200, 350, and 500 bars, and the $scCO_2$ flow rate at 2, 3, and 4 mL/min) and pre-treatment (sun drying and freeze drying, and unground and ground) as parameters, Taher et al. (2014) developed a study on optimization of $scCO_2$ extraction of microalgae (*Scenedesmus* sp.) lipids. They found lyophilization coupled with grinding prior to $scCO_2$ extraction significantly improved the yield of microalgae lipids; as such, $scCO_2$ extraction of microalgae lipids depended strongly on the matrix pre-treatment to enhance internal mass transfer (Taher et al., 2014). Also, Cheng et al. (2011) observed the pre-treatment of bead-beating significantly enhanced the recovery efficiency of FAMEs (98.7%) in the $scCO_2$ extraction, whereas untreated samples just recovered 34.0 % of FAMEs (Cheng et al., 2011). Furthermore, Taher et al. (2014) reported ground and unground freeze-dried samples (73.0% & 72.9 %) yielded almost the same triglycerides content, but Crampon et al. (2013) observed all triglycerides were extracted in respect of samples with smallest particle size smaller than 0.160 mm, while extraction of samples with biggest particle size bigger than 0.315 mm but smaller than 1 mm was limited requiring longer extraction time or more $scCO_2$ feeded. Conversely, some studies

implied the chanelling effect resulted from reducing sample particle size lowered extraction yield (Egerrs, 1996; Liu et al., 2011).

Besides matrix treatment like milling and bead-beating, drying method is of equal importance in the context of pre-treatments. Currently, air drying (under a continuously flowing hot stream of air at 40-45 °C for 48 h) is the most common method at industrial scale due to the economic and energy-saving purpose, though the shape and color of hot air-dried products deteriorates comparing with the original ones (Ratti, 2001). Recently, several studies have applied freeze drying under the vacuum condition to dehydrate *Stichopus japonicus*, obtaining dried sea cucumbers with higher quality and weaker odor (Duan et al., 2010; Z. Li et al., 2004; Yun et al., 2006). Shao and Jiao (2009) pointed out, except for lyophilized powder, nutrients in hot air-dried sea cucumbers and salted sea cucumbers cannot be fully absorbed by human; and the lipid content in freeze-dried sea cucumbers increases to 20% (Shao & Jiao, 2009). Nevertheless, a study aiming to explore the influences of drying methods on $scCO_2$ extraction of lipids from microalgae (*Nannochloropsis oculata*) found lipids extraction from freeze-dried samples consumed more $scCO_2$ to recover all the neutral lipids comparing with hot air-dried samples, indicating freeze drying protected the structure of microalgae cells (Crampon et al., 2013).

Static time is also worth discussing in the extraction process, which enables sufficient contact between feeding materials and $scCO_2$. A fixed static extraction time of over 10 min was often used, allowing their samples to soak in $scCO_2$ for adequate time (Jiao & Kermanshahi pour, 2018; Sánchez-Camargo et al., 2011, 2012; Woźniak et al., 2017), but it still requires to be determined whether the static status has significant influence on the extraction efficiency.

The patents associated with $scCO_2$ extraction of lipids from sea cucumbers involved five steps in the process: cleaning, freeze drying, grinding, sieving, and $scCO_2$ extraction. They recommended extraction conditions: (1) pressure: 25-35 MPa, (2) temperature: 30-60 °C, (3) dynamic extraction time: 60-180 min, (4) $scCO_2$ flow rate: 20-30 L/h (Li et al., 2016; Shao & Jiao, 2009; Sun et al., 2017; Sun et al., 2017). Under such conditions, yields on the dry weight basis and the wet weight basis were reported as around 10% and approximately 2.5%, respectively. Similarly, a recent study by Zhu et al. (2010) isolated lipids from the gonad of sea urchin (*Strongylocentrotus nudus*), another echinoderm, using

the $scCO_2$ extraction method. In this study, the fresh gonad tissue of sea urchin was freeze-dried and ground into powder, followed by loading the powder into the supercritical fluid apparatus extracting under 28 MPa at 50 °C for 80 min. The flow rate of $scCO_2$ was controlled at 20 L/h. Such extraction conditions were reported to yield 53.7 ± 2.6% of lipids (Zhu et al., 2010).

On the other hand, the results of $scCO_2$ extraction are usually compared to those obtained from conventional extraction methods to evaluate the performance of $scCO_2$ extraction. In general, the lipid recovery efficiency from marine organisms using $scCO_2$ is relatively lower (Sánchez-Camargo et al., 2011). Yamaguchi et al. (1986) reported a 57% recovery of lipids from krill at 245 bar and 60 °C (Yamaguchi et al., 1986). The result was supported by Létisse et al. (2006) who pointed out the maximum recovery of $scCO_2$ extraction of lipids from sardine heads was 50% of that of hexane extraction, and corresponding concentration of PUFAs (31.2%) was also slightly lower than that obtained from the hexane extraction (35.4%) and the Bligh and Dyer extraction (32.7%) (Létisse et al., 2006). Conversely, some studies about isolating lipids from microalgae indicated $scCO_2$ extraction was superior to other extraction techniques, exhibiting the maximum lipid yield (Cheng et al., 2011; Taher et al., 2014). So, the comparison between the $scCO_2$ extraction and conventional solvent extractions is considered as a straightforward method to assess the performance of SFE. Additionally, although conventional methods have been proven to be more efficient for lipids extraction in some investigations, Treyvaud Amiguet et al. (2012) specified $scCO_2$ showed high selectivity to Omega-3 PUFAs. Each gram of shrimp oil derived from SFE contained 795 mg of fatty acids including 78 mg of EPA and 79.7 mg of DHA, while the acetone extraction yields 627 mg of fatty acids per gram of shrimp oil among which contained 72.0 mg of EPA and 69.7 mg of DHA (Treyvaud Amiguet et al., 2012).

Table 2.3 A summary table of scCO2 extraction of lipids from marine sources.

Materials	Pre-treatment	Temperature	Pressure	Extraction time	Co-solvent	Maximum global extraction yield	Recovery efficiency	Ref.
Northern shrimp processing by-products (*Pandalus borealis Kreyer*)	Hot air dry, ground to powder (~2 mm mesh)	40 and 50 °C	15 and 35 MPa	10-90 min	\	13.7% dwb. (fatty acids: 10.89% dwb.)	66.5% (fatty acids: 76.97%)	Treyvaud Amiguet et al., 2012
Brazilian redspotted shrimp wastes (*Farfantepenaeus paulensis*)	Freeze dry, ground to powder (24-400 mesh)	40-60 °C	20-40 MPa	Static: 20 min Dynamic: 200 min	\	2.2% dwb.	64% (hexane), 45% (the Bligh & Dyer)	Sánchez-Camargo et al., 2011
Brazilian redspotted shrimp wastes (*Farfantepenaeus paulensis*)	Freeze dry ground to powder (24-400 mesh)	50 °C	30 MPa	Static: 20 min Dynamic: 100 min	Ethanol	4.63% dwb.	93.8%	Sánchez-Camargo et al., 2012

Antarctic krill (*Euphausia superba*)	Freeze dry, ground to powder	40-80 °C	24.5-39.2 MPa	120-240 min	\	11.7 % dwb.	59.4% (the Bligh &Dyer)	Yamaguchi et al., 1986
Rainbow trout (*Salmo gairdneri*)	Freeze dry, ground to particles (1-2 mm diameter)	40-50 °C	13.8-34.5 MPa	540 min or 360 min (with co-solvent)	Ethanol	78% dwb. (without co-solvent) 97% dwb. (with co-solvent)	\	Hardardottir & Kinsella, 1988
Sardine heads	Freeze dry	40-80 °C	10-35 MPa	Static: 2 min Dynamic: 9-55 min	\	10.5 %	50% (hexane) 16.67% (the Bligh & Dyer)	Létisse et al., 2006
Fish head of Longtail tuna (*Thunnus tonggol*)	Freeze dry, vacuum treatment at 0.133 bar for three days	45-65 °C	20-40 MPa	1.8, 2, 3, 5, 16 hrs	Ethanol (20-80% v/v)	35.6% dwb.	98.34%	Ferdosh et al., 2013
Fresh sardine	Freeze dry, vacuum treatment at 0.133 bar	40-70 °C	20-40 MPa	Static: 30 min Dynamic: 80 min	Ethanol	8.16% dwb.	>100% (Soxhlet)	Gedi et al., 2015

Microalgae (*Nannochloropsis salina*)	Microwave oven dry	40-80 °C	20-37 MPa	60 min	Hexane + Ethanol (1:1)	31.37% dwb.	\	Patil et al., 2018
Microalgae (*Pavlova* sp.)	Spray dry, bead-beating to disrupt the cells of microalgae	60 °C	30.6 MPa	6 h	\	17.9% dwb. (FAMEs: 15.7% dwb.)	40.04% (mix solvents) >100% (Soxhlet hexane) (FAMEs: 98.7%)	Cheng et al., 2011
Brown Seaweed (*Sargassum hemiphyllum*)	Freeze dry, ground to pass through a 1 mm sieve	40-50 °C	24.1-37.9 MPa	1 h	\	55.8% dwb.	>100% (Soxhlet)	Cheung et al., 1998
Microalgae (*Scenedesmus* sp.)	Freeze dry, ground	35-65 °C	20-50 MPa	Static: 15 min Dynamic: unstated	\	7.41 % dwb.	>100%	Taher et al., 2014
Sea urchin (*Strongylocentrotus nudus*)	Freeze-drying, and then ground to powder	50 °C	28 MPa	80 min	\	53.7 % dwb.		Zhu et al., 2010

Sea cucumbers and their by-products	Freeze dry, ground to powder (20-60 mesh)	50-60 °C	25-30 MPa	60-100 min	\	\	\	Shao & Jiao, 2009
Sea cucumbers	Freeze dry, ground to powder (60 mesh)	55 °C	30 MPa	2 h	\	~10% dwb.	\	Li et al., 2016
Sea cucumbers	Cook, cut, freeze dry, ground to powder (20-100 mesh)	32-35 °C	25-30 MPa	3-3.5 h	\	2.54% dwb. of Cerebrosides (glycosphingolipids)	\	Sun et al., 2017
Sea cucumbers internal organs (*Cucumaria frondosa*)	Cut, boil, mix with water (1:1-1:1.5 (w/w)), enzyme treatment, freeze dry	37-45 °C	25-35 MPa	1.5-3 h	\	2.19% wbb.	\	Sun et al., 2017

Note: dwb: dry weight basis, wwb: wet weight basis.

2.4. Design of Experiments: An Overview of Response Surface Methodology

Response surface methodology (RSM) is a series of mathematical and statistical tools for modelling and evaluating problems, in which responses are affected by multiple process variables and the goal is to maximize/minimize the response or meet a target value (Montgomery, 2017). As a second-order design, RSM contains cube (corner) points, axial points, and center points. Each part appears for different purposes:

(1) cube points: examine linear effect and interaction;

(2) axial points: explore quadratic effect and increase precision on linear effect;

(3) center points: investigate quadratic effect and obtain sigma hat information.

Design methods include central composite circumscribed (CCC) design, central composite inscribed (CCI) design, central composite face-centered (CCF) design, and Box-Behnken design; among which, central CCF (a special CCC design, a is chosen as 1) and Box-Behnken designs require 3 levels for each factor, but the others require 5 levels for each factor. Central composite design comprises an imbedded factorial or fractional factorial design with center points that is augmented with a group of axial points for estimation of curvature, while Box-Behnken design is an independent quadratic design rather than an embedded factorial or fractional factorial design. Also, central composite design includes extreme conditions (corner points), axial points (at the center of each face of the factorial space), and center points, while Box-Behnken design only covers midpoints of the edges of process spaces and center points. Therefore, central composite design is more reliable for a better quadratic model (a full quadratic model), although Box-Behnken design may yield relatively less runs (Heckert et al., 2012).

For central composite design (shown as **Figure 2.4**), CCC design provides high quality predictions over the entire design space, but its axial points are all outside the range of the factors establishing new extremes for all factors. As such, it is not a good choice for the study with limited range selection of process variables. CCI design only uses points within the setting ranges and utilizes low and high levels of each factor as axial points; but its prediction ability is not as strong as CCC design. In CCF design, axial points are at the center of each factorial space with a specific a as ±1. So, it is not a rotatable design and not able to provide constant prediction variance at all points that are equidistant from the design center. Also, it gives poor precision for estimating pure quadratic coefficients (Heckert et

al., 2012). Therefore, CCI design is regarded as the preferred in a case required each factor falling in a certain range. This method was also been used in $scCO_2$ extraction of lipids from Brazilian redspotted shrimp wastes (Sánchez-Camargo et al., 2011).

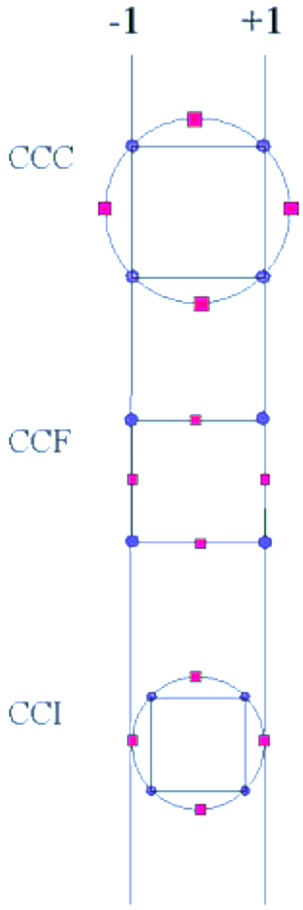


Figure 2.4 Comparison of the three different central composite designs.

2.5. Knowledge Gap

Nutrition composition (e.g., lipid content and fatty acid composition) of sea cucumber species is very sensitive to external changes, like seasons, diet patterns, locations, and gender (Abuzaytoun, 2017; Gianasi et al., 2017; Mamelona et al., 2010). Knowledge of fatty acid profiles of *Cucumaria frondosa* viscera is not only important in evaluating its nutritional values but also in assessing the performance of $scCO_2$ extraction of fatty acids from *Cucumaria frondosa* viscera. That means it is critical to obtained fatty acids profiles for our own experimental subjects. Research studies from the current literature have mainly focused on the optimization of crude oils yields rather than Omega-3 fatty acids, and most studies used the gravimetrical method to express the lipid contents of samples under

investigation. Although, the use of the gravimetrical method is more common than other means of analytical instruments and chromatographic methods, it results in some non-lipid or undesired compounds being taken into account. Cheng et al. (2011) found the microalgae crude lipids yield obtained from Soxhlet extraction was 18.5 g/100 g of the spray-dried samples while FAMEs yield that was quantified by GC was 9.8 g/100 g of the spray-dried samples only. The significant differences between global extraction yields and FAMEs yields implied the extraction of considerable amount of impurities from microalgae, like lipid-soluble pigments (Cheng et al., 2011). Furthermore, Abuzaytoun (2017) reported lipid extracts of *Cucumaria frondosa* viscera were highly pigmented due to the presence of carotenoids. We can conclude that it is not accurate to use the global extraction yield as the lipids content, and the fatty acids/FAMEs yield is a more appropriate index for marine oils, particularly for nutraceutical applications. Moreover, use of chromatographic techniques allows for generating insights into the profile of fatty acid and evaluating different extraction methods with respect to the extraction of individual fatty acids.

Besides, to the best of our knowledge, existing investigations related to lipids extraction from *Cucumaria frondosa* viscera mostly used conventional methods. $ScCO_2$ extraction process development and optimization for recovery of lipids from shrimp by-products, fish and fish by-products, microalgae, and sea urchin provide valuable insight, however, the effect of process parameters on lipid recovery from these feedstocks cannot be extended to sea cucumber. To the best of our knowledge, there is very limited work on $scCO_2$ extraction of lipids from sea cucumber viscera and the effects of process parameters on extraction yield of Omega-3 from sea cucumber viscera is not well understood. Moreover, extraction yield optimization and comparison between SFE and conventional extraction methods have not been previously evaluated.

The goal of this research is to gain deeper understandings on the effect of process variables, optimization of extraction process, expression of lipidic profiles, selection of pre-treatments, and performance of $scCO_2$ extraction compared with conventional methods.

Chapter 3: Materials and Methods

This chapter covers materials and methods relevant to the conventional methods and $scCO_2$ extraction as well as transesterification and Gas Chromatography method for extract characterization. Design of experiments and data analysis are also described.

3.1. Materials

Hot air-dried *Cucumaria frondosa* viscera were obtained from AKSO Marine Biotech Inc. (Halifax, Nova Scotia). *Cucumaria frondosa* was harvested in Fishing Area FAO 21 from July to November 2019 and kept frozen until processed in the plant. After being thawed and washed with sea water, the collected sea cucumbers were eviscerated viscera and their tentacles were cut off, leaving the cylindrical bodies. Those three parts were collected separately and dried under hot air at 40-45˚C for 48h. Samples were stored at room temperature and delivered to the Biorefining and Remediation Laboratory at Dalhousie University.

Frozen fresh *Cucumaria frondosa* viscera were also provided by from AKSO Marine Biotech Inc. After harvesting and processing, the viscera were frozen, stored in a cooler, and delivered to the Biorefining and Remediation Laboratory.

Ultra-high purity liquid carbon dioxide, nitrogen, helium, air, and hydrogen were purchased from Praxair, Dartmouth, Nova Scotia, Canada. The liquid carbon dioxide was used as the solvent in $scCO_2$ extraction. Internal standard methyl nonadecanoate (C19:0 methyl ester) and external standards 37 FAME mix standard and PUFA NO.3, butylated hydroxytoluene (BHT), sodium chloride (NaCl), potassium chloride (KCl), sodium sulfate (Na_2SO_4), anhydrous methanol, agent grade acetyl chloride, Whatman #1 filter paper, and glass wool were purchased from Sigma Aldrich Ltd (Oakville, Ontario, Canada). ACS grade toluene, sodium bicarbonate ($NaHCO_3$), GC grade hexane, and GC grade chloroform were purchased from VWR International Ltd (Mississauga, Ontario, Canada). 95% ethanol was purchased from Tupper Med Store at Dalhousie University (Halifax, Nova Scotia, Canada). All chemicals were used as received.

3.2. Methods

3.2.1. Sample Preparation

According to the literature review on pre-treatments, it was evident that particle size reduction increased the surface area and enhanced the contact between $scCO_2$ and samples. Thus, dehydrated samples were ground in an electric grinder (Shardor Technology Ltd., China) and sieved using an 18 mesh-sized sieve. The ground samples were packed in airtight zippered freezer bags and stored at -20 ˚C until required.

3.2.2. Sample Pre-treatment

To evaluate the effects of different drying methods on $scCO_2$ extraction, frozen fresh sea cucumber viscera were freeze dried using a bench-top freeze dryer (Labconco FreeZone 2.5 L Bench-top Freeze Dry System, Kansas City, MO, USA). Hot air-dried samples that had already been prepared by AKSO Marine Biotech Inc., were directly used.

The freeze drying pre-treatment method was adopted from Liu et al., (2021). Thawed *Cucumaria frondosa* viscera were separated into several portions putting in 20 mL tubes for freezing overnight at -20 ˚C and then transferred to freeze dryer flasks. The flasks were loaded onto the freeze dryer and set the temperature and pressure at around -87 ˚C and 0.014 mBar for two days. After freeze drying, samples were ground in an electric grinder and separated using sieves to obtain the size smaller 18 mesh (<1 mm). The milled samples were sealed hermetically using airtight zippered freezer bags to avoid moisture and oxidation and stored at -20 ˚C until required.

3.2.3. Moisture Content Determination

Moisture content was measured to determine the lipid content on dry weight basis, which makes all the response factors in the same unit for comparison. Moisture content of sea cucumber viscera samples was determined gravimetrically using a vacuum oven according to a method modified from (AOAC), 2006. Dried empty aluminum dishes in a vacuum oven at 105 ˚C for 3 hours, cooled down in a desiccator, and then weighed them using an analytical balance (Mettler Toledo AT261 Analytical Balance, Columbus, Ohio, United States). Approximately 1.5 g of hot air-dried and freeze-dried samples were weighed in the aluminum dishes and dried at 105 ˚C until the weight did not change. After drying, dishes were moved to a desiccator cooling down to a constant weight. The

measurement was carried out in triplicate for each sample. Reweighed dishes with dried samples and calculated the moisture content (%) using the following equation:

$$Moisture\ (\%) = \frac{(Weight\ of\ samples\ before\ drying - weight\ of\ samples\ after\ drying)}{Weight\ of\ samples\ before\ drying} \times 100 \quad (1)$$

3.2.4. The Bligh and Dyer Method

Cucumaria frondosa viscera were extracted according to a modified Bligh & Dyer method (Bligh & Dyer, 1959; Christie, 1982). Aliquots of samples (approximately 1.4900-1.5099 g) in quadruplicate were accurately weighed into labelled glass tubes on an analytical scale, followed by adding 6 mL of distilled water to make up to 80% of moisture content (6/7.5*100=80%). 7 mL of chloroform with 0.01% BHT and 15 mL of methanol (chloroform: methanol: water (v/v/v) =1:2:0.8) were also added into each tube. After covering with a nitrogen stream, the mixture was vortexed vigorously until the mixture formed a single phase. The well-homogenized mixture was poured into a prepared filter paper cone, and then the tissue residue, the tube, and the filter paper were rinsed with 7.5 mL of chloroform. The filtrate and the rinsing solution were collected in a clean centrifuge tube, and 7.5 mL of 0.88% KCl solution was added. The addition of aqueous 0.88% KCl led to the solution breaking into two phases (salt-induced phase separation). The top phase contained water, methanol, and non-lipid constituents and the bottom phase contained chloroform and lipid. The resulting proportions of chloroform: methanol: water (v/v/v) was 2:2:1.8 in the final mixture. After centrifugation, the top layer was discarded until almost touching the interface. The lower layer was pipetted onto the Na_2SO_4 in the filter paper cone. After the lower phase had been filtrated, the filter paper cone with Na_2SO_4 was rinsed using 3-4 mL of chloroform. The lipids-rich chloroform was collected in a pre-weighed clean tube and evaporated using a rotary evaporator (Buchi, Switzerland) under 45 °C, 258mbar. The extracts were further dried under the nitrogen, weighed, and stored at -20 °C. The extract was considered as the global extract, which was determined using Eq. (2):

$$Global\ extraction\ yield\ (g/100\ g\ of\ samples\ used) = \frac{Mass\ of\ the\ extract}{Mass\ of\ dehydrated\ sea\ cucumber\ viscera} \times\ 100 \quad (2)$$

An improved ultrasonic-assisted Bligh & Dyer method was employed. After adding water, chloroform, and methanol to the ratio of 1:2:0.8 (v/v/v), the mixture was covered with a stream of nitrogen and placed in an ultrasonic water bath (Branson 2510 sonicator, Brookfield, Connecticut, United States) with a frequency of 40 kHz for 10 min. The subsequent steps are consistent with the previous description.

3.2.5. $ScCO_2$ Extraction

The $scCO_2$ extraction apparatus (SFE-110, Supercritical Fluid Technologies Inc, USA) comprised of a CO_2 cylinder, a CO_2 pump, a co-solvent pump, a heating chamber, a sample vessel, collection vials, and a flow meter (**Figure 3.1**). Weighed *Cucumaria frondosa* viscera (1.4900-1.5099 g) along with co-solvent were loaded in a 10 mL sample vessel directly. The tightly sealed vessel was connected to the system. The extraction procedure included static and dynamic phase. Once the temperature and pressure achieved the setting in the closed system, the static stage was recorded. This step allows contact between the sample and the supercritical solvent. After the pre-determined time of static soaking, the static/dynamic valve was opened, and the restrictor valve was slightly adjusted to ensure $scCO_2$ was continuously drained through the vessel at approximately 10 mL/min. That is the dynamic extraction, and the extraction time was recorded as well. Two pre-weighed vials (a collection vial and a trap vial) were used for the extract collection. After extraction, the system was vented until the pressure dropped to 0 MPa and the pressure reached the room temperature. The vessel was opened to remove the residue and then reconnected to the system for washing. 95 % ethanol was purged using the co-solvent pump to rinse the line and the vessel to collect the remaining extract. The washing solution was evaporated in a rotary evaporator until no solvent was trapped. The wash extract after evaporation and the original extract were regarded as the global extract, weighed, covered with an inert atmosphere of nitrogen, and stored at -20 ˚C. The global extraction yield was calculated as shown in Eq. (2).

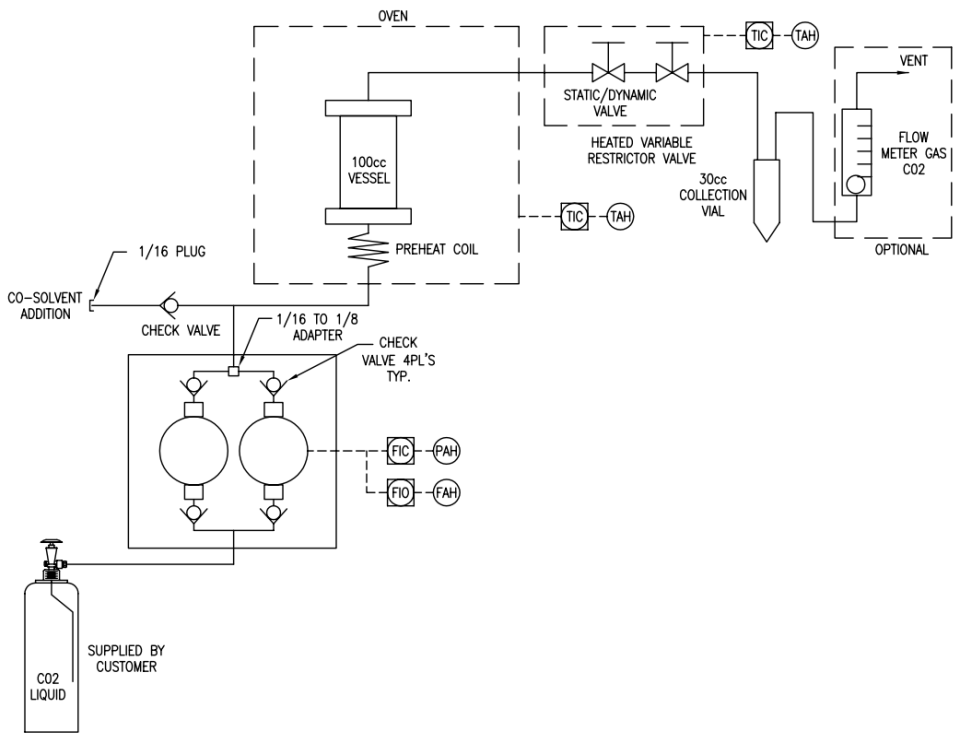


Figure 3.1 Process flow diagram of the SFE-110. Retrieved from SFE-110 handbook.

3.2.6. Transesterification After Extraction

The fatty acids were converted into FAMEs using the methanolic HCl transesterification. Briefly a certain amount of toluene with 0.01% BHT was added into the extract to reach a concentration of 100 mg/mL, followed by transferring 1.5 mL of extract-rich toluene into a 12 mL clean tube with dried methyl C19:0 (10 mg/mL, 0.75 mL). 3 mL of 5% methanolic HCl was added to the mixture and transesterification was performed at 100 ˚C for 1 hour. In that process, fatty acids were methylated to FAMEs. After reaction completion, tubes were removed from the heating block and cooled to a room temperature. 2 mL of 5% NaCl solution was added. After centrifugation, the mixture was separated into an organic and aqueous phase. The organic phase was collected in a new tube, while the aqueous phase was re-extracted using 1 mL of hexane for three times. With the assistance of centrifugation, the separated hexane layer was collected. 2 mL of 2% $NaHCO_3$ solution was added to the pooled organic layers to remove excess acid, and then the mixture was centrifuged. The upper layer was transferred to another new tube; whereas the aqueous layer was washed three times with 1 mL hexane, centrifuged, and transferred. A spoonful of Na_2SO_4 was added to absorb the water. A filter prepared by placing a plug of glass wool

in a disposable Pasteur glass pipette was used to filter the water-free mixture. The filtrate was collected in a pre-weighed tube and dried under a stream of nitrogen. The dried, derivatized product was weighed, covered with nitrogen, and stored at -20 ˚C.

3.2.7. *In-situ* Transesterification

In *in-situ* transesterification, methanolic HCL was applied to four replicates of identical *Cucumaria frondosa* viscera (~ 500 mg) with 10mg/mL of methyl C19:0 added as internal standard. The transesterification reaction was the same as described in **Section 3.2.6**.

An improved method with the assistance of an ultrasonic water bath was adopted. Prior to the addition of methanolic HCl, the sample along with 1.5 mL of toluene containing 0.01% BHT was treated in an ultrasonic water bath for 10 min. The next steps all followed the procedure in **Section 3.2.6**.

3.2.8. Fatty Acids Profiles in GC

Sample preparation: The methyl esters obtained in two-step extraction-transesterification (**Section 3.2.6**) and *in-situ* transesterification (**Section 3.2.7**) were diluted using hexane to a concentration of 20 mg/mL and transferred 1.5 mL of the diluted samples to a pre-labelled amber GC vial covering with nitrogen.

Standards preparation: Methyl C19:0 was used as the internal standard. It was prepared by dissolving 100 mg of methyl C19:0 in 10 mL of hexane, resulting in a solution with a concentration of 10 mg/mL.

The external standards – 37 FAME mix standard and PUFA NO.3 standard – were used as reference for qualitative analysis. 37 FAME mix standard was received as a 10 mg/mL solution in dichloromethane. In order to eliminate matrix effects, it was dried under the nitrogen and re-homogenized in 1 mL of hexane. PUFA NO.3 standard was received as a solid of 100 mg/package, which was further dissolved in 1 mL of hexane. After dissolving both standards in hexane, these were stored at -20 °C. 0.1 mg/mL of 37 FAME mix standard and 1.0 mg/mL PUFA NO.3 standard were prepared, respectively.

GC analysis: To specify the order of peaks in the external standards and identify some uncertain peaks presented in the chromatograph of samples, gas chromatography equipped with mass spectrometry (Agilent 7890B GC equipped with MSD, California, United States) and DB-23 column (60 m x 0.25 mm x 0.15 um) was used firstly. The oven

initial temperature was 50 ˚C holding for 1 min, and increased to 175 ˚C at a rate of 25 ˚C/min, and then raised to 230 ˚C at a rate of 4 ˚C/min. The temperature of 230 ˚C was hold for 5 min. The inlet and transfer line were set at 250 ˚C and 280 ˚C, respectively. Details are listed in Table **3.1**. The fatty acids were identified by the NIST (National Institute of Standards and Technology) library.

Table 3.1 GC-MS analysis conditions for the extract of *Cucumaria frondosa* viscera.

Chromatographic system	Agilent 7890B GC equipped with MSD
Column	DB-23, 60 m x 0.25 mm x 0.15 mm
Inlet temperature	250 °C
Injection volume	1 mL
Split ratio	50:1
Carrier gas	Helium
Head pressure	Constant pressure, 33 cm/sec at 50 °C
Oven temperature	50 °C (1 min), 25 °C/min to 175 °C, 4 °C/min to 230 °C (5 min)
Transfer line temperature	280 °C
Ion source	EI
Scan Mass	40-450 amu
Scan Speed	1.80 scans/sec

GC fitted with flame ionization detector (FID), and DB-23 column was applied to analyze samples and standards. The carrier gas was helium, using a split ratio of 1:50. The detector and injector temperatures were 280 and 250 °C, respectively. The column was maintained at 50 °C for 1 min, and raised from 50 to 215 °C at 25 °C/min, and increased to 230 ˚C at 4 ˚C/min, and finally maintained at 230 °C for 5 min. One microliter of the methyl esters was injected onto the column, and the fatty acids were identified by a comparison of the retention times with those of the methyl ester standards. Some uncertain peaks that occurred in the chromatograph of samples, followed the suggestions provided by the NIST library. The condition was detailed in **Table 3.2**.

Table 3.2 GC-FID analysis conditions for the extract of *Cucumaria frondosa* viscera.

Chromatographic system	Agilent 7890B GC equipped with FID
Column	DB-23, 60 m x 0.25 mm x 0.15 mm
Inlet temperature	250 °C
Injection volume	1 mL
Split ratio	50:1
Carrier gas	Helium
Head pressure	Constant pressure, 33 cm/sec at 50 °C
Oven temperature	50 °C (1 min), 25 °C/min to 175 °C, 4 °C/min to 230 °C (5 min)
FID detector temperature	280 °C
Detector gases	Air: 450 mL/min Hydrogen: 40 mL/min Helium make-up gas: 30 mL/min

The identification of the order of peaks in the external standards from GC analysis are presented in **Appendix**.

3.2.9. Fatty Acids Content Calculation

Fatty acid content was quantified by comparison of peak area from GC analysis between a known amount of added methyl C19:0 as internal standard:

$$Amount\ of\ a\ fatty\ acid = (peak\ area_{a\ fatty\ acid} \times amount_{methyl\ C19:0}) \div peak\ area\ _{methyl\ C19:0} \quad (3)$$

$$Amount\ of\ total\ fatty\ acids = \Sigma(peak\ area_{each\ fatty\ acid} \times amount_{methyl\ C19:0}) \div peak\ area\ _{methyl\ C19:0} \quad (4)$$

Results of fatty acids composition were expressed as the amount of a fatty acid in the total fatty acids (g/100 g of the total fatty acids). The total fatty acid content was calculated as:

$$Fatty\ acids\ content\ (g/100\ g\ of\ samples\ used) = \frac{Amount\ of\ total\ fatty\ acids}{Amount\ of\ dried\ biomass\ used} \times 100 \quad (5)$$

Otherwise, comparing the total fatty acids with the fatty acids extracted by conventional methods, the recovery efficiency was calculated as:

$$Fatty\ acids\ recovery\ efficiency\ (\%\ recovery) = \frac{Fatty\ acid\ yields\ of\ scCO_2\ extraction}{Fatty\ acid\ yields\ of\ conventional\ extraction} \times 100\% \quad (6)$$

3.2.10. Morphological Imaging

Hitachi S-4700 FE Scanning Electron Microscope (Chiyoda, Tokyo, Japan) was used to investigate structure morphological changes due to different pre-treatments. To avoid charging of the surface and promote the emission of secondary electrons, samples were mounted on aluminum stubs, coated with gold-palladium alloy for 260 s, obtaining 20-nanometers coated layers. The coated samples were scanned using the scanning electron microscope at an accelerating voltage of 1.5 kV.

3.2.11. Experimental Design and Statistical Analysis

In order to optimize the global extract from the $scCO_2$ extraction of *Cucumaria frondosa* viscera, a preliminary screening was carried out using single-factor experiments by fixing all parameters except one variable. In the preliminary screening, temperature of 35, 55, and 80 ˚C, pressure of 20, 35, and 50 MPa, dynamic extraction time at 30, 60, 135, and 210 min, static time at 0, 10, 20, and 30 min, and co-solvent to biomass mass ratio at 0 (no co-solvent added), 1 (1.5 g of dehydrated samples with 1.5 g of ethanol), and 2 (1.5 g of dehydrated samples with 3.0 g of ethanol) were tested. The preliminary screening experiments were performed in duplicate. According to the results from the preliminary screening, the selection and the range of process variables were specified.

A response surface method, central composite inscribed (CCI) design, with four parameters – temperature (35-75 ˚C), pressure (20-50 MPa), dynamic extraction time (30-70 min), and mass ratio of co-solvent to biomass (0-2) – was created in Minitab 19 (Minitab Inc., State College, PA, USA). Each parameter was examined in five levels: -a, -1, 0, +1, and + a (**Table 3.3**). Here, a was 2 when the amount of involving factors is 4 ($a = (2^4)^{1/4}=2$). The design included centerpoint that runs seven times, also 16 cube points and 8 axial point, resulting in 31 trials (**Table 3.4**). The total fatty acid yields and selected Omega-3 fatty acid yields were evaluated as the response variables for optimization.

Table 3.3 Process variables and their coded levels

Process variables	Levels				
	-a	**-1**	**0**	**+1**	**+a**
Temperature (˚C)	35	45	55	65	75
Pressure (MPa)	20	27.5	35	42.5	50
Dynamic extraction time (min)	30	40	50	60	70
Ratio of co-solvent to biomass (w/w)	0	0.5	1	1.5	2

Overall, the response surface methodology combines regression modeling and analysis of variance (ANOVA), resulting in a model:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} {X_i}^2 + \sum_{i=1} \sum_{i<j} \beta_{ij} X_i X_j \quad (7)$$

where Y represents the response, X_i and X_j are the independent variables, β_0 is the intercept coefficient, and β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interaction coefficients.

ANOVA was employed to assess the four process variables and their quadratic effects and interactions, using F-test and its associated probability, p(F), whereas t-test was performed to determine the significance of the estimated coefficients in the model. The test of statistical significance was based on the total error criteria within a confidence level of 95%. The determination coefficient (R^2) and its adjusted value (*adj.* R^2) were used to evaluate the goodness of the fit to the regression model. In order to validate the model, additional runs at the optimum conditions, suggested by Response Optimizer in Minitab 19, was conducted and the experimental result was compared with the model prediction. Three-dimensional surface plots and contour plots were generated by varying two variables within the studied range and holding the other two constant.

Table 3.4 Central composite experimental design (inscribed) for SFE of lipids from sea cucumber viscera.

Run #	Temperature (˚C)	Pressure (MPa)	Dynamic Extraction Time (min)	Ratio of co-solvent to biomass (w/w)
1	45 (-1)	27.5 (-1)	40 (-1)	0.5 (-1)
2	65 (+1)	27.5 (-1)	40 (-1)	0.5 (-1)
3	45 (-1)	42.5 (+1)	40 (-1)	0.5 (-1)
4	65 (+1)	42.5 (+1)	40 (-1)	0.5 (-1)
5	45 (-1)	27.5 (-1)	60 (+1)	0.5 (-1)
6	65 (+1)	27.5 (-1)	60 (+1)	0.5 (-1)
7	45 (-1)	42.5 (+1)	60 (+1)	0.5 (-1)
8	65 (+1)	42.5 (+1)	60(+1)	0.5 (-1)
9	45 (-1)	27.5 (-1)	40 (-1)	1.5 (+1)
10	65 (+1)	27.5 (-1)	40 (-1)	1.5 (+1)
11	45 (-1)	42.5 (+1)	40 (-1)	1.5 (+1)
12	65 (+1)	42.5 (+1)	40 (-1)	1.5 (+1)
13	45 (-1)	27.5 (-1)	60 (+1)	1.5 (+1)
14	65 (+1)	27.5 (-1)	60 (+1)	1.5 (+1)
15	45 (-1)	42.5 (+1)	60 (+1)	1.5 (+1)
16	65 (+1)	42.5 (+1)	60 (+1)	1.5 (+1)
17	35 (-2)	35 (0)	50 (0)	1 (0)
18	75 (+2)	35 (0)	50 (0)	1 (0)
19	55 (0)	20 (-2)	50 (0)	1 (0)
20	55 (0)	50 (+2)	50 (0)	1 (0)
21	55 (0)	35 (0)	30 (-2)	1 (0)
22	55 (0)	35 (0)	70 (+2)	1 (0)
23	55 (0)	35 (0)	50 (0)	0 (-2)
24	55 (0)	35 (0)	50 (0)	2 (+2)
25-31	55 (0)	35 (0)	50 (0)	1 (0)

Chapter 4: Conventional Extraction of Fatty Acids from *Cucumaria frondosa* Viscera: Comparison and Improvements

In this chapter, conventional extraction method, followed by chromatographic methods were employed to analyze the fatty acids profile of *Cucumaria frondosa* viscera. Additionally, ultrasonication prior to the conventional methods was implemented to determine whether it will result in improving the extraction yield.

4.1. Fatty Acids Content and Profiles Obtained Using the Bligh & Dyer and the Ultrasonic-Assisted Bligh & Dyer Methods

The extract obtained demonstrated reddish brown color and good fluidity in the room temperature (**Figure 4.1**). The global extraction yield and the fatty acids content for the Bligh & Dyer method were 33.116 g/100 g of the hot air-dried samples and 8.473 g/100 g of the hot air-dried samples, respectively; while global extraction yield and the fatty acids content for the case of ultrasonic-assisted Bligh & Dyer method were 37.101 g/100 g of the hot air-dried samples and 12.718 g/100 g of the hot air-dried samples, accordingly (**Table 4.1**). The significant increase of almost 50% in fatty acids content indicated the ultrasonication improved the fatty acid recovery (P-value = 0.006 < 0.05), but the improvement did not make significant difference on the global extraction yields (P-value = 0.072 > 0.05). It was likely that the solvents system of the Bligh & Dyer method had highly polar nature and weakened the sample structure, leading to a great deal of crude lipids being extracted. Although the assistance of ultrasound disrupted cells, making samples release lipids towards the chloroform-methanol-water system, the increase was insignificant. The gap between the global extraction yield and the fatty acids content emphasized the extraction of impurities and/or undesired compounds.

The global extraction content of the *Cucumaria frondosa* viscera used in this study was far above those of freeze-dried *Cucumaria frondosa* viscera collected from Rimouski, Quebec, Canada (2.0 g/100 g of samples on dried weight basis) and hot air-dried viscera harvested in Newfoundland, Canada (25.69 g/100 g of samples on dried weight basis) (Liu et al., 2021; Mamelona et al., 2010). It suggested that the by-products of this species grew in Nova Scotia might be a better natural source for commercial sea cucumber oils. Also, these summer harvested samples investigated in this study yielded more sea cucumber oils

than those reported in Abuzaytoun's study (23.83 g of global extract/100 g of samples on dried weight basis) although they were all collected from waters near Nova Scotia (Abuzaytoun, 2017). That reemphasized the variability of this sea cucumber species.

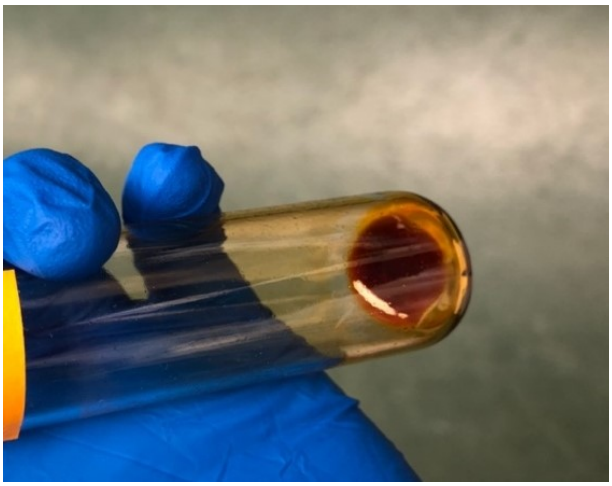


Figure 4. 1 The extract after evaporation.

Weight amount of EPA and DHA in the total fatty acids was provided in **Table 4.1.** According to the 2-sample t-test results, the Bligh & Dyer method extracted significantly higher amount of EPA in the total fatty acids comparing with the ultrasonic-assisted Bligh & Dyer method (P-value = 0.024 <0.05), whereas no significant difference was observed for the mass fraction of DHA (P-value = 0.164 >0.05). That might be because the ultrasonication disrupted cells, resulting in the extraction of more other fatty acids. Accordingly, the portion of EPA in the total fatty acids decreased. However, the standard deviation of the amount of EPA in the total fatty acids obtained in the Bligh & Dyer method was almost ten times to that of the ultrasonic-assisted Bligh & Dyer method.

Table 4.1 Global extraction yields (g of the extract/100 g of the hot air-dried samples), fatty acids contents (g of fatty acids/100 g of the hot air-dried samples), and the amount of EPA and DHA in the total fatty acids (g/100 g of the total fatty acids) for the Bligh & Dyer method and the ultrasonic-assisted Bligh & Dyer method (n=4, Mean±SD).

Methods	Global extraction yield [1]	Fatty acids content [2]	EPA [3]	DHA [4]
The Bligh & Dyer method	33.116±2.725 [a]	8.473±1.457 [a]	23.454±0.458 [a]	1.177±0.057 [a]
Ultrasonic-assisted Bligh & Dyer method	37.101±2.427 [a]	12.718±0.744 [b]	22.486±0.043 [b]	1.231±0.015 [a]

Note: Values in the same column with different letters (i.e., a & b) are significantly different at $P < 0.05$.

[1]: g of the extract/100 g of the hot air-dried samples

[2]: g of fatty acids/100 g of the hot air-dried samples

[3 4]: g/100 g of the total fatty acids

4.2. Fatty Acids Content and Profiles Obtained from In-Situ Transesterification and Ultrasonic-assisted In-Situ Transesterification

In order to explore the advantage of direct transesterification and further examine the improvement of ultrasonication in the fatty acid recovery, *in-situ* transesterification and the ultrasonic-assisted *in-situ* transesterification were applied, and the fatty acid content and the EPA and DHA composition were listed in **Table 4.2**. It is notable that the fatty acids content increased significantly in the case of ultrasonication-assisted pre-treatment (P-value = 0.002 < 0.05), while the EPA amount in the total fatty acids was comparable under those two scenarios (P-value = 0.092 > 0.05). It also showed a significant decrease on the DHA composition of those two methods (P-value = 0.002 < 0.05). It was likely that the ultrasonication promoted the extraction of other fatty acids like the branched fatty acid, 12-methyltetradecanoic acid (ai-C15:0, 12-MTA; increasing from 20.229 g/100 g of the

total fatty acids to 22.062 g/100 g of the total fatty acids), leading to the decrease of the portion of DHA in the total fatty acids.

Table 4.2 Fatty acids contents (g/100 g of the hot air-dried samples) and the amount of EPA and DHA in the total fatty acids (g/100 g of the total fatty acids) for *in-situ* transesterification and the ultrasonic-assisted *in-situ* transesterification (n=4, Mean±SD).

Methods	Fatty acids content [1]	EPA [2]	DHA [3]
In-situ transesterification	14.914±0.327 [a]	22.303±0.448 [a]	0.967±0.021 [a]
Ultrasonic-assisted *in-situ* transesterification	16.304±0.188 [b]	21.735±0.254 [a]	0.876±0.028 [b]

Note: Values in the same column with different letters are significantly different at $P < 0.05$.

[1]: g of fatty acids/100 g of the hot air-dried samples

[2 3]: g/100 g of the total fatty acids

4.3. Comparison of Fatty Acids Composition Obtained from Two-Step Transesterification (Extraction Followed by Transesterification) and In-Situ Transesterification

In the previous two section, it was noteworthy that the fatty acids contents were different for the two-step transesterification and the *in-situ* transesterification. Aiming to further validate that finding, comparisons between them were conducted, as demonstrated in **Table 4.3**. According to the Tukey pairwise comparison, significant differences on the fatty acids content were observed, which indicated the advantage of the *in-situ* transesterification over the two-step protocol (P-value = 0.000 < 0.05). Also, it revealed the ultrasonication assisted to extract significantly more fatty acids through cell disruption (P-value = 0.000 <0.05) (**Table 4.3**). In the study conducted by Cavonius et al. (2014), they found the Bligh and Dyer method recovered significantly less total fatty acids than methanolic HCl *in situ* transesterification for some microalgae species (Cavonius et al., 2014). Also, the result was supported by Dickey et al. (2002). They compared the direct transesterification and the Bligh and Dyer method through determining the fatty acid

content in striped bass tissues and found the direct method recovered more fatty acids (Dickey et al., 2002).

In comparison of the amount of EPA and DHA in the total fatty acids under those four situations (the Bligh & Dyer followed by transesterification, the ultrasonic-assisted Bligh & Dyer followed by transesterification, *in-situ* transesterification, and the ultrasonic-assisted *in-situ* transesterification), we saw the mass fraction of EPA (P-value = 0.001 < 0.05) and DHA (P-value =0.002 < 0.05) obtained from the two-step transesterification was significantly higher than those resulted from the *in-situ* transesterification. The significant differences indicated more other fatty acids were methylated using the direct transesterification method, resulting in the decrease of EPA and DHA in the total fatty acids. Besides, it was reported that the ultrasonication contributed significantly to change the portion of EPA in the total fatty acids (P-value = 0.002 < 0.05), but it was the opposite for DHA (P-value = 0.477 > 0.05) (**Table 4.3**). That differed from the results obtained in the previous sections using two-sample t-test because Tukey comparison of two-way ANOVA adjusted the P-value for multiple testing and controlled the family-wise error rate yielding more precise and more reliable results. Overall, the ultrasonic-assisted *in-situ* transesterification performed better in the characterization of sample lipids.

Table 4.3 Fatty acids contents (g/100 g of the hot air-dried samples) and the amount of EPA and DHA in the total fatty acids (g/100 g of the total fatty acids) for two-step transesterification and *in-situ* transesterification (n=4, Mean±SD)

Response	Method	Ultrasonication	
		No	Yes
Fatty acids contents [1]	Two-step transesterification	8.473±1.457 [a]	12.718±0.744 [c]
	In-situ transesterification	14.914±0.327 [b]	16.304±0.188 [d]
EPA [2]	Two-step transesterification	23.454±0.458 [a]	22.486±0.043 [c]
	In-situ transesterification	22.303±0.448 [b]	21.735±0.254 [d]
DHA [3]	Two-step transesterification	1.177±0.057 [a]	1.231±0.015 [a]
	In-situ transesterification	0.967±0.021 [b]	0.876±0.028 [b]

Note: Fatty acids contents and the amount of EPA and DHA in the total fatty acids under different methods was compared by Tukey test at 95% confidence level. Values with different letters (i.e., a, b, c, & d) are significantly different at $P < 0.05$.

[1]: g of fatty acids/100 g of the hot air-dried samples

[2 3]: g/100 g of the total fatty acids

The fatty acids profiles of samples processed using ultrasonic-assisted methods were detailed in **Table 4.4.** The fatty acids composition of listed two methods were comparable, among which, EPA was the predominant fatty acid, followed by 12-MTA and palmitoleic acid. Zhong et al. (2007), Mamelona et al. (2010), Abuzaytoun (2017), and Liu et al. (2021) also noticed the amount of EPA in every gram of fatty acids was the highest among other fatty acids.

Limited research done on identification of FAMEs present in sea cucumber found the presence of branched fatty acid 12-MTA. 12-MTA can be used for tumor cell treatment

individually or in combination with other nutritional or anti-cancer compounds through inducing cell apoptosis related metabolites or activities in cancer proliferation (Collin et al., 2003). Collin et al, (2003) and Abuzaytoun (2017) also detected 12-MTA in *Cucumaria frondosa* and its by-products, with 21.27 g/100 g of the total fatty acids in winter-harvested viscera, 19.17 g/100 g of the total fatty acids in spring-harvested viscera, and 11.79 g/100 g of the total fatty acids in summer-harvested viscera (Abuzaytoun, 2017; Collin et al., 2003). This shows the importance of identification of individual fatty acid presented in sea cucumber species.

Table 4.4 Comparison of fatty acids composition (g/100 g of the total fatty acids) of Cucumaria frondosa viscera treated with the ultrasonic-assisted two-step transesterification and the ultrasonic-assisted direct transesterification.

Type	Isomer	Systematic name	Two-step	Direct
Saturated	C14:0	Myristic acid	1.744±0.009	1.869±0.046
	C15:0	Pentadecylic acid	2.476±0.002	2.544±0.049
	C16:0	Palmitic acid	1.443±0.003	1.433±0.021
	C17:0	Heptadecanoic acid	0.267±0.012	0.297±0.021
	C18:0	Stearic acid	2.990±0.017	2.803±0.035
	C20:0	Arachidic acid	0.531±0.011	0.500±0.011
	C21:0	Henicosanoic acid	0.196±0.003	0.192±0.005
	C22:0	Behenic acid	0.619±0.011	0.527±0.022
Total			10.266	10.165
Monounsaturated	C16:1n7	Palmitoleic acid	16.514±0.061	16.845±0.293
	C17:1	10-Heptadecenoic acid	0.438±0.004	0.446±0.015
	C18:1n9 cis	Oleic acid	1.302±0.005	1.305±0.013
	C18:1n7	Vaccenic acid	2.903±0.002	2.899±0.030
	C20:1n9	11-Eicosenoic acid	0.544±0.014	0.544±0.006
	C22:1n9	Erucic acid	0.502±0.017	0.53±0.034
	C24:1n9	Nervonic acid	1.857±0.036	1.727±0.061
Total			24.061	24.297
Polyunsaturated	C16:2n4	Hexadecadienoic acid	0.928±0.035	1.014±0.049
	C18:2n6 trans	Linolelaidic acid	0.119±0.001	0.134±0.002
	C18:2n6 cis	Linoleic acid	0.279±0.000	0.276±0.004
	C18:2n4	11,14-Octadecadienoic acid	0.469±0.002	0.473±0.005
	C20:2n6	11,14-eicosadienoic acid	0.269±0.001	0.283±0.006
	C22:2n6	13,16-Docosadienoic acid	0.112±0.004	0.138±0.006
	C16:3n4	6,9,12-hexadecatrienoic acid	0.418±0.006	0.437±0.016
	C18:3n4	8,11,14-octadecatrienoic acid	0.101±0.010	0.098±0.001
	C18:3n3	α-Linolenic acid (ALA)	0.121±0.023	0.146±0.002
	C16:4n1	6,9,12,15-hexadecatetraenoic acid	1.223±0.005	1.257±0.021
	C18:4n3	Stearidonic acid	0.836±0.003	0.891±0.050
	C20:4n6	Arachidonic acid	0.571±0.002	0.598±0.008
	C20:4n3	Eicosatetraenoic acid (ETA)	0.278±0.010	0.297±0.008
	C20:5n3	Eicosapentaenoic acid (EPA)	22.486±0.043	21.735±0.254
	C21:5n3	Heneicosapentaenoic acid (HPA)	0.393±0.031	0.366±0.006
	C22:5n3	Docosapentaenoic acid (DPA)	0.278±0.004	0.284±0.006
	C22:6n3	Docosahexaenoic acid (DHA)	1.231±0.015	0.876±0.028
Total of Omega-3			25.623	24.594
Total of Omega-6			1.350	1.429
Total			30.112	29.302
Branched	4,8,12-Me-13:0	4,8,12-Trimethyltridecanoic acid	2.475±0.005	2.576±0.048
	Me-14:0 isomer 1		0.466±0.002	0.581±0.012
	Me-14:0 isomer 2	Methyltridecanoic acid	0.414±0.023	0.411±0.010
	Me-14:0 isomer 3		0.377±0.001	0.382±0.008
	i-16:0	14-methylpentadecanoic acid	4.349±0.010	4.399±0.071
	i-17:0	15-methylhexadecanoic acid	0.289±0.008	0.379±0.030
	ai-15:0	12-methyltetradecanoic acid	21.346±0.029	22.062±0.470
	ai-17:0	14-methylhexadecanoic acid	0.866±0.024	0.823±0.025
Total			30.582	31.614
Others *			4.980	5.062

**Note: Others refer to fatty acid isomers presenting in sample GC chromatograph other than external standards, which were suggested through GC-MS and NIST library.*

Recent studies pointed out an excessive intake of Omega-6 fatty acids was widely observed in the daily diet, leading to a higher ratio of Omega-6/Omega-3 (15-20:1) (Simopoulos, 2002). Excess Omega-6 fatty acids is one of the main factors of inflammation in human bodies, while Omega-3 fatty acids can inhibit inflammation through limiting the metabolism of Omega-6 fatty acids and the generation of associated inflammatory substances (Calder, 2015; Wall et al., 2010). A recommended ratio of Omega-6/Omega-3 was reported as 1-5:1 (Simopoulos, 2008). In *Cucumaria frondosa* viscera, the ratio was approximately 0.05, which could balance the extra Omega-6 to achieve a proper ratio of Omega-6/Omega-3.

Comparing with the crude marine oils recovered from fish by-products, *Cucumaria frondosa* viscera contained more outstanding amount of Omega-3 fatty acids and lower portion of Omega-6 fatty acids in every gram of the total fatty acids. For instance, 20.77 g of Omega-3 fatty acids/100 g of the total fatty acids (EPA accounted for 8.52 g/100 g of the total fatty acids) and 2.29 g of Omega-6 fatty acids/100 g of the total fatty acids were found in the crude cod liver oil, resulting in a ratio of Omega-6/Omega-3 as 0.11 (Dave et al., 2014). Therefore, *Cucumaria frondosa* viscera also possess great marketable features for commercializing as a natural source of nutritional supplements.

On the other hand, apart from the extraction of undesired compounds like lipid-soluble pigments resulting in the remarkable difference between the crude oil content and the fatty acids yield, the extraction of water-soluble impurities might also contribute that difference. The upper layer resulted from the transesterification process contained FAMEs, toluene, and hexane, while the lower layer comprised an aqueous phase. However, the color of the aqueous layer of the two-step transesterification was darker than that of the *in-situ* transesterification, implying more water-soluble impurities were extracted by the mixed solvents system of the Bligh & Dyer method.

4.4. Conclusion

Cucumaria frondosa viscera are Omega-3 fatty acid enriched natural sources, containing approximately 37 g of crude oils/100 g of the hot air-dried samples and 16.304 g of fatty acids/100 g of the hot air-dried samples in this study. In this chapter, we found that the ultrasonication supported the recovery of fatty acids, and *in-situ* transesterification helped to characterize the *Cucumaria frondosa* viscera with maximum fatty acids content.

Therefore, amongst the methods tested, the average maximum fatty acid content of *Cucumaria frondosa* viscera was acquired in the ultrasonic-assisted *in-situ* transesterification as 16.304 g/100 g of the hot air-dried samples, which would be used as a reference to evaluate the recovery efficiency of $scCO_2$ extraction.

Chapter 5: Supercritical Carbon Dioxide Extraction of Fatty Acids from *Cucumaria frondosa* Viscera: A Preliminary Screening for Process Variables Determination

Design of experiments (DoE) consists of three steps: screening experiments for important factors, response surface design for regression models and optimization, and model validation. A preliminary screening is regarded as an effective and economical way to identify a certain number of factors that contribute to a process, from many potential candidates (Heckert et al., 2012).

In this chapter, a preliminary screening using single factor experiments were employed to identify important factors that should be considered in a response surface design for $scCO_2$ extraction of fatty acids from *Cucumaria frondosa* viscera. Based on the literature review in Chapter 2 and the specific requirements for supercritical status ($T \geq 31$ ˚C; $P \geq 7.38$ MPa), temperature (35, 55, and 75 ˚C), pressure (20, 35, 50 MPa), static extraction time (0, 10, 20, 30 min), dynamic extraction time (30, 60, 135. 210 min), and mass ratio of co-solvent to biomass (0, 1:1, 2:1) were tested separately.

5.1. Effect of Process Parameters on Extraction Yield

The yields of target compounds (fatty acids, EPA, selected Omega-3, and crude oils) versus temperature are shown in **Figure 5.1**. It was notable that as temperature increased, yields of fatty acids and the global extracts changed marginally. According to one-way ANOVA analysis, the P-values suggested temperature had no significant effect on fatty acids yields (P-values > 0.05), but it significantly influenced the global extracts yields (P-values < 0.05). However, the solubility of target compounds depends on $scCO_2$ density, which is as a function of temperature and pressure; as such, temperature might have interactive effects with other variables, like pressure or co-solvents. Therefore, it cannot be concluded that the effect of temperature was an insignificant variable for fatty acids extraction.

The change of yields as the function of pressure is shown in **Figure 5.2**. As pressure increased, fatty acids yields increased and then decreased, but the global extraction yields increased and then reached plateau. That indicated there was an inflection point for fatty acids yields in the selected range. The one-way ANOVA proposed pressure had significant

effects on all the responses with P-values smaller than 0.05. Also, the Tukey pairwise comparisons supported a turn-around point for fatty acids yields at around 35 MPa, exhibiting the runs under the pressure of 35 MPa was significantly different from the other two pressures at 20 MPa and 50 MPa respectively (P-values < 0.05), but there was no significant difference between the other two runs under 20 MPa and 50 MPa (P-values > 0.05).

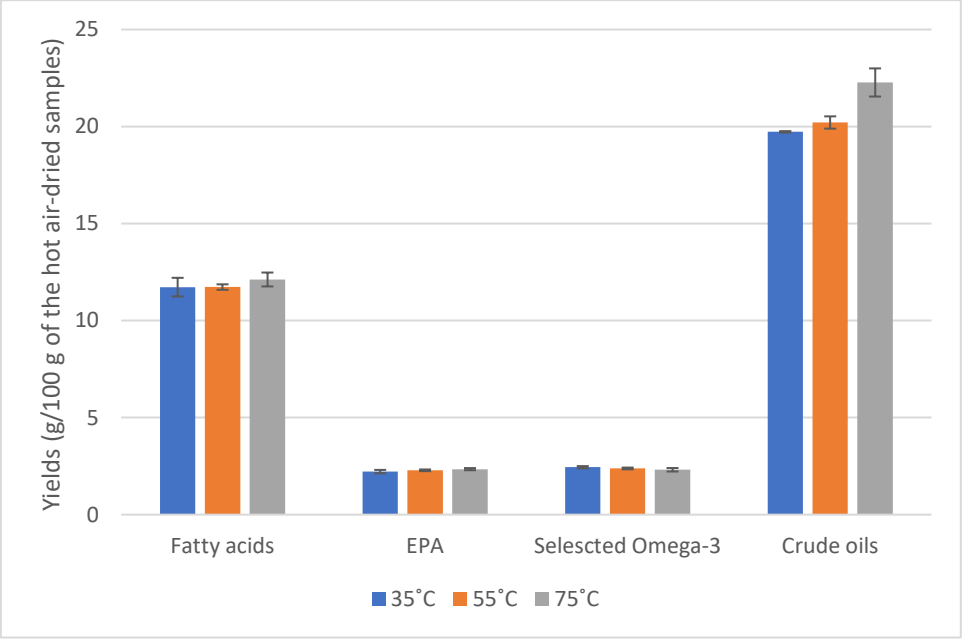


Figure 5.1 Effects of temperature on yields of fatty acids, EPA, selected Omega-3 fatty acids, and crude oils at 50 MPa of pressure, 20 min of static extraction time, 60 min of dynamic extraction time, and 0:1 of co-solvent to biomass ratio. Error bars show the range of error for duplicate samples, n=2.

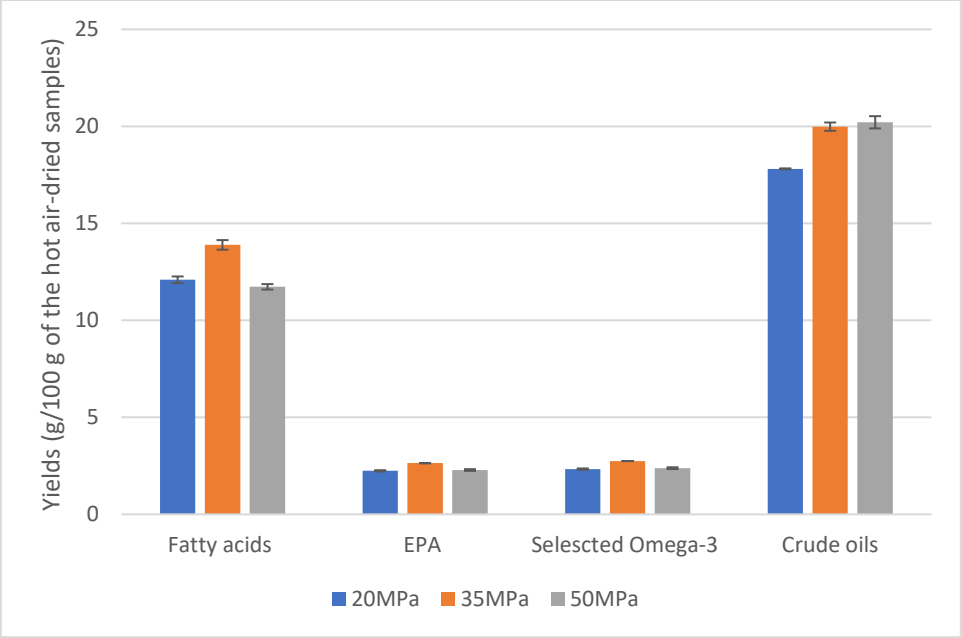


Figure 5.2 Effects of pressure on yields of fatty acids, EPA, selected Omega-3 fatty acids, and crude oils at 55 ˚C of temperature, 20 min of static extraction time, 60 min of dynamic extraction time, and 0:1 of co-solvent to biomass mass ratio. Error bars show the range of error for duplicate samples, n=2.

It was observed that with increasing the static extraction time from 0 to 10 min to 20 min to 30 min, the extracts elute immediately when the static/dynamic valve and the restrictor valve were opened after the 20 minutes' soaking. **Figure 5.3** demonstrated that yields did not change in response to increasing static extraction time from 20 min to 30 min. One-way ANOVA also supported this conclusion, revealing no significant changes in yields of fatty acids and the global extract in response to increasing the static time from 20 to 30 min (P-values > 0.05). Therefore, it was concluded that the static time of 20 min, allows for sufficient contact between samples and $scCO_2$.

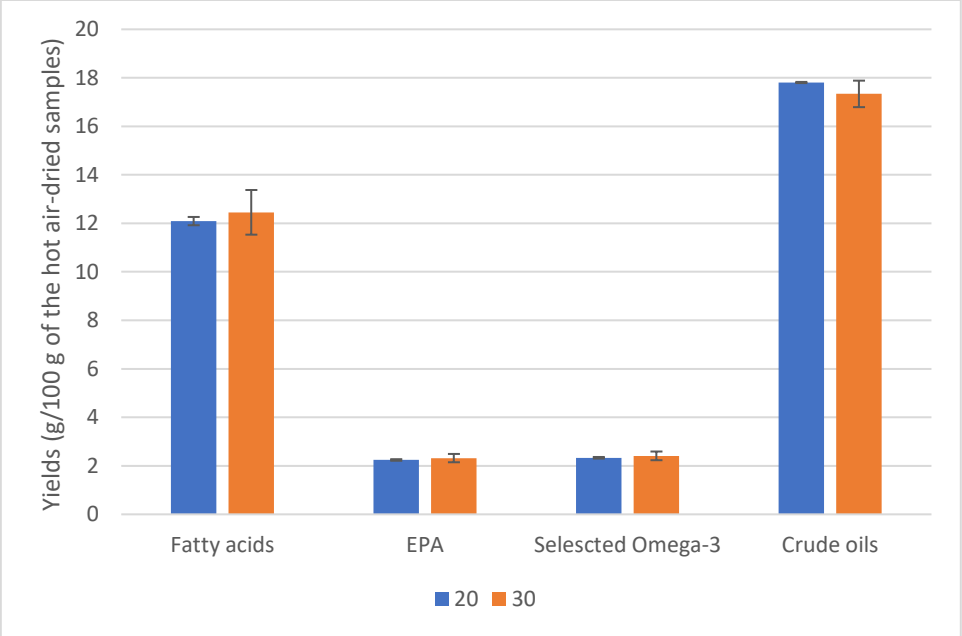
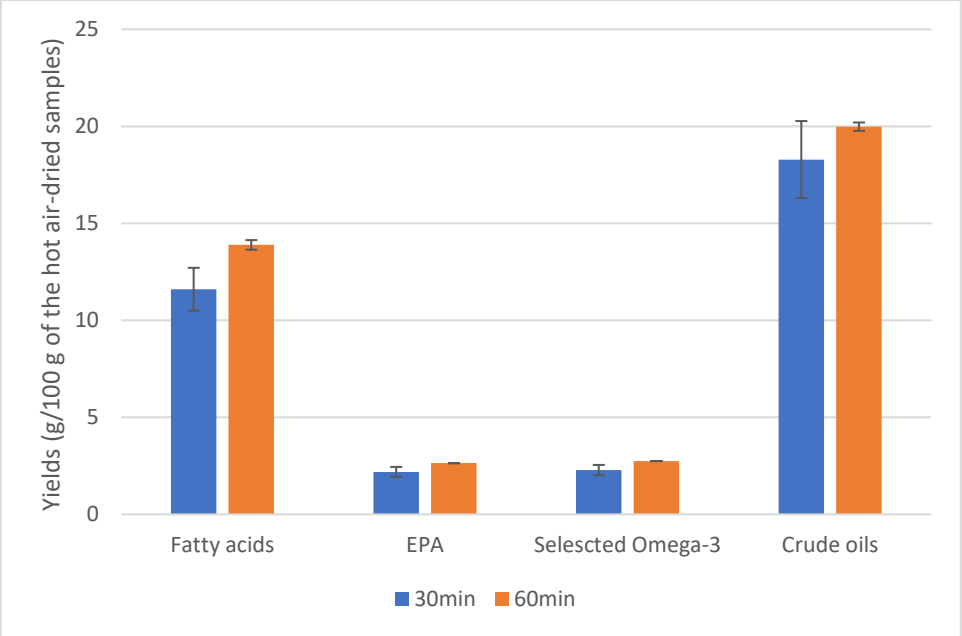
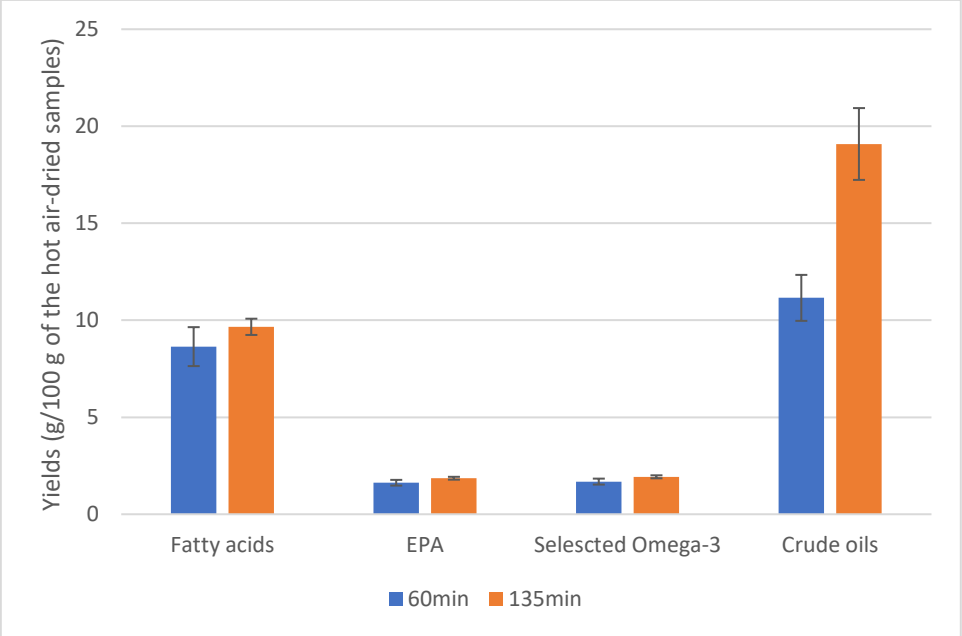


Figure 5.3 Effects of static extraction time on yields of fatty acids, EPA, selected Omega-3 fatty acids, and crude oils at 55 ˚C of temperature, 35 MPa of pressure, 60 min of dynamic extraction time, and 0:1 of co-solvent to biomass mass ratio. Error bars show the range of error for duplicate samples, n=2.

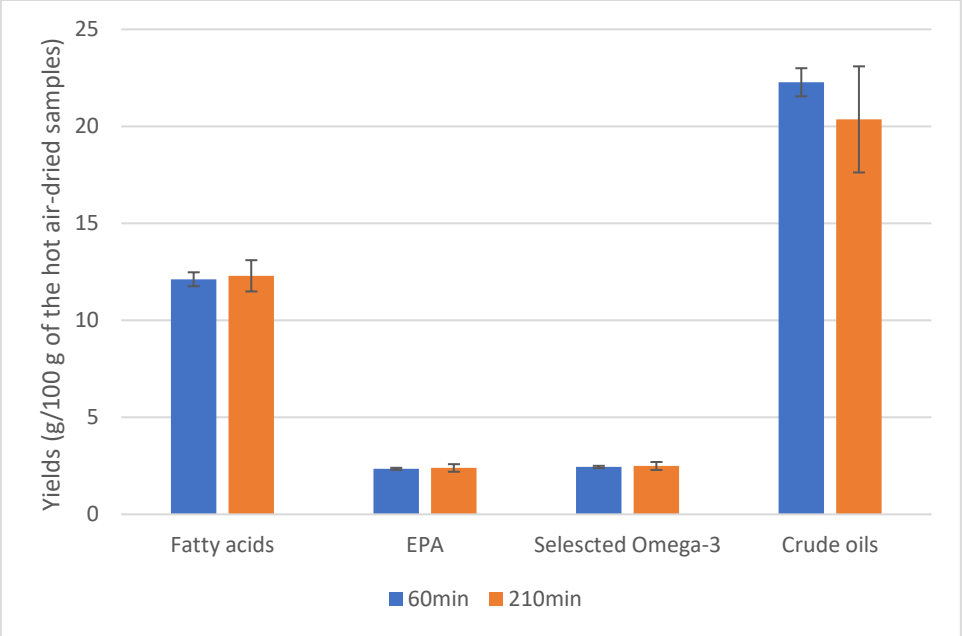
The range of dynamic extraction time derived from existing literature, 30-210 min, was evaluated in this study. **Figure 5.4 (a)-(c)** demonstrates yields of target compounds in response to change in dynamic extraction time. Results suggested that marginally significant change in yields of the crude oil and fatty acids were spotted before 60 min ($0.05 < \text{P-values} < 0.1$, **Figure 5.4 (a)**), while more than 60 min dynamic time did not significantly impact the extraction yields (P-values > 0.05, **Figure 5.4 (b)&(c)**). The change in global extract yields in response to increase in dynamic extraction time from 60 min to 210 min was not statistically significant **(Figure 5.4 (b) & (c))**. Dynamic extraction time may need to be adjusted based on the actual sample weights used; as such, in order to design more complex models with better predictors (allowed to cover insignificant effects), dynamic extraction time from 30 min to 75 min was selected as a variable in the surface response design of this study.



(a) Dynamic extraction time increased from 30 min to 60 min at 55 ˚C of temperature, 35 MPa of pressure, 20 min of static extraction time, and 0:1 of co-solvent to biomass mass ratio.



(b) Dynamic extraction time increased from 60 min to 135 min at 30 ˚C of temperature, 10 MPa of pressure, 0 min of static extraction time, and 0:0 of co-solvent to biomass mass ratio.



(c) Dynamic extraction time increased from 60 min to 210 min at 80 ˚C of temperature, 50 MPa of pressure, 20 min of static extraction time, and 0:0 of co-solvent to biomass mass ratio.

Figure 5.4 Effects of dynamic extraction time on yields of fatty acids, EPA, selected Omega-3 fatty acids and crude oils. Error bars show the range of error for duplicate samples, n=2.

Figure 5.5 showed trends of all the responses as mass ratio of co-solvent to biomass increased. One-way ANOVA suggested that with mass ratio of co-solvent to biomass increased from 0:1 to 2:1, it showed significant differences were observed in yields of selected Omega-3 fatty acids and the global extracts (P-values < 0.05,). Besides, a near-marginal significant effect on the total fatty acids yields was also found (P-values = 0.055 > 0.05). However, the Tukey pairwise comparisons further revealed increasing mass ratio of co-solvent to biomass from 1:1 to 2:1 significantly affected the total fatty acids yields (P-values < 0.05). Thus, it suggested that we were supposed to include mass ratio of co-solvent to biomass increased from 0:1 to 2:1 in the following response surface design.

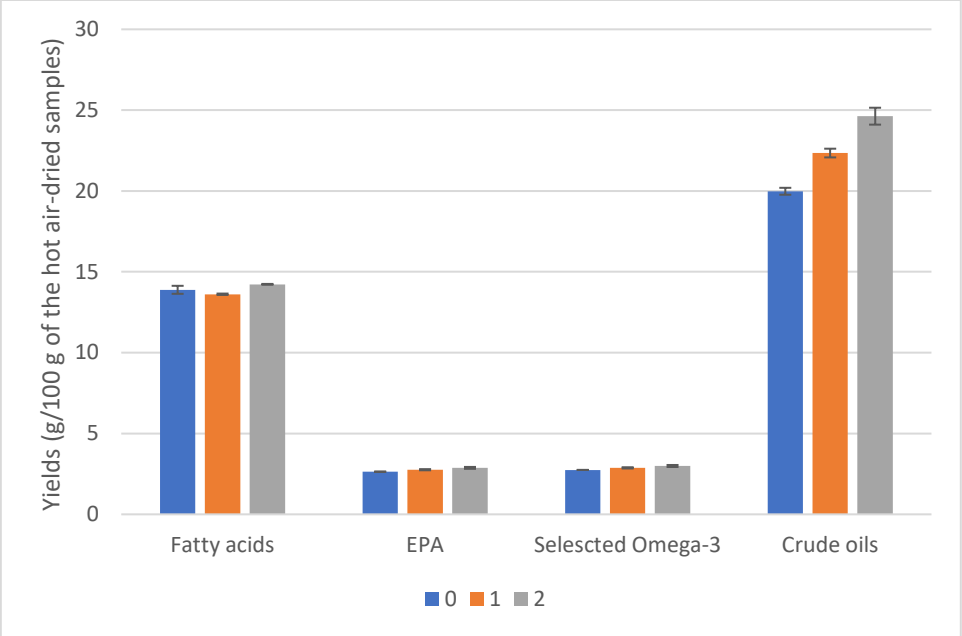


Figure 5.5 Effects of mass ratio of co-solvent to biomass on yields of fatty acids, EPA, selected Omega-3 fatty acids, and crude oils at 55 ˚C of temperature, 35 MPa of pressure, 20 min of static extraction time, and 60 min of dynamic extraction time. Error bars show the range of error for duplicate samples, n=2.

5.2. Conclusion

In conclusion, temperature (35-75 ˚C), pressure (20-50 MPa), dynamic extraction time (30-70 min), and mass ratio of co-solvent to biomass (0-2:1) were used for a response surface design, while static extraction was fixed as 20 min. Although a preliminary screening using single-factor experiments yields less experimental runs and reflects more straightforward effects of variables under tested, it still suffers apparent shortcomings in this study, particularly assuming no interaction between independent factors. That usually draws some incorrect results, leading to variables with significant interactive impacts being cut out. Therefore, a test for curvature using 2^k factorial design should be incorporated following a single factor test in the future study for a more comprehensive preliminary screening. However, the interactive effects would be further identified in the next chapter in this study.

Chapter 6: Investigation of The Impacts of Process Variables on Yields of Target Compounds Using Response Surface Methodology

In chapter 5, we have already specified the selection of process variables and their corresponding ranges through single factor preliminary screening. In this chapter, the effect of process variables: temperature (35-75 ˚C), pressure (20-50 MPa), dynamic extraction (30-70 min), mass ratio of co-solvent to biomass (0-2) on yields of total fatty acids and selected Omega-3 fatty acids (EPA and DHA) were studied. Through a response surface design in this chapter, regression models for the prediction of those target compounds were developed. Based on the experimental results and the predictive models, optimal conditions for maximum target value-added substances were suggested and further validated through the actual practice.

6.1. Analysis of Variance and Regression Model Development

Based on the results from the preliminary screening, yields of the crude oils, fatty acids, EPA, and DHA were examined to be affected by process variables – temperature (˚C, $\mathbf{X_1}$), pressure (MPa, $\mathbf{X_2}$), extraction time (min, $\mathbf{X_3}$), and ratio of co-solvent to biomass (w/w, $\mathbf{X_4}$) - in the $scCO_2$ extraction. A response surface method, CCI design involving those four parameters was conducted in five levels (-a, -1, 0, +1, and +a), yielding 31 experimental runs: seven center points, sixteen cube points, and eight axial points. The complete design matrix coupled with corresponding response data (the global extract yields, fatty acids yields, EPA yields, DHA yields, and selected Omega-3 fatty acids yields, as well as corresponding recovery efficiencies) was summarized in **Table 6.1**.

From **Table 6.1**, different combinations of extraction conditions resulted in different yields of the global extracts, fatty acids, EPA, DHA, and selected Omega-3 fatty acids (g/100 g of the hot air-dried samples), varying from 21.041 g/100 g of the hot air-dried samples to 26.352 g/100 g of the hot air-dried samples, from 11.780 g/100 g of the hot air-dried samples to 16.129 g/100 g of the hot air-dried samples, from 2.274 g/100 g of the hot air-dried samples to 2.917 g/100 g of the hot air-dried samples, from 0.100 g/100 g of the hot air-dried samples to 0.128 g/100 g of the hot air-dried samples, from 2.376 g/100 g of the hot air-dried samples to 3.038 g/100 g of the hot air-dried samples, respectively. The independent variables along with experimental values were used to develop regression

models for those responses. The full quadratic regression models for yields of those compounds were suggested by Minitab 19.0, and the following models were obtained:

The global extraction yield regression model in uncoded levels is expressed in Eq. (1):

$$Y_{\text{the global extracts}} = 0.161 - 0.00188\ X_1 + 0.00609\ X_2 - 0.00133\ X_3 + 0.0554\ X_4 + 0.000015\ X_1^2 - 0.000052\ X_2^2 - 0.000002\ X_3^2 + 0.00198\ X_4^2 - 0.000035\ X_1X_2 + 0.000036\ X_1X_3 - 0.000054\ X_1X_4 - 0.000000\ X_2X_3 - 0.000425\ X_2X_4 - 0.000362\ X_3X_4$$

The global extraction yield regression model (Eq. (1)) refined by stepwise backward elimination of non-significant terms at a significance level of 5% is expressed in **Eq. (2)**:

$$Y_{\text{the global extracts}} = 0.21230 + 0.02337\ X_4$$

The fatty acids yield regression model in uncoded levels is expressed in **Eq. (3)**:

$$Y_{\text{FA yields}} = -0.0339 - 0.00004\ X_1 + 0.00405\ \mathbf{X_2} + 0.00164\ X_3 + 0.0666\ \mathbf{X_4} + 0.000003\ X_1^2 - 0.000066\ \mathbf{X_2^2} - 0.000009\ X_3^2 - 0.00090\ X_4^2 + 0.000030\ X_1X_2 + 0.000000\ X_1X_3 - 0.000674\ \mathbf{X_1X_4} - 0.000011\ X_2X_3 + 0.000100\ X_2X_4 - 0.000358\ X_3X_4$$

The fatty acids yield regression model (Eq. (3)) refined by stepwise backward elimination of non-significant terms at a significance level of 5% is expressed in **Eq. (4)**:

$$Y_{\text{FA yields}} = -0.0012 + 0.000253\ X_1 + 0.00350\ \mathbf{X_2} + 0.000336\ X_3 + 0.0683\ \mathbf{X_4} - 0.000064\ \mathbf{X_2^2} + 0.000030\ X_1X_2 - 0.000674\ \mathbf{X_1X_4} - 0.000358\ X_3X_4$$

The EPA yield regression model in uncoded levels is expressed in **Eq. (5)**:

$$Y_{\text{EPA yields}} = -0.0034 - 0.000040\ X_1 + 0.000841\ X_2 + 0.000313\ X_3 + 0.00963\ X_4 + 0.000002\ X_1^2 - 0.000009\ \mathbf{X_2^2} - 0.000001\ X_3^2 - 0.000514\ X_4^2 + 0.000002\ X_1X_2 - 0.000001\ X_1X_3 - 0.000077\ X_1X_4 - 0.000005\ X_2X_3 - 0.000012\ X_2X_4 - 0.000031\ X_3X_4$$

The EPA yield regression model (Eq. (5)) refined by stepwise backward elimination of non-significant terms at a significance level of 5% is expressed in **Eq. (6)**:

$$Y_{\text{EPA yields}} = 0.00710 + 0.000074\ X_1 + 0.000689\ X_2 + 0.002423\ X_4 - 0.000009\ \mathbf{X_2^2}$$

The DHA yield regression model in uncoded levels is expressed in **Eq. (7)**:

$$Y_{\text{DHA yields}} = -0.00028 - 0.000002\ X_1 + 0.000024\ X_2 + 0.000038\ X_3 + 0.000145\ X_4 + 0.000000\ X_1^2 - 0.000000\ X_2^2 - 0.000000\ X_3^2 + 0.000009\ X_4^2 + 0.000000\ X_1X_2 - 0.000000\ X_1X_3 - 0.000002\ X_1X_4 - 0.000000\ X_2X_3 - 0.000003\ X_2X_4 + 0.000001\ X_3X_4$$

However, stepwise backward elimination of non-significant terms at a significance level of 5% removes all terms in the DHA yield regression model (Eq. (7)).

The selected Omega-3 fatty acids yield regression model in uncoded levels is expressed in **Eq. (8)**:

$Y_{\text{the selected Omega-3 fatty acids yields}} = -0.0037 - 0.000042\ X_1 + 0.000866 + 0.000351\ X_3 + 0.00977\ X_4 + 0.000002\ X_1^2 - 0.000009\ \mathbf{X_2^2} - 0.000001\ X_3^2 - 0.000505\ X_4^2 + 0.000002\ X_1X_2 - 0.000001\ X_1X_3 - 0.000079\ X_1X_4 - 0.000005\ X_2X_3 - 0.000014\ X_2X_4 - 0.000029\ X_3X_4$

The EPA yield regression model (Eq. (8)) refined by stepwise backward elimination of non-significant terms at a significance level of 5% is expressed in **Eq. (9)**:

$Y_{\text{the selected Omega-3 fatty acids yields}} = 0.00794 + 0.000074\ X_1 + 0.000701\ X_2 + 0.002461\ X_4 - 0.000009\ \mathbf{X_2^2}$

X_1, X_2, X_3, and X_4 represent temperature, pressure, dynamic extraction time, and mass ratio of co-solvents to biomass, respectively.

According to the experimental data derived from **Table 6.1**, an ANOVA was carried out to explore the influence of process parameter on responses, as presented in **Table 6.2**. Model terms with P-value smaller than 0.05 were significant, which implied strong evidence (more than 95% of probability) on the statistical significance of the independent variables (X_1, X_2, X_3, and X_4) in terms of linear, quadratic, and interactive effects. As for the fatty acids yield regression model, model items – X_2, X_4, X_2^2, and X_1X_4 – were significant with P-values less than 0.05; among which, the quadratic effect of X_2^2 was most dominant than others, with lower P-values and higher F-values. The quadratic effect of X_2^2 significantly influenced the extraction of EPA and selected Omega-3 fatty acids (P-values<0.05). However, all the process variables were insignificant (P-values>0.05) in the crude oil extraction and the DHA recovery, and no reduced model for DHA yields was refined.

Some existing studies on the crude marine oils corroborated the findings for the regression model of the crude sea cucumber oil. Sánchez-Camargo et al. (2011, 2012) highlighted that the selected range of the pressure (20-40 MPa) and the temperature (40-60 ˚C) showed a very low significant effect (P-value>0.05) on the $scCO_2$ extraction of lipids, but the addition of co-solvent ethanol improved lipids extraction yields. Similarly, Charest et al. (2001) in their preliminary study found that the pressure and the temperature under the selected range had little effect on the lipids extraction while ethanol showed significantly positive effect.

Table 6.1 The central composite inscribed (CCI) design of process variables, corresponding experimental responses (yields of the global extracts, fatty acids, EPA, DHA, and selected Omega-3 fatty acids in g/100 g of the hot air-dried samples), predicted values and their recovery efficiencies (compared with fatty acid contents obtained from *in-situ* transesterification).

#	X_1 (°C)	X_2 (MPa)	X_3 (min)	X_4 (w/w)	The global extraction yields			FA yields			EPA yields			DHA yields			Selected Omega-3 FA yields		
					Exp.	Predict	Recovery %	Exp.	Predict	Recovery %	Exp.	Predict	Recovery %	Exp.	Predict	Recovery %	Exp.	Predict	Recovery %
1	45	27.5	40	0.5	21.202	21.544	57.147	12.154	11.882	74.549	2.383	2.308	67.241	0.108	0.106	75.538	2.491	2.414	67.563
2	65	27.5	40	0.5	22.045	22.053	59.419	13.422	13.338	82.326	2.522	2.525	71.158	0.109	0.110	76.146	2.630	2.635	71.351
3	45	42.5	40	0.5	21.093	22.580	56.854	12.512	12.452	76.743	2.289	2.454	64.604	0.100	0.114	69.999	2.389	2.568	64.813
4	65	42.5	40	0.5	21.041	22.044	56.712	14.768	14.798	90.577	2.714	2.722	76.589	0.123	0.123	85.816	2.837	2.845	76.946
5	45	27.5	60	0.5	21.090	21.457	56.844	11.780	12.359	72.251	2.334	2.393	65.875	0.113	0.114	79.426	2.448	2.507	66.400
6	65	27.5	60	0.5	23.136	23.421	62.360	13.948	13.826	85.549	2.545	2.576	71.831	0.106	0.109	74.509	2.652	2.685	71.934
7	45	42.5	60	0.5	22.358	22.488	60.262	12.359	12.592	75.802	2.341	2.402	66.067	0.109	0.112	76.577	2.451	2.514	66.474
8	65	42.5	60	0.5	21.621	23.407	58.276	14.910	14.948	91.451	2.525	2.636	71.263	0.106	0.112	74.505	2.632	2.748	71.389
9	45	27.5	40	1.5	25.606	24.615	69.018	13.869	14.175	85.065	2.684	2.667	75.752	0.112	0.112	78.272	2.796	2.779	75.850
10	65	27.5	40	1.5	24.259	25.017	65.387	14.565	14.284	89.336	2.774	2.729	78.285	0.115	0.113	80.677	2.889	2.842	78.378
11	45	42.5	40	1.5	24.411	25.013	65.797	14.821	14.896	90.906	2.810	2.795	79.308	0.119	0.116	83.140	2.929	2.912	79.457
12	65	42.5	40	1.5	23.943	24.370	64.534	16.129	15.894	98.925	2.873	2.908	81.085	0.117	0.122	82.004	2.991	3.031	81.121
13	45	27.5	60	1.5	23.921	23.805	64.476	14.014	13.936	85.953	2.682	2.691	75.692	0.123	0.123	86.103	2.805	2.813	76.095
14	65	27.5	60	1.5	26.352	25.661	71.028	13.650	14.055	83.724	2.790	2.719	78.741	0.123	0.115	86.069	2.913	2.834	79.025
15	45	42.5	60	1.5	23.410	24.197	63.097	13.892	14.319	85.204	2.592	2.682	73.136	0.112	0.117	78.593	2.704	2.799	73.348
16	65	42.5	60	1.5	24.463	25.009	65.936	15.103	15.327	92.631	2.672	2.762	75.403	0.111	0.113	77.479	2.783	2.875	75.484
17	35	35	50	1	24.057	23.594	64.841	13.730	13.273	84.210	2.679	2.595	75.588	0.124	0.119	87.120	2.803	2.714	76.034
18	75	35	50	1	26.136	24.916	70.445	15.577	15.738	95.543	2.917	2.891	82.322	0.121	0.120	84.436	3.038	3.011	82.404
19	55	20	50	1	21.434	22.295	57.773	12.077	11.999	74.074	2.274	2.383	64.173	0.102	0.109	71.556	2.376	2.491	64.459
20	55	50	50	1	25.222	22.679	67.981	14.061	13.842	86.243	2.789	2.572	78.717	0.128	0.115	89.810	2.918	2.687	79.147
21	55	35	30	1	24.282	23.306	65.449	13.666	14.076	83.821	2.648	2.674	74.735	0.115	0.110	80.336	2.763	2.784	74.952
22	55	35	70	1	24.565	23.858	66.210	14.692	13.987	90.114	2.748	2.613	77.533	0.111	0.110	77.991	2.859	2.722	77.551
23	55	35	50	0	23.372	21.510	62.996	12.999	12.976	79.728	2.513	2.387	70.917	0.122	0.113	85.735	2.636	2.499	71.491
24	55	35	50	2	26.003	26.183	70.087	15.923	15.649	97.661	2.854	2.871	80.548	0.117	0.120	81.878	2.971	2.992	80.599
25	55	35	50	1	23.609	23.649	63.633	14.679	14.402	90.032	2.753	2.680	77.682	0.113	0.116	79.296	2.866	2.796	77.745
26	55	35	50	1	24.190	23.649	65.200	14.460	14.402	88.692	2.718	2.680	76.694	0.117	0.116	82.015	2.835	2.796	76.900
27	55	35	50	1	23.016	23.649	62.036	14.794	14.402	90.740	2.627	2.680	74.139	0.112	0.116	78.735	2.740	2.796	74.317
28	55	35	50	1	24.008	23.649	64.709	14.809	14.402	90.833	2.790	2.680	78.731	0.118	0.116	82.961	2.908	2.796	78.895
29	55	35	50	1	22.554	23.649	60.791	14.052	14.402	86.189	2.521	2.680	71.139	0.110	0.116	76.974	2.631	2.796	71.365
30	55	35	50	1	23.180	23.649	62.477	13.600	14.402	83.417	2.589	2.680	73.071	0.120	0.116	83.786	2.709	2.796	73.486
31	55	35	50	1	24.984	23.649	67.341	14.420	14.402	88.444	2.765	2.680	78.028	0.118	0.116	82.917	2.883	2.796	78.217

Table 6.2 Analysis of variance (ANOVA) for response surface model (including all model items; significant model items are in bold fonts).

Source of variation	Regression models including all model terms										Reduced regression models							
	The global extraction yields		Fatty acids yields		EPA yields		DHA yields		Selected Omega-3 fatty acids yields		The global extraction yields		Fatty acids yields		EPA yields		Selected Omega-3 fatty acids yields	
	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
Linear	0.92	0.475	4.10	0.018	1.75	0.189	1.23	0.336	1.7	0.2	24.30	0.000	11.05	0.000	15.77	0.000	14.76	0.000
X_1	0.27	0.610	0.00	0.972	0.01	0.908	0.02	0.903	0.01	0.907	\	\	0.23	0.638	10.87	0.003	9.98	0.004
X_2	1.75	0.205	6.55	**0.021**	3.86	0.067	0.96	0.342	3.72	0.072	\	\	8.24	0.009	7.34	0.012	6.94	0.014
X_3	0.14	0.711	1.85	0.193	0.92	0.352	3.91	0.065	1.05	0.321	\	\	2.27	0.146	\	\	\	\
X_4	0.80	0.383	9.87	**0.006**	2.82	0.113	0.19	0.671	2.65	0.123	24.30	0.000	19.60	0.000	29.10	0.000	27.37	0.000
Square	0.50	0.739	5.00	0.008	1.48	0.253	0.56	0.694	1.42	0.273	\	\	22.68	0.000	6.14	0.020	5.79	0.023
X_1^2	0.37	0.550	0.09	0.766	0.46	0.506	0.48	0.497	0.47	0.501	\	\	\	\	\	\	\	\
X_2^2	1.37	0.259	18.88	**0.001**	4.87	**0.042**	0.43	0.521	4.59	**0.048**	\	\	22.68	0.000	6.14	0.020	5.79	0.023
X_3^2	0.00	0.947	1.19	0.292	0.16	0.692	1.12	0.305	0.2	0.663	\	\	\	\	\	\	\	\
X_4^2	0.04	0.845	0.07	0.796	0.31	0.585	0.03	0.866	0.27	0.608	\	\	\	\	\	\	\	\
2-way interaction	0.39	0.873	2.61	0.058	0.55	0.762	0.80	0.581	0.55	0.761	\	\	6.10	0.003	\	\	\	\
X_1X_2	0.62	0.443	3.81	0.069	0.17	0.684	0.44	0.515	0.19	0.671	\	\	4.64	0.042	\	\	\	\
X_1X_3	1.20	0.289	0.00	0.983	0.07	0.791	1.64	0.218	0.11	0.747	\	\	\	\	\	\	\	\
X_1X_4	0.01	0.936	8.75	**0.009**	1.57	0.228	0.21	0.650	1.49	0.239	\	\	10.66	0.004	\	\	\	\
X_2X_3	0.00	0.997	0.55	0.471	1.23	0.284	2.11	0.166	1.3	0.271	\	\	\	\	\	\	\	\
X_2X_4	0.23	0.637	0.11	0.746	0.02	0.890	0.31	0.587	0.03	0.871	\	\	\	\	\	\	\	\
X_3X_4	0.30	0.593	2.47	0.135	0.25	0.626	0.11	0.746	0.21	0.655	\	\	3.01	0.097	\	\	\	\
Lack-of-Fit	3.60	0.065	1.11	0.469	1.74	0.258	5.42	**0.025**	1.87	0.229	2.27	0.156	0.83	0.645	1.21	0.435	1.30	0.397

Note: X_1 = temperature; X_2 = pressure; X_3 = dynamic extraction time; X_4 = mass ratio of co-solvent/biomass

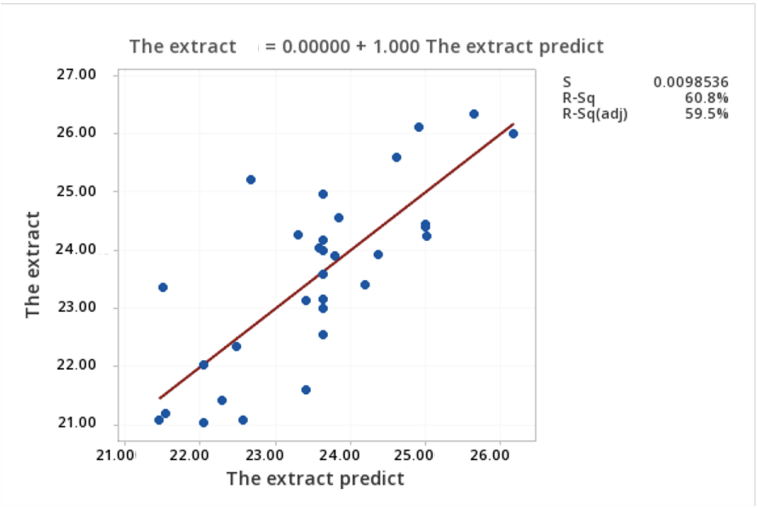
Table 6.3 Fitting summary of regression models

Response variables	Regression models including all model terms				Reduced regression models			
	F-value	**P-value**	**R-Square**	***Adj* R-Square**	**F-value**	**P-value**	**R-Square**	***Adj* R-Square**
The global extraction yields	1.77	0.136	60.82%	26.53%	24.30	0.000	45.59%	43.71%
Fatty acids yields	11.12	0.000	90.68%	82.53%	23.41	0.000	89.49%	85.67%
EPA yields	3.21	0.014	73.77%	50.83%	12.63	0.000	66.03%	60.80%
DHA yields	0.72	0.729	38.64%	0.00%	\	\	\	\
Selected Omega-3 fatty acids yields	3.04	0.018	72.67%	48.75%	11.86	0.000	64.60%	59.16%

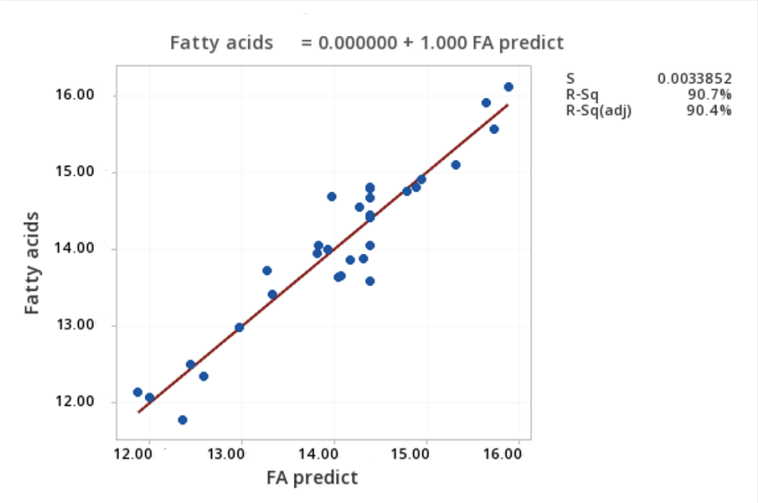
Table 6.3 showed the Fisher test values (F-value), P-value, Regression coefficient of determination R-square (R^2), and adjusted R-square (*adj* R^2) of regression models of yields of the global extraction, fatty acids, EPA, DHA, and the selected Omega-3 fatty acids. Those statistical indicators were used to examine the goodness of fit for those models. A well-fitted regression model suggests the response factors and independent variables are correlated positively or negatively. Except for the global extracts yield regression model and the DHA yield regression model, other models in this study had P-values less than 0.05, confirming the validity of those generated models: the experimental values fitted into those models well with good precision and reliability. Also, the ANOVA showed that the P-value of Lack-of-Fit of the models excluding the DHA yield regression model was insignificant (P-values>0.05). That indicated those models adequately explained the relationship between dependent variables and independent factors and the suggested model fitted the experimental data well. However, for the regression model of the global extract yields, there was a conflict between model P-value (P-value>0.05) and Lack-of-Fit P-value (P-value>0.05). The predictors had limited and incomprehensive effects on the global extracts, but the proposed model fitted the experimental data to some extent in coded levels. Overall, the regression models of the global extracts and DHA were not predictive and only qualitative and evaluative response surfaces could be developed in coded levels. The fitted line plots of the actual values and the predicts that generated by the regression models (**Figure 6.1 (a)&(d)**) further proved the unpredictability of the regression models of the global extracts and DHA. The unpredictability of the model for global extract yield may be attributed to the fact that global extracts contain not only lipids and pigments but some unspecified compounds. Therefore, these unspecified compounds need to be identified and the parameters that influence their extraction using $scCO_2$ should be understood prior to developing a model that is able to predict the global extract yield. The unpredictability of the DHA model may be due to the fact that DHA is only present at a very limited quantity in biomass.

R^2 reveals the percent of variation in the response that the model can account for; while *adj* R^2 adjusts R^2 for the number of predictors in the model, also showing whether regression models are well fitted. The greater the R^2, the more accurately the regression model fits experimental data. On the basis of R^2 and *adj* R^2, the fitted models for fatty acids

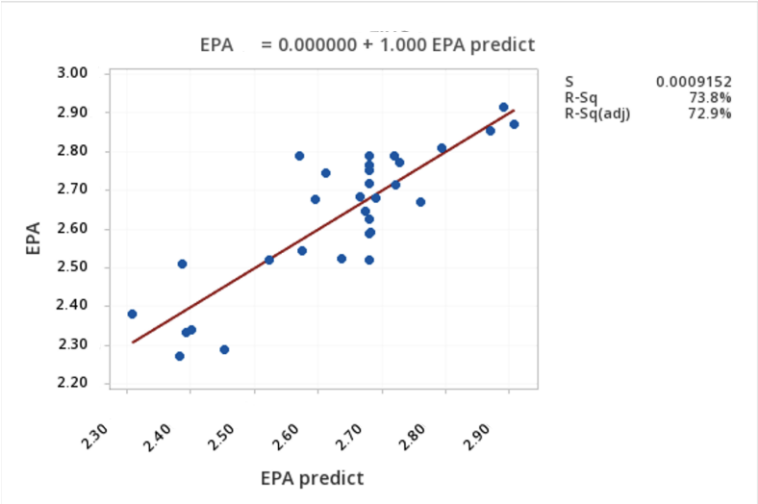
yields were valid, with of R^2 = 90.68% and 89.49%, and *adj* R^2 = 82.54% and 85.67%, respectively, indicating that those models could describe about 90% of the variability in fatty acids yields or they could explain approximately 90% of results of fatty acids yields. **Figure 6.1 (b)** graphically examined the experimental fatty acids yields well fitted into their corresponding predicted values against the linear regression line, with an R^2 of 90.7%. However, the R^2 and *adj* R^2 for the regression model EPA yields and selected Omega-3 fatty acids were less satisfactory, which meant only 73-74% of results could be described by their regression models. That was also supported by their fitted line plots (**Figure 6.1 (c)&(e)**). Those plots suggested that the regression models for EPA and the selected Omega-3 yields did not cover experimental data sufficiently due to the relatively lower R^2 resulting from the non-ideal correlation between actual values and predicted values. This could be due to various systematic, random, or human errors introduced in experimental process, or might be caused by inherent characteristics like very low yields of EPA and DHA introducing more errors into the data collected. Also, fatty acids yields were obtained through a series of operations including extraction, transesterification, and GC analysis; as a result, fatty acids loss or degradation was easy to happen during the process. Moreover, the lower R^2 and *adj* R^2 of the global extracts and DHA regression models additionally backed their poor predictability and fitness (**Figure 6.1 (a)&(d)**).



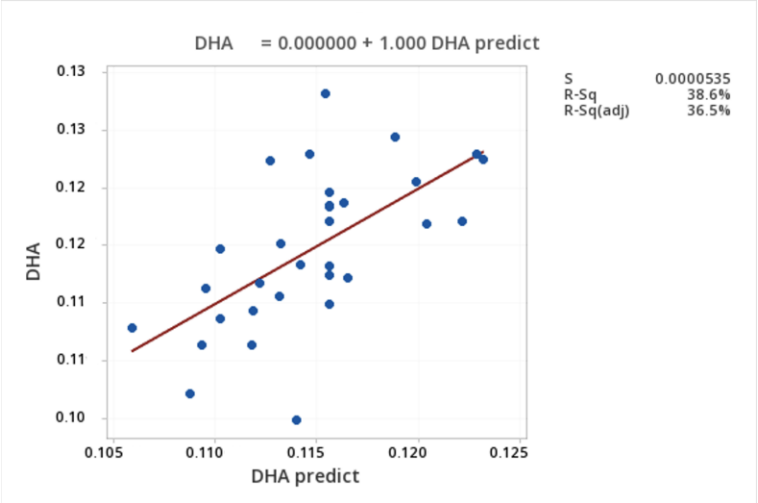
(a)


Fatty acids = 0.000000 + 1.000 FA predict
S 0.0033852
R-Sq 90.7%
R-Sq(adj) 90.4%
Fatty acids
FA predict
16.00
15.00
14.00
13.00
12.00
12.00
13.00
14.00
15.00
16.00

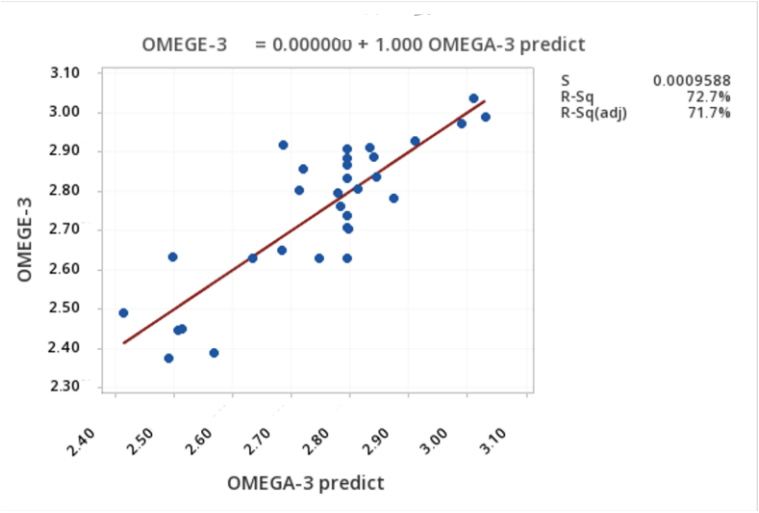
(b)


EPA = 0.000000 + 1.000 EPA predict
S 0.0009152
R-Sq 73.8%
R-Sq(adj) 72.9%
EPA
EPA predict
3.00
2.90
2.80
2.70
2.60
2.50
2.40
2.30
2.20
2.30
2.40
2.50
2.60
2.70
2.80
2.90

(c)


DHA = 0.000000 + 1.000 DHA predict
S 0.0000535
R-Sq 38.6%
R-Sq(adj) 36.5%
DHA
DHA predict
0.13
0.13
0.12
0.12
0.11
0.11
0.10
0.105
0.110
0.115
0.120
0.125

(d)



(e)

Figure 6.1 Actual vs. Predicted values of yields of the global extracts (a), fatty acids (b), EPA (c), DHA (d), and selected Omega-3 (e).

Thus, the regression models of fatty acids yield and the selected Omega-3 fatty acids yield established in this study were reliable with relatively satisfactory correlations between responses and process variables. On the other hand, because of the lack of fitness and predictivity, the global extract yields and DHA yields regression models were not discussed and used for optimization.

6.2. Effect of Process Parameters

6.2.1. Linear and Quadratic Effects on Yields

The main effects plots showing how process factors affected responses differently were illustrated as **Figure 6.2, Figure 6.3**, and **Figure 6.4**. **Figure 6.2** showed as temperature increased, the fatty acid yields slightly fluctuated; and as mass ratio of co-solvent/biomass and dynamic extraction time increased separately, fatty acid yields increased as well. Adding ethanol modified the selectivity and polarity of $scCO_2$; as such, some polar lipids that could not be extracted by neat $scCO_2$ were recovered. Liu et al. (2021) found the polar lipids – phospholipids – were one of the predominant lipid classes in air-dried *Cucumaria frondosa* viscera, accounting for 27.77 g in 100 g of the total lipids. Longer extraction time enhanced target compounds dissolution enabling a relatively complete extraction; while as time increased, the increase of fatty acids flattened out. Similar growing trend was observed when pressure increased in a certain range (**Figure**

6.2); but further increase of pressure from an inflection point led to an obvious reduction in fatty acids yields. This was most likely owing to the rising density and capacity of $scCO_2$ resulting from the increase of pressure, which facilitated its penetration into samples, hence enhancing the interaction between supercritical fluids and biomass. However, following an inflection point, the selectivity of $scCO_2$ changed and then fatty acids solubility declined. A study on the selectivity of $scCO_2$ to fish oils also detected the amount of EPA and DHA in the total fatty acids increased as the pressure increased from 10 MPa to 30 MPa at temperature of 40 ˚C, whereas their composition decreased as the pressure raised to 40 MPa (Lopes et al., 2012). In comparison of each trendline, mass ratio of co-solvent/biomass and pressure seemed to be more sensitive for fatty acids extraction yields. Those findings were compatible with the ANOVA analysis (**Table 6.2**) and the results obtained from the preliminary screening.

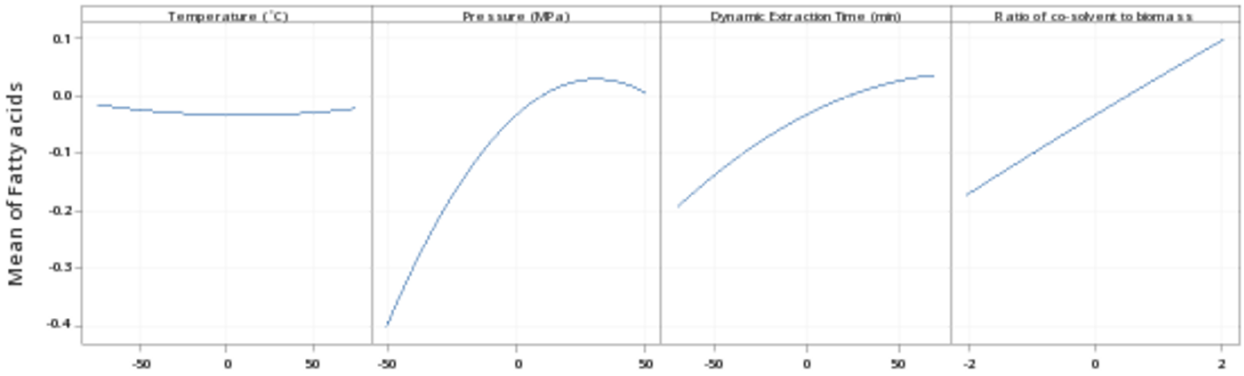


Figure 6.2 Main effects plots of process variables – temperature, pressure, dynamic extraction time, and mass ratio of co-solvent/biomass – on response of FA yields.

As illustrated in **Figure 6.3**, all those four process variables had similar effects on EPA yields as they did on fatty acid yields. Moreover, their effects on selected Omega-3 fatty acid yields were the same as those on EPA yields (**Figure 6.4**). That is due to the fact that EPA constitute the major portion of Omega-3 fatty acids. From these two plots (**Figure 6.3** & **6.4**), it can be seen that pressure played more important roles in the recovery of EPA and selected omega-3 fatty acids comparing with other parameters as its incline was the steepest.

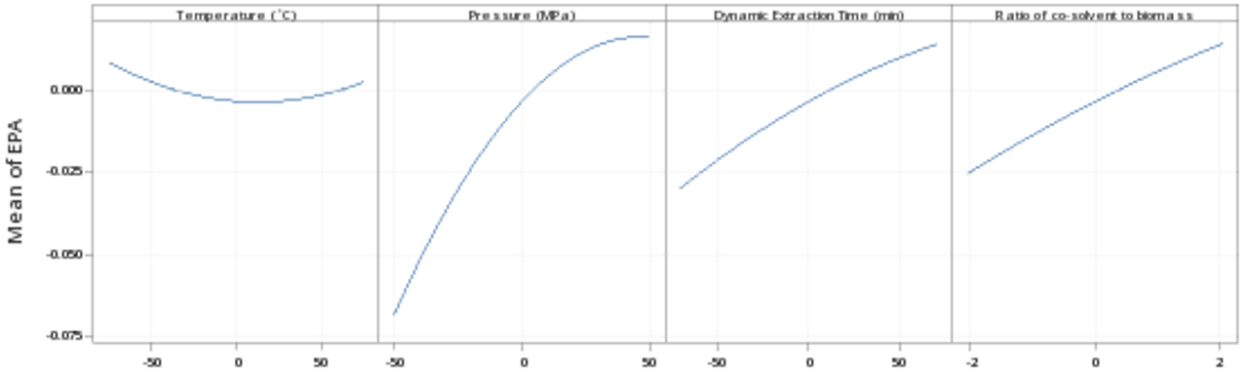


Figure 6.3 Main effects plots of process variables – temperature, pressure, dynamic extraction time, and mass ratio of co-solvent/biomass – on response of EPA yields.

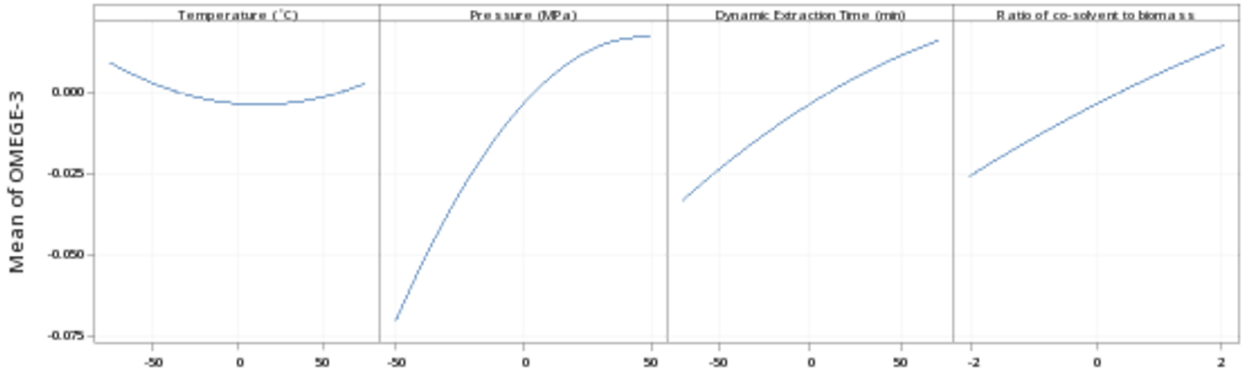


Figure 6.4 Main effects plots of process variables – temperature, pressure, dynamic extraction time, and mass ratio of co-solvent/biomass – on selected Omega-3 fatty acids.

The significance of each independent variable on yields of fatty acids, EPA, and the selected Omega-3 fatty acids was presented in **Table 6.2**. The significance of linear effects on fatty acids yields based on P-values was: mass ratio of co-solvent/biomass > pressure > dynamic extraction time > temperature. The first two model items were significant with P-

values < 0.05, and the mass ratio of co-solvent/biomass was the most principal processing variable for the fatty acids extraction, as evidenced by its higher F-value and lower P-value. The significance of linear effects on EPA yields based on P-values was: pressure > mass ratio of co-solvent/biomass > dynamic extraction time > temperature; however, none of them was significant for EPA recovery. Also, those four factors were all insignificant for the selected Omega-3 fatty acids recovery either, their significance based on P-values ranked as: pressure > mass ratio of co-solvent/biomass > dynamic extraction time > temperature. The ranking suggested that the density/selectivity of solvents, which was primarily regulated by pressure and co-solvents, were critical in the recovery of fatty acids. Addition of co-solvent is expected to improve the polarity of $scCO_2$ resulting in enhanced extraction of more polar fatty acids. Longer extraction did not result in a significant increase in fatty acid dissolution in $scCO_2$. That indicated most fatty acids were extracted within 30 min. Temperature theoretically has negative correlation with $scCO_2$ densities and this study showed its impact was smallest among those four process variables. The insignificant influence of temperature on fatty acid yield was also observed at the stage of preliminary experiments. Moreover, all the responses were significantly affected by the quadratic effects of pressure.

6.2.2. Interaction Effects on Compounds Yields

In terms of the interaction model terms, only the two-way interaction of mass ratio of co-solvent/biomass and temperature was regarded as the most significant interaction model term on fatty acids yields. That reemphasized co-solvent/biomass mass ratio was the predominant process factor for the fatty acids extraction. **Figure 6.5** plotted the interaction relation between temperature and co-solvent to biomass mass ratio for fatty acids yields, on which the crossed line suggested there was an interaction effect. A general trend that could be clearly observed, that was increasing the mass ratio of co-solvent to biomass increased the fatty acids yields when holding the temperature at a constant level. We hypothesized that modifying the non-polar nature of $scCO_2$ after adding the co-solvent improved the extraction of polar fatty acids, which meant higher yields of fatty acids could be obtained through adjusting the selectivity of $scCO_2$. However, no significant interaction effect between any two of the four parameters (i.e., temperature, pressure, dynamic

extraction time, and co-solvent to biomass ratio) was observed for EPA and the selected Omega-3 fatty acids yields.

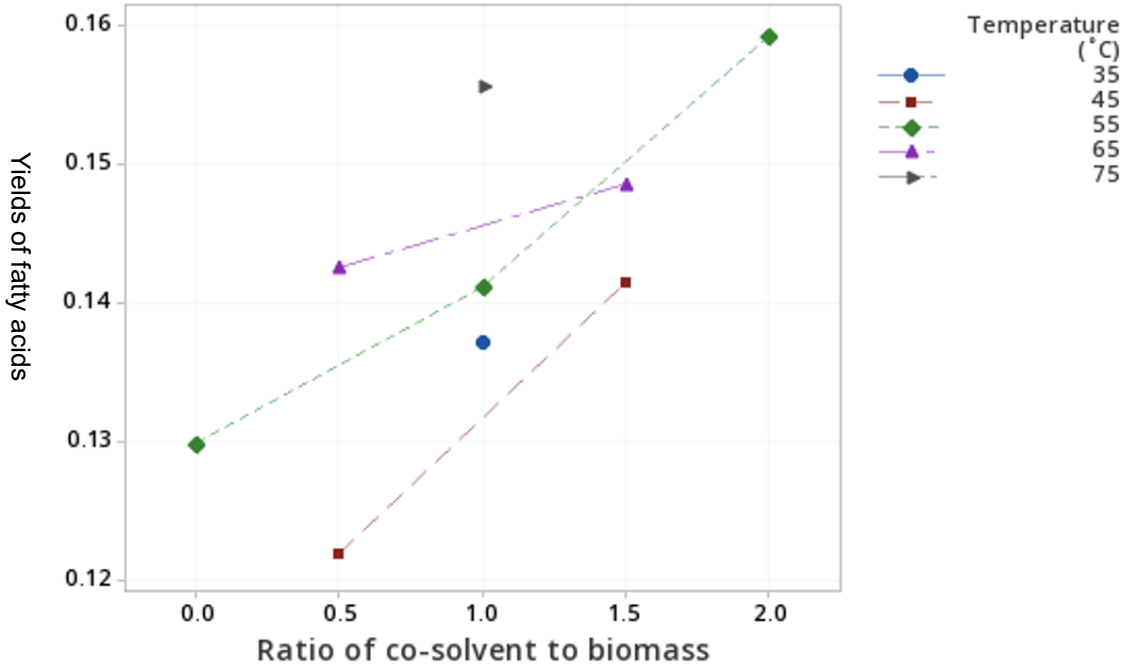


Figure 6.5 The interaction of temperature and mass ratio of co-solvent to biomass. X-axis and Y-axis represent mass ratio of co-solvent to biomass and fatty acids yields (g/g of the hot air-dried samples),

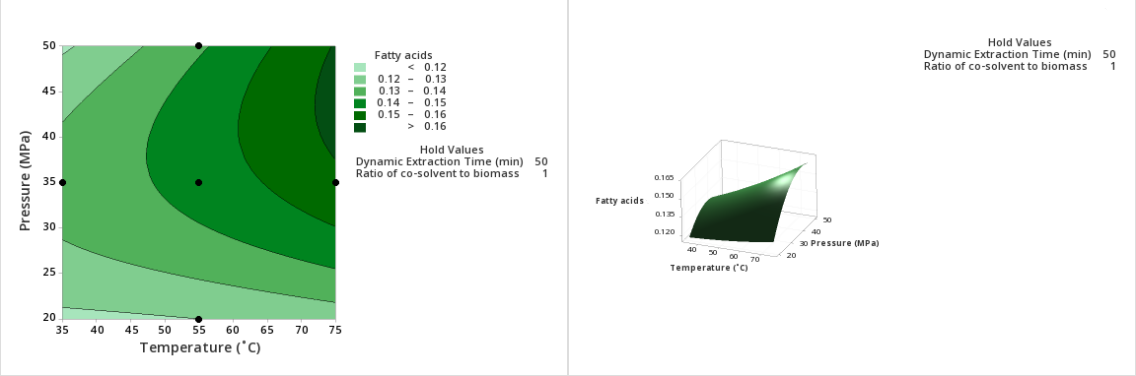
The three-dimensional response surface plots and contour plots for those three responses of interest were plotted in **Figure 6.6-6.8**, respectively. In each diagram, two factors were held for better understanding on how the other two factors interacted. Normally, those non-targeted variables are maintained at their median values, like the temperature of 55 ˚C, the pressure of 35 MPa, the dynamic extraction time of 50 min, the co-solvent/biomass mass ratio of 1. Contour lines link all the points with the same response, and colored contour bands with different shades represent different ranges of responses: the darker the color, the greater the yields.

The only interaction effect that significantly affected fatty acids yields was the interaction of temperature and co-solvent/biomass mass ratio (X_1X_4), as plotted in **Figure 6.6 (c)**. The experimental points were marked in the plot, showing fatty acids yields increased from 13.73 g/100 g of the hot air-dried samples to 15.58 g/100 g of the hot air-dried samples when temperature increased from 35 ˚C to 75 ˚C under consistent co-

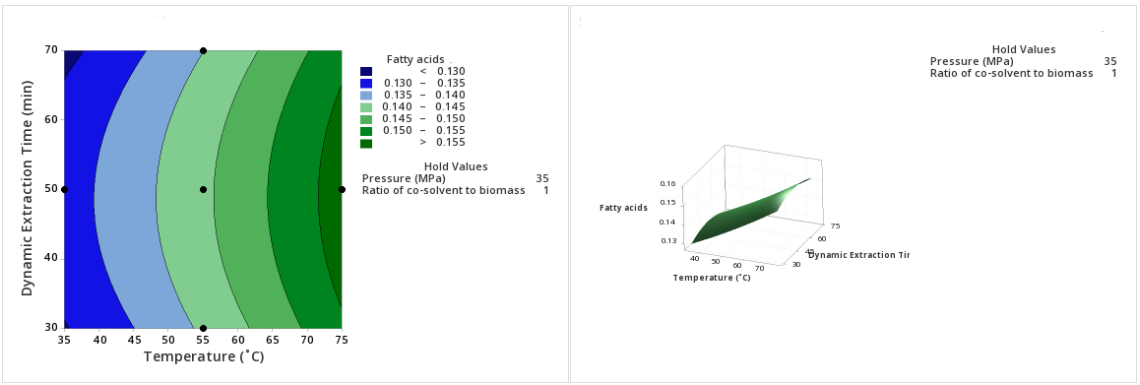
solvent/biomass mass ratio at 1.0. Also, as mass ratio of co-solvent/biomass increased from 0 to 2.0 under 55 ˚C, fatty acids yields were remarkably improved by almost 3%. Notably, co-solvent addition not only enhances the polarity of $scCO_2$ favoring the extraction of polar fatty acids in polar lipids but modifies its selectivity with better fatty acids solubility particularly under the temperature lower than about 70 ˚C. Fatty acids yields increased outstandingly as temperature increased with mass ratio of co-solvent/biomass at 0; However, the yield showed no notable increase as temperature increased with mass ratio of co-solvent/biomass higher than approximately 1.3, implying adding ethanol assisted in maintaining desired solubilities for higher fatty acids yields and also it reduced and even eliminated the negative effect of temperature on $scCO_2$ densities.

Figure 6.6 (a), (b), and **(d)-(f)** show the effects of the insignificant interaction factors on FA yields. The interaction between temperature and pressure positively affected fatty acids yields – the color bands became darker as the predictor in x-axis increased – as displayed in **Figure 6.6 (a)**. That means FA yields increased as temperature increased at a fixed pressure above approximately 23 MPa. Also, the positive effect of temperature seemed to become more pronounced at pressure higher than 35 MPa. However, as temperature maintained as a constant level while simultaneously increasing pressure, fatty acids yields would increase and then decrease. For $scCO_2$, its solubility is associated with its density, which is negatively correlated with temperature and positively correlated with pressure. As such, fatty acids yields can achieve the highest range when the supercritical fluid possesses certain densities (highest yields did not obtain at the highest density). Therefore, based on the analysis of **(a)** and **(c)**, it can be concluded there was an antagonist effect between temperature and pressure and between temperature and mass ratio of co-solvent/biomass (the effects of temperature that are inhabited by the effects of pressure and mass ratio of co-solvent to biomass). Also, that indicated the higher temperature enhanced mass transfer of solvents into samples through increasing the diffusion of fluids. In **(b)**, fatty acids yields increased with rising temperature at a fixed dynamic extraction time; but, for some fixed temperatures, as dynamic extraction time increased, fatty acids yields remained in the same color ramp, and almost all contour lines intersected on x-axis. That explained how insignificant the interaction effect had. **(d)** showed an interaction pattern that fatty acids yields raised sharply and then dropped when the pressure increased at a

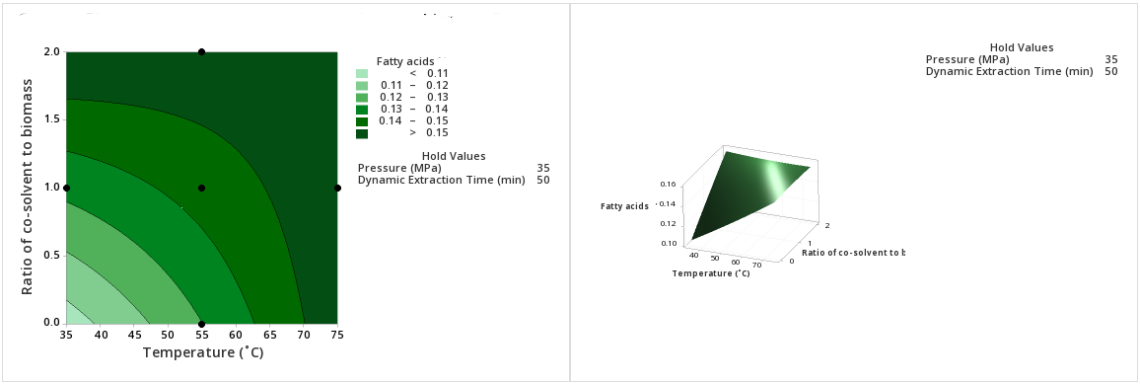
constant dynamic extraction time. Also, the nearly vertical contour lines intersected to x-axis reemphasizing the importantly positive role of pressure in the fatty acid extraction. **(e)** depicted the interaction effect of pressure and mass ratio of co-solvent/biomass that fatty acids yields were continuously increased by increasing co-solvent/biomass mass ratio at any pre-determined value of pressure and the increase in pressure also lead to an improvement on fatty acids yields while holding co-solvent/biomass mass ratio at any point within experimental scale. The plots further examined the solubility of fatty acids in $scCO_2$ was associated with a certain range of density regulated by pressure, mass ratio of co-solvent/biomass, even also temperature. Therefore, only weak interaction was spotted in above circumstances. In **(f)**: fatty acids yields increased with increasing co-solvent/biomass mass ratio while holding dynamic extraction time at a constant value; while fatty acids yield almost remained at the same level as dynamic extraction increased at a fixed co-solvent/biomass mass ratio. Additionally, the contour lines, especially those representing fatty acids yields (g/g of the hot air-dried samples) of 0.13, 0.14, 0.15, and 0.16, were nearly perpendicular to the y-axis suggested fatty acids yields were more sensitive to mass ratio of co-solvent/biomass comparing with dynamic extraction time.



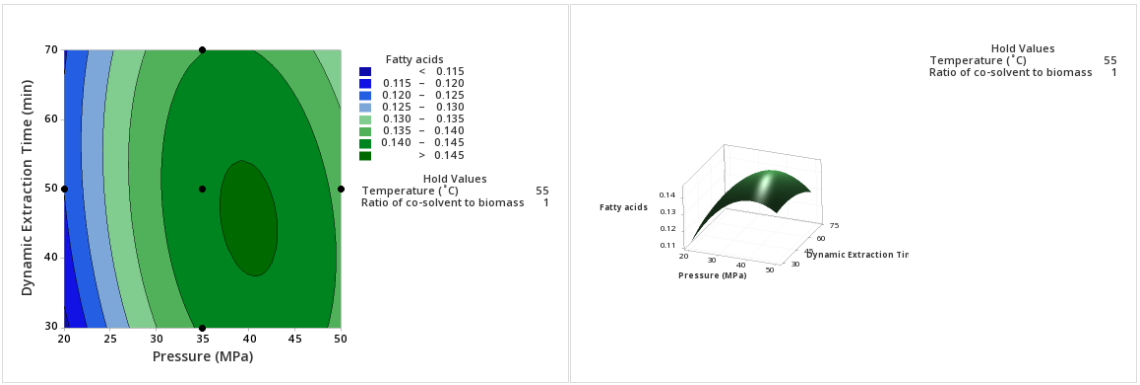
(a) the effect of pressure and temperature on fatty acids yields in per gram of the hot air-dried samples with constant dynamic extraction time of 50 min and co-solvent/biomass mass ratio of 1.



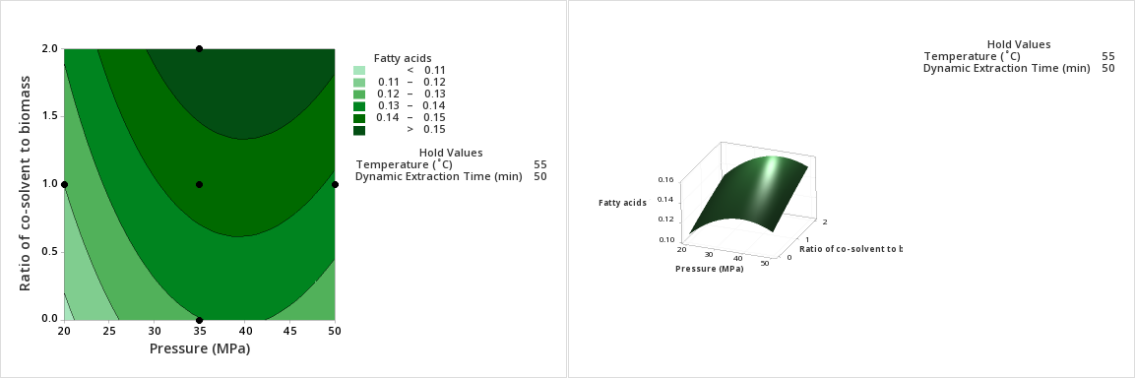
(b) the effect of dynamic extraction time and temperature on fatty acids yields in per gram of the hot air-dried samples with constant pressure of 35 MPa and co-solvent/biomass mass ratio of 1.



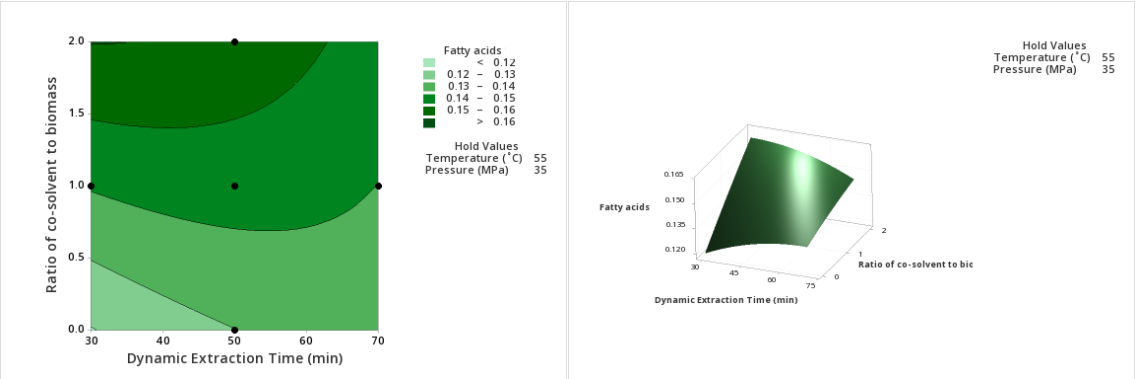
(c) the effect of co-solvent/biomass mass ratio and temperature on fatty acids yields in per gram of the hot air-dried samples with constant pressure of 35 MPa and dynamic extraction time of 50 min.



(d) the effect of dynamic extraction time and pressure on fatty acids yields in per gram of the hot air-dried samples with constant temperature of 55 ˚C and co-solvent/biomass mass ratio of 1.



(e) the effect of co-solvent/biomass mass ratio and pressure on fatty acids yields in per gram of the hot air-dried samples with constant temperature of 55 ˚C and dynamic extraction time of 50 min.

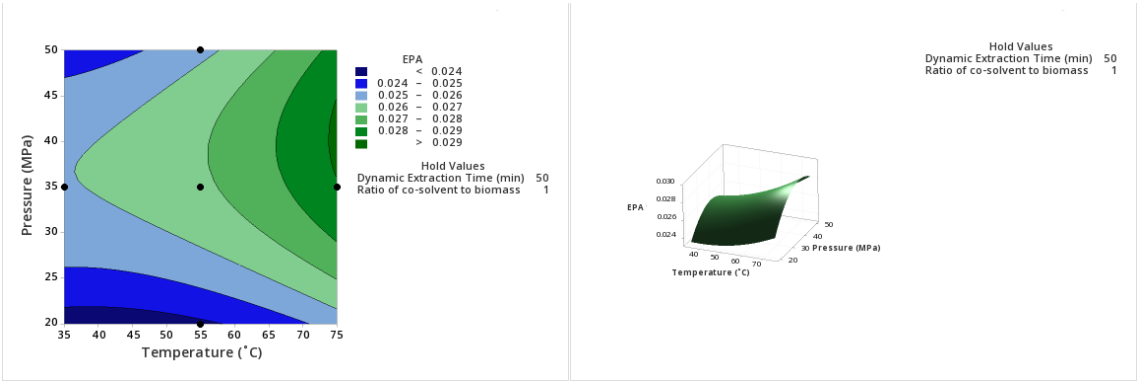


(f) the effect of co-solvent/biomass mass ratio and dynamic extraction time on fatty acids yields in per gram of the hot air-dried samples with constant temperature of 55 ˚C and pressure of 35 MPa.

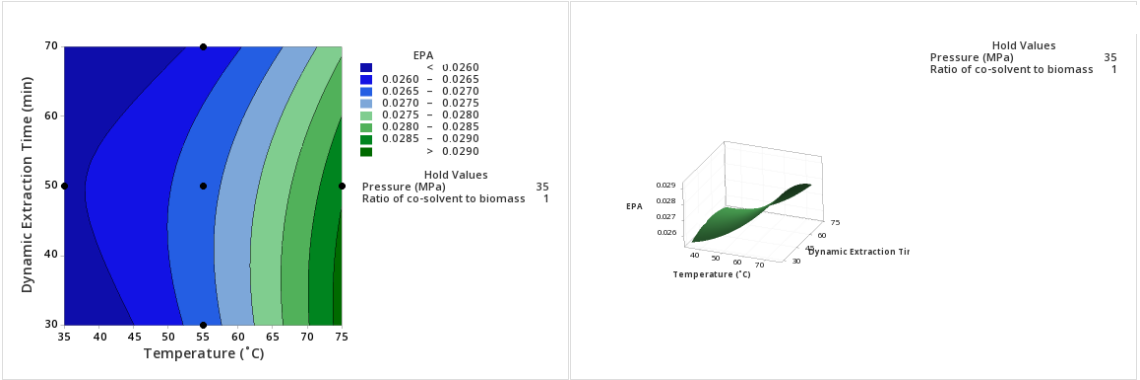
Figure 6.6 Surface plots and contour plots for fatty acids yields.

Unlike fatty acids yields, which were significantly impacted by an interaction between process variables, EPA yields and the selected Omega-3 fatty acids were not significantly affected by any interaction effect, and those insignificant interactions were plotted in **Figure 6.7** and **Figure 6.8**. Notably, those interactions had similar patterns comparing to the interaction effects of fatty acids yields. For instance, **Figure 6.7 (a)** and **Figure 6.8 (a)**, the interaction patterns of temperature and pressure on EPA yields and the selected Omega-3 fatty acids, resembled to **Figure 6.6 (a)**, and hence temperature and pressure exhibited comparable interaction effects on Omega-3 fatty acids yields as they did on the total fatty acids yields. It can be noted that the selected fatty acids yields fell in light-

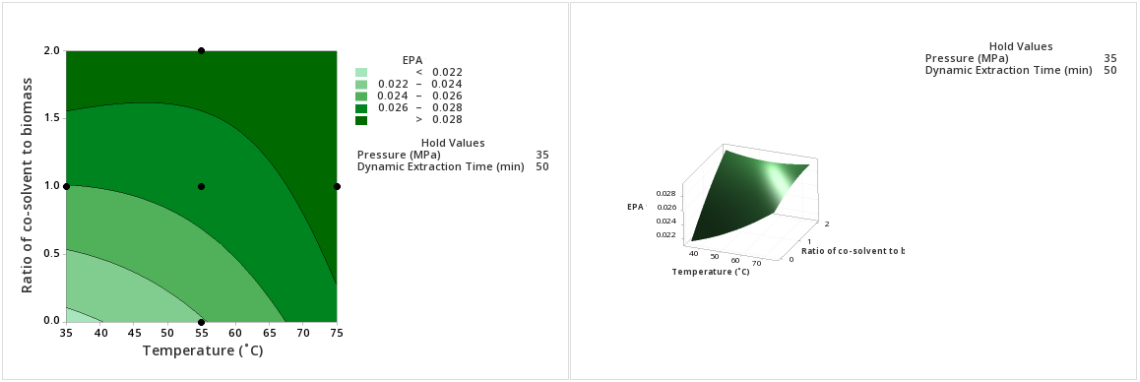
colored region of contour plot when a high temperature and a low pressure or a low temperature and a high pressure were used. However, by increasing temperature and pressure at the same time, the highest yields could be obtained. Therefore, it can be concluded that typical fatty acids had their own solubility patterns in $scCO_2$, and its recovery efficiency was related to a certain range of $scCO_2$ densities. Also, the interaction patterns demonstrated in **Figure 6.7 (a)** and **Figure 6.7 (c)** as well as **Figure 6.8 (a)** and **Figure 6.8 (c)** indicated antagonist effects between temperature and pressure and between temperature and mass ratio of co-solvent/biomass (the effects of temperature that are inhabited by the effects of pressure and mass ratio of co-solvent to biomass). That also reemphasized the higher temperature enhanced mass transfer and diffusion of fluids.



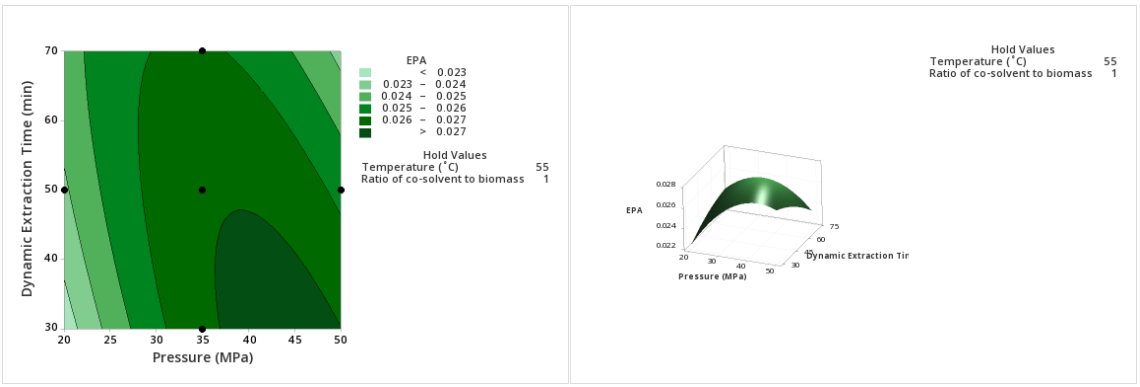
(a) the effect of pressure and temperature on EPA yields in per gram of the hot air-dried samples with constant dynamic extraction time of 50 min and co-solvent/biomass mass ratio of 1.



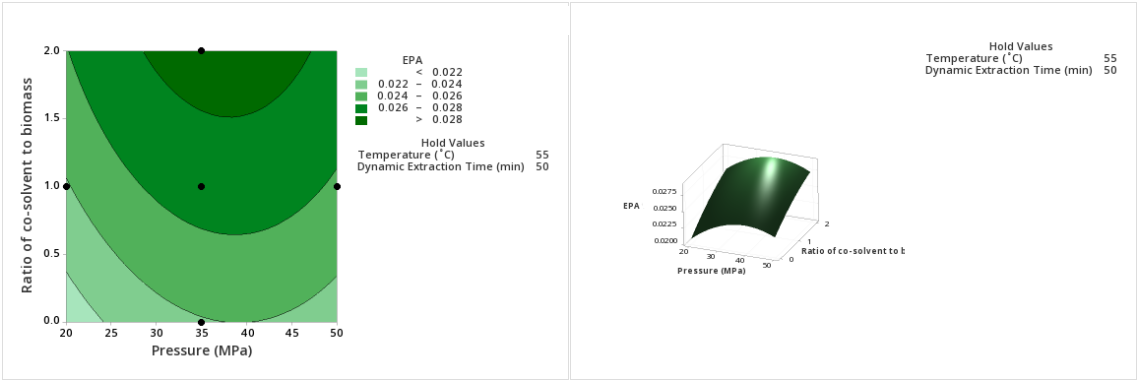
(b) the effect of dynamic extraction time and temperature on EPA yields in per gram of the hot air-dried samples with constant pressure of 35 MPa and co-solvent/biomass mass ratio of 1.



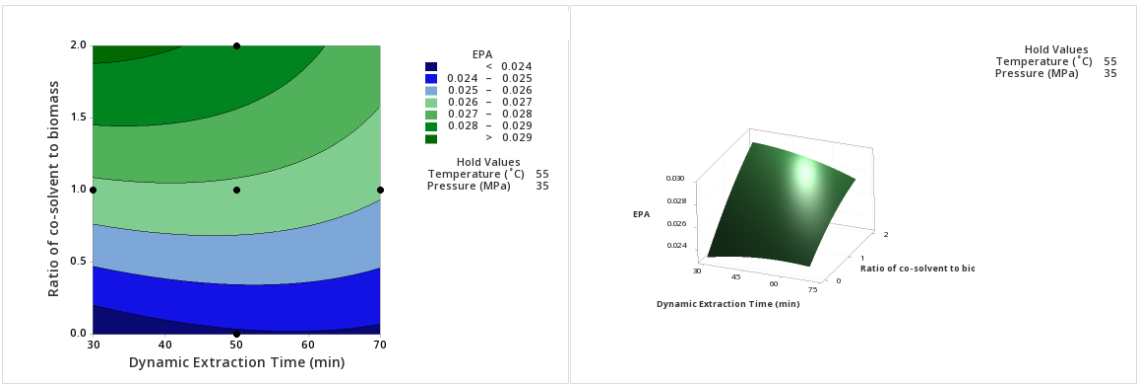
(c) the effect of co-solvent/biomass mass ratio and temperature on EPA yields in per gram of the hot air-dried samples with constant pressure 0f 35 MPa and dynamic extraction time of 50 min.



(d) the effect of dynamic extraction time and pressure on EPA yields in per gram of the hot air-dried samples with constant temperature of 55 ˚C and co-solvent/biomass mass ratio of 1.

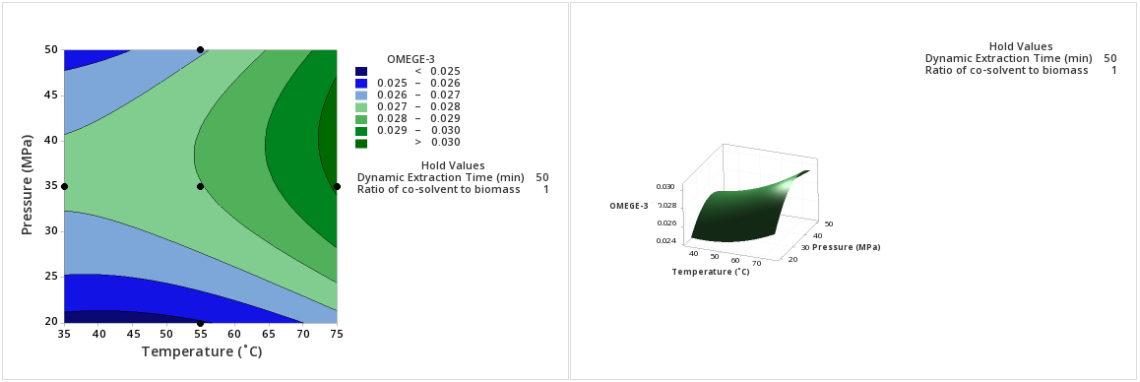


(e) the effect of co-solvent/biomass mass ratio and pressure on EPA yields in per gram of the hot air-dried samples with constant temperature of 55 ˚C and dynamic extraction time of 50 min.

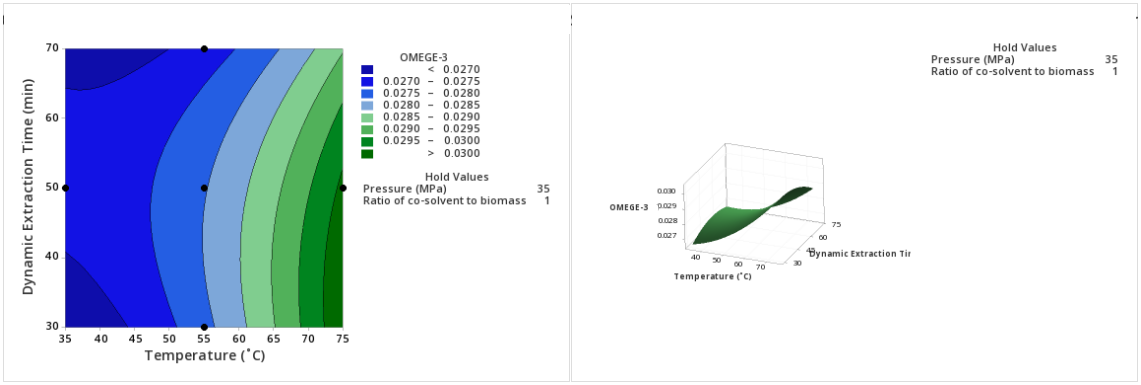


(f) the effect of co-solvent/biomass mass ratio and dynamic extraction time on EPA yields in per gram of the hot air-dried samples with constant temperature of 55 ˚C and pressure of 35 MPa.

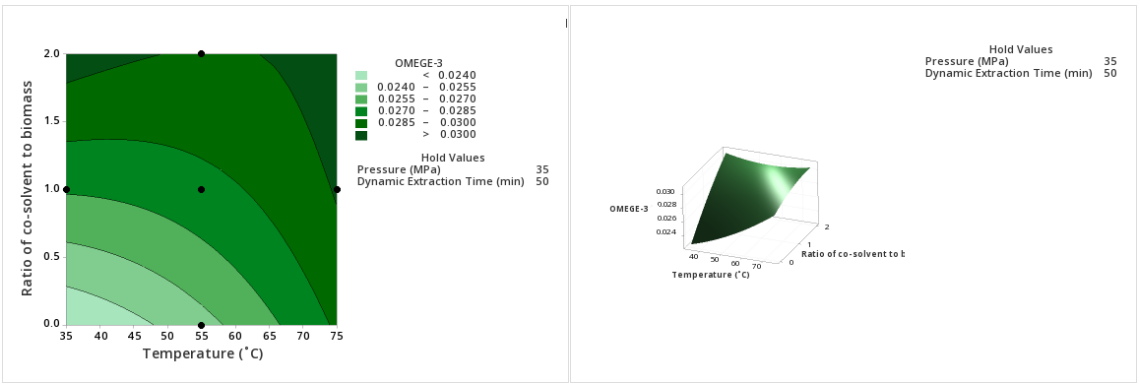
Figure 6.7 Surface plots and contour plots for EPA yields.



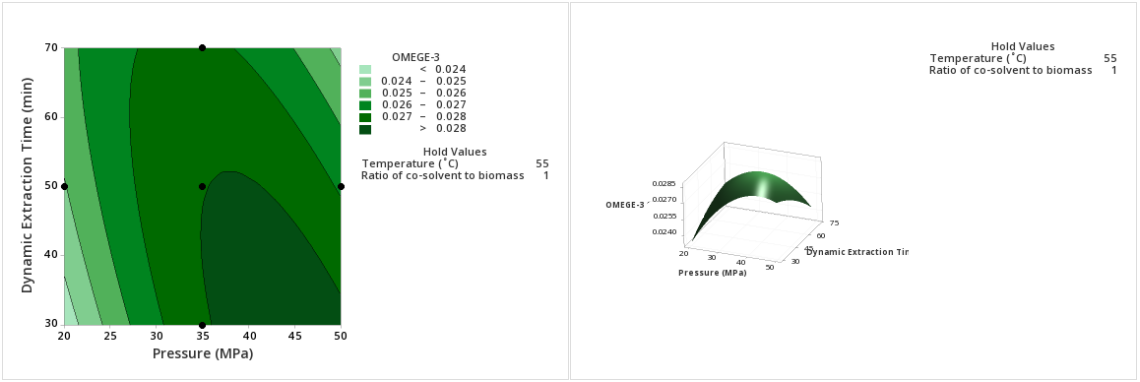
(a) the effect of pressure and temperature on the selected Omega-3 fatty acids yields in per gram of the hot air-dried samples with constant dynamic extraction time of 50 min and co-solvent/biomass mass ratio of 1.



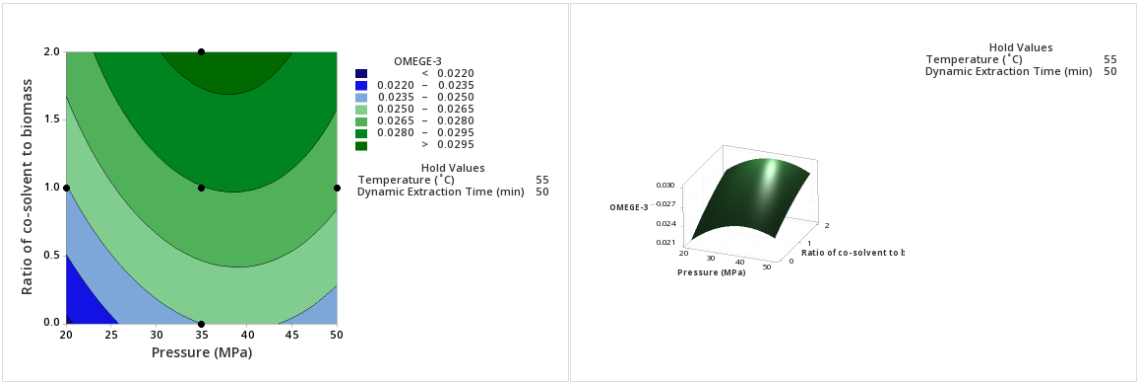
(b) the effect of dynamic extraction time and temperature on the selected Omega-3 fatty acids yields in per gram of the hot air-dried samples with constant pressure of 35 MPa and co-solvent/biomass mass ratio of 1.



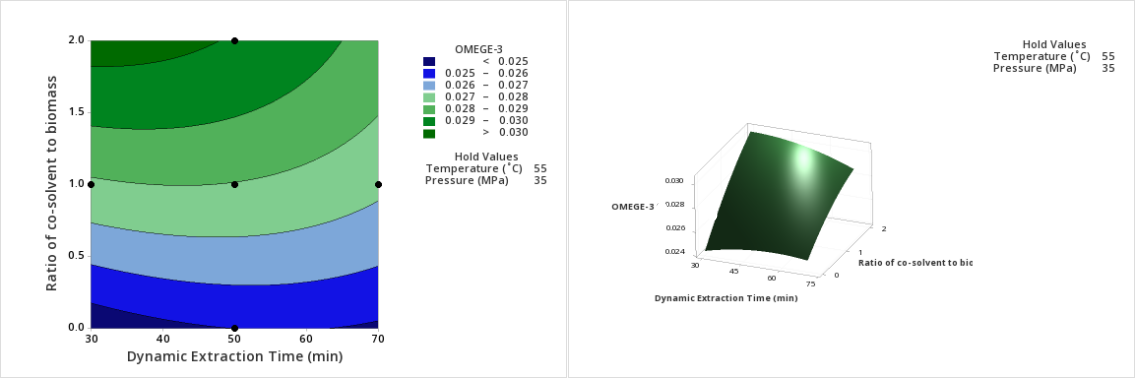
(c) the effect of co-solvent/biomass mass ratio and temperature on the selected Omega-3 fatty acids yields in per gram of the hot air-dried samples with constant pressure of 35 MPa and dynamic extraction time of 50 min.



(d) the effect of dynamic extraction time and pressure on the selected Omega-3 fatty acids yields in per gram of the hot air-dried samples with constant temperature of 55 ˚C and co-solvent/biomass mass ratio of 1.



(e) the effect of co-solvent/biomass mass ratio and pressure on the selected Omega-3 fatty acids yields in per gram of the hot air-dried samples with constant temperature of 55 ˚C and dynamic extraction time of 50 min.



(f) the effect of co-solvent/biomass mass ratio and dynamic extraction time on the selected Omega-3 fatty acids yields in per gram of the hot air-dried samples with constant temperature of 55˚C and pressure of 35 MPa.

Figure 6.8 Surface plots and contour plots for the selected Omega-3 fatty acids yields.

6.3. Optimization of $ScCO_2$ Extraction of Fatty Acids from Hot Air-Dried *Cucumaria frondosa* Viscera

In order to maximize yields of fatty acids and/or the selected Omega-3 fatty acids, the optimal extraction conditions were determined using the response optimizer in Minitab 19.0 and presented in **Table 6.4**. The optimal extraction conditions for maximum fatty acids are suggested to be temperature of 75 ˚C, pressure of 46.667 MPa, dynamic extraction time of 30 min, and mass ratio of ethanol/biomass of 2. The predicted yields of fatty acids are 16.908 g/100 g of the hot air-dried samples. However, the fatty acid content for *Cucumaria frondosa* viscera according to the *in-situ* transesterification was 16.304 g/100 g of the hot air-dried samples as reported in Chapter 4; as such, the predicted yields theoretically should be smaller than 16.304 g/100 g of the hot air-dried samples falling in the 95% predicted interval as (14.178, 16.304) g/100 g of the hot air-dried samples. The optimal reaction conditions for maximum selected Omega-3 fatty acids are suggested to be temperature of 75 ˚C, pressure of 44.849 MPa, dynamic extraction time of 30 min, and mass ratio of ethanol/biomass of 2. The predicted yields of EPA and DHA are 3.102 g/100 g of the hot air-dried samples and 0.131 g/100 g of the hot air-dried samples, respectively. Based on the results of *in-situ* transesterification, the EPA and DHA contents were reported as 3.544 g/100 g of the hot air-dried samples and 0.143 g/100 g of the hot air-dried samples,

respectively; as a result, their 95% predicted interval should be adjusted to (2.267, 3.544) g/100 g of the hot air-dried samples and (0.097, 0.143) g/100 g of the hot air-dried samples, correspondingly. Moreover, the optimal reaction conditions for maximum fatty acids and the selected Omega-3 fatty acids are anticipated to be temperature of 75 ˚C, pressure of 44.849 MPa, dynamic extraction time of 30 min, and mass ratio of ethanol/biomass of 2. The predicted yields of fatty acids, EPA, and DHA are 16.890 g/100 g of the hot air-dried samples, 3.102 g/100 g of the hot air-dried samples, and 0.131 g/100 g of the hot air-dried samples, respectively. Also, due to the fatty acids amount obtained from conventional method smaller than the upper limit of software-suggested predicted interval, the predicted yields for fatty acids, EPA, and DHA should fall in the 95% predicted intervals as (13.837, 16.304) g/100 g of the hot air-dried samples, (2.267, 3.544) g/100 g of the hot air-dried samples, and (0.097, 0.143) g/100 g of the hot air-dried samples accordingly. In addition, the optimization plots for those target compounds were shown in Appendix **Figure A.3 (a)**, **Figure A.4 (a)**, and **Figure A.5 (a)**, respectively.

In comparison of those three scenarios, it is evident that temperature of 75 ˚C, pressure of 44.849 MPa, dynamic extraction time of 30 min, and mass ratio of ethanol/biomass of 2 is the ideal setting for maximizing yields of all responses (max all in **Table 6.4**). Nevertheless, in pursuit of economic benefits, streamlining production plays an important role at the industrial level. Extraction without introducing co-solvents is regarded as a preference to avoid purification process. On the basis of that, the mass ratio of co-solvent/biomass was held at a value of 0 in the response optimizer. Corresponding predicted yields were listed in **Table 6.4**, and optimization plots were presented in Appendix **Figure A.3 (b)**, **Figure A.4 (b)**, and **Figure A.5 (b)**. Comparing with the optimization with or without the co-solvent, it could be noted there was an overlap between their 95% predicted intervals. As such, extraction under the optimal conditions without the co-solvent added would be a better choice for industrial practice; but further verification for that conclusion using two-sample T test was required. Therefore, experiments were conducted in duplicate under such optimized circumstances to further verify whether a good agreement between experimental values and predicted values was achieved.

Table 6.4 Optimal operation conditions of supercritical fluid extraction for maximum FA, EPA, and DHA.

Situation	Optimal reaction conditions				Predicted product yield (%, g/100 g of the hot air-dried samples)		
	X_1 (˚C)	X_2 (MPa)	X_3 (min)	X_4 (w/w)	FA	EPA	DHA
Max FA	75	46.667	30	2	16.908	\	\
	75	42.121	64	0	16.298	\	\
Max Omega-3	75	44.849	30	2	\	3.102	0.131
	75	46.364	30	0	\	2.828	0.141
Max all	75	44.849	30	2	16.890	3.102	0.131
	75	43.636	41	0	15.843	2.802	0.138

Note: Given that the DHA yield regression model was not predictive, the optimization process used the value of maximum selected Omega-3 yields subtracting maximum EPA yields as DHA yields.

Experiments under optimal conditions with/without co-solvents that maximize yields of fatty acids and selected Omega-3 fatty acids at the same time – 75 ˚C of temperature, 45 MPa of pressure, 30 min of dynamic extraction time, and 2 of co-solvent to biomass mass ratio; as well as 75 ˚C of temperature, 44 MPa of pressure, 41 min of dynamic extraction time, and 0 of co-solvent to biomass mass ratio – were carried out in duplicate, respectively. Corresponding results were summarized in **Table 6.5**. The recovery efficiencies for these two conditions were 88.32% of fatty acids and 79.71% of the selected Omega-3 fatty acids as well as 85.27% of fatty acids and 73.18% of the selected Omega-3 fatty acids, respectively. The acquired experimental yields of fatty acids and selected Omega-3 fatty acids all fell in the 95% predicted intervals; as such, a good agreement between experimental values and model-determined values has been obtained, indicating that the generated models are reliable. Remarkably, the experimental design allowed us to tune the optimized temperature at 75 °C. Although a study mentioned the main Omega-3 fatty acids, EPA, and DHA were significantly degraded at 50 °C or above and the suggested

temperature seemed to be high (Hădărugă et al., 2016), we did not observe a deterioration of PUFAs, implying $scCO_2$ might protect the extract from degradation. Létisse et al. (2006) found higher temperature (75 ˚C) yielded more EPA and DHA without degradation as well. However, that should be notice the mean yields of the duplicate experiments did not fall within the 95% confident intervals. It is likely due to the small sample size of each condition that cannot represent the sample population, and the uncertain variation of biological samples.

However, based on the two-sample T test (a test is used to examine whether the difference between the means of two independent populations is equal to a specific value), the results showed the addition of ethanol significantly affect the yields of fatty acids and selected Omega-3 fatty acids ($P_{\text{(fatty acids yields)}} = 0.032 < 0.05$, $P_{\text{(selected Omega-3 fatty acids)}} = 0.05 = 0.05$). Moreover, the P-value for fatty acids yields was consistent with the ANOVA of the generated models (**Table 6.2**), in which co-solvent to biomass mass ratio-related linear terms were significant with P-values small than 0.05. In order to make a decision for the incorporation of co-solvent to improve the extraction yield, a techno-economic analysis of the whole process is required.

Table 6.5 Experimental yields of fatty acids and selected Omega-3 (g/100 g of the hot air-dried samples) under optimal conditions with/without co-solvents added (n=2, Mean±SD).

Optimal conditions		Fatty acids yields	Selected Omega-3 fatty acids yields
75 ˚C of temperature, 45 MPa of pressure, 30 min of dynamic extraction time, and co-solvent to biomass mass ratio of 2	Experimental value	14.400±0.106 [a]	2.939±0.073 [a]
	Predicted value [1]	16.304	3.233
	Predicted interval [2]	(14.270, 16.304)	(2.492, 3.687)
	Recovery efficiency	88.322%	79.713%
75 ˚C of temperature, 44 MPa of pressure, 41 min of dynamic extraction time, and co-solvent to biomass mass ratio of 0	Experimental value	13.903±0.073 [b]	2.698±0.030 [b]
	Predicted value	15.843	2.940
	Predicted interval [2]	(13.817, 16.304)	(2.366, 3.687)
	Recovery efficiency	85.274%	74.176%

Note: Values in the same row with different letter are significantly different at $P \leq 0.05$.

[1]: The predicted value for the optimal condition with co-solvent/biomass as 2:1was adjusted to the reported maximum values obtained from in-situ transesterification because the suggested value was higher than 16.304%.

[2]: Predicted interval represents 95% predicted interval. The upper limit of the interval was adjusted to the reported maximum values obtained from in-situ transesterification.

6.4 Conclusion

In this chapter, a CCI of RSM was designed to investigate the effects of selected process variables on yields of fatty acids and selected Omega-3 fatty acids. With respect to the conventional methods, the maximum efficiency of SFE evaluated using experimental fatty acids yields was approximately 97% under 55 ˚C of temperature, 35 MPa of pressure, 50 min of dynamic extraction time, and 2:1 of co-solvent to biomass ratio, while the average recovery efficiencies of fatty acids and selected Omega-3 under the optimal conditions was about 88% and 80%. It was also found that the linear effect of pressure and co-solvent to biomass ratio, the quadratic effect of pressure, and the interaction between temperature and mass ratio of co-solvent to biomass significantly affected fatty acids yields but selected

Omega-3 fatty acids regression model only contained one dominant model term as the quadratic effect of pressure. On the other hand, to simplify the industrial practice, the necessary of co-solvent were evaluated under optimal conditions. The results suggested it was necessary to use co-solvents to improve fatty acids yields.

To the best of our knowledge, the majority of studies only focused on using response surface design to investigate the global extraction yields, followed by GC analysis of fatty acids composition. This is the first attempt to use a response surface design to develop relevant regression models and optimize the $scCO_2$ extraction of fatty acids and selected Omega-3 fatty acids from sea cucumber internal organs.

Chapter 7: Comparison Between Conventional and Supercritical Carbon Dioxide Methods for Fatty Acids Extraction

In previous chapters, we have obtained an understanding on the extraction of liposoluble constituents from Atlantic sea cucumber viscera using conventional and $scCO_2$ methods. Comparing with the conventional methods, maximum efficiency of $scCO_2$ extraction under 55 ˚C of temperature, 35 MPa of pressure, 50 min of dynamic extraction time, and 2:1 of co-solvent to biomass ratio achieved 97.7% of fatty acids content resulted from the *in-situ* transesterification. In order to gain deeper insights on $scCO_2$ extraction of fatty acids from the studied subjects, this chapter would compare $scCO_2$ technique with conventional methods.

7.1. Comparison of Lipids Content and Fatty Acids Profile

Both organic solvent and supercritical fluids have been used to extract lipids from solid matrices. In this study, sc-CO_2 extraction of liposoluble substances from sea cucumber viscera under the optimal condition (75 ˚C of temperature, 45 MPa of pressure, 30 min of dynamic extraction time, and 2:1 of co-solvent to biomass mass ratio) was compared with those obtained by conventional methods. The extraction efficiency of each method was demonstrated as yields of crude oils and fatty acids, shown in **Figure 7.1**. It was clear that the maximum crude oils yield was achieved using the conventional Bligh & Dyer method, but $scCO_2$ performed much better on the extraction of fatty acids comparing with the Bligh & Dyer method (P-value = 0.021 < 0.05). Although the mix solvents system of the Bligh & Dyer method possessed stronger solvating power and could extract more lipids, the fatty acids content of samples using the Bligh & Dyer method were not significantly higher than that obtained by the $scCO_2$ extraction method. That might be because of the co-extraction of other undesired compounds in the conventional extraction process. The addition of co-solvent is expected to enhance the polarity of $scCO_2$, and the higher diffusivity and lower viscosity of $scCO_2$ allowed for penetration of solvent more effectively. Therefore, more fatty acids yielded initially. Nonetheless, directly adding co-solvent to the extraction vessel, rather than using co-solvent pump to keep pumping ethanol, limited the effect of co-solvents on enhancing the solubility of polar lipids. As soon as ethanol was removed from the system, the extraction process would turn to $scCO_2$

extraction without co-solvent assistance (Eller & King, 1996). As such, such a $scCO_2$ method without using co-solvent pump may not be able to boost the extraction yields of the selected Omega-3 fatty acids comparing with conventional methods; but it still requires further examination on whether continuous pumping of co-solvents will enhance the extraction of the selected Omega-3 fatty acids.

Fatty acids contents achieved by *in-situ* transesterification were represented the average maximum value. In comparison of the average maximum fatty acids contents, we found the extraction efficiencies of fatty acids recovered by $scCO_2$ and the Bligh & Dyer were 88% and 78%, respectively; while the extraction efficiency of selected Omega-3 PUFAs was approximately 80% for both methods. Moreover, conventional methods required solvent separation and purification steps following the extraction whereas the $scCO_2$ extraction required only depressurization of the system to collect the extract. Therefore, the superiority of $scCO_2$ extraction over the conventional extraction technique was examined. In the context of $scCO_2$ extraction, developing more efficient pre-treatment (i.e., ultrasonic probe, microwave) may further enhance the lipid extraction and eliminate the need for the use of co-solvent.

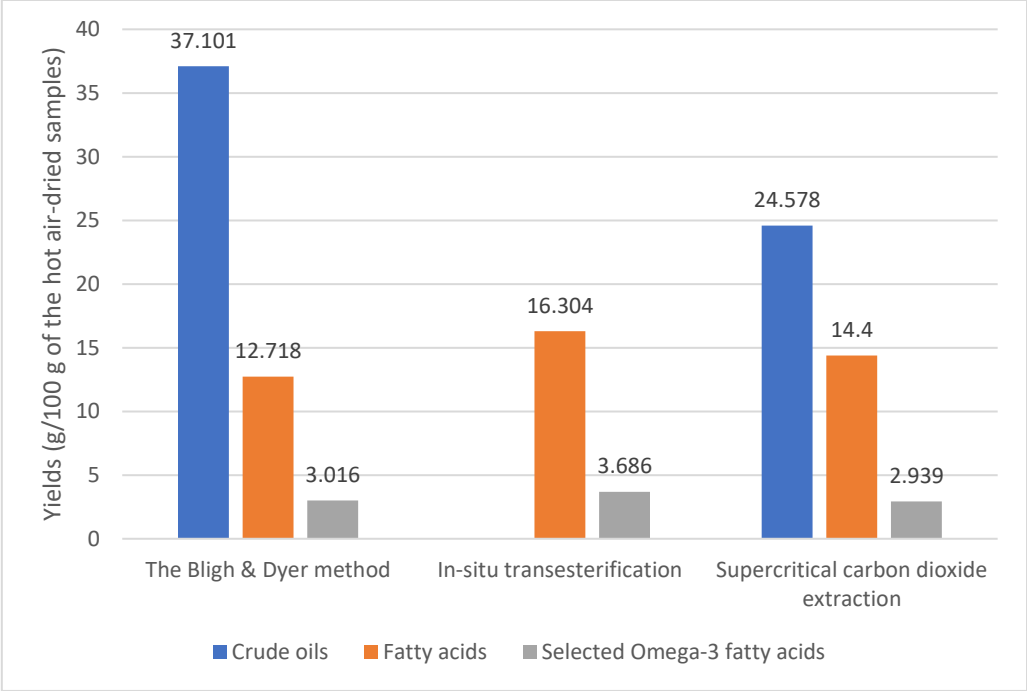


Figure 7.1 Crude oils yields and fatty acids yields of *Cucumaria frondosa* viscera (g/100 g of the hot air-dried samples) by different methods (scCO2 extraction under the optimal condition).

Table 7.1 exhibited the lipidic profiles of the extract found by each method, which was determined by GC-FID. We found MTA-12, EPA, and Palmitoleic acid were the predominant fatty acids for all the extracts, but $scCO_2$ showed higher selectivity to MTA-12, instead of our target compounds Omega-3 PUFAs. Also, negligible differences were observed when the fatty acid composition of scCO2 extracted lipids was compared to that extracted by the conventional methods.

Table 7.1 Fatty acids composition (g/100 of the total fatty acids) of extracted lipids using different methods.

Type	Isomer	Systematic name	*In-situ*	Bligh & Dyer	scCO2
Saturated	C14:0	Myristic acid	1.869±0.046	1.744±0.009	1.923±0.053
	C15:0	Pentadecylic acid	2.544±0.049	2.476±0.002	2.751±0.022
	C16:0	Palmitic acid	1.433±0.021	1.443±0.003	1.522±0.061
	C17:0	Heptadecanoic acid	0.297±0.021	0.267±0.012	0.283±0.008
	C18:0	Stearic acid	2.803±0.035	2.990±0.017	2.709±0.058
	C20:0	Arachidic acid	0.500±0.011	0.531±0.011	0.472±0.077
	C21:0	Henicosanoic acid	0.192±0.005	0.196±0.003	0.158±0.014
	C22:0	Behenic acid	0.527±0.022	0.619±0.011	0.500±0.014
Total			10.165	10.266	10.319
Monounsaturated	C16:1n7	Palmitoleic acid	16.845±0.293	16.514±0.061	17.821±0.046
	C17:1	10-Heptadecenoic acid	0.446±0.015	0.438±0.004	0.474±0.004
	C18:1n9 cis	Oleic acid	1.305±0.013	1.302±0.005	1.332±0.024
	C18:1n7	Vaccenic acid	2.899±0.030	2.903±0.002	2.898±0.045
	C20:1n9	11-Eicosenoic acid	0.544±0.006	0.544±0.014	0.495±0.009
	C22:1n9	Erucic acid	0.53±0.034	0.502±0.017	0.463±0.013
	C24:1n9	Nervonic acid	1.727±0.061	1.857±0.036	1.631±0.016
Total			24.297	24.061	25.114
Polyunsaturated	C16:2n4	Hexadecadienoic acid	1.014±0.049	0.928±0.035	0.981±0.020
	C18:2n6 trans	Linolelaidic acid	0.134±0.002	0.119±0.001	0.126±0.000
	C18:2n6 cis	Linoleic acid	0.276±0.004	0.279±0.000	0.276±0.005
	C18:2n4	11,14-Octadecadienoic acid	0.473±0.005	0.469±0.002	0.468±0.008
	C20:2n6	11,14-eicosadienoic acid	0.283±0.006	0.269±0.001	0.202±0.012
	C22:2n6	13,16-Docosadienoic acid	0.138±0.006	0.112±0.004	0.118±0.010
	C16:3n4	6,9,12-hexadecatrienoic acid	0.437±0.016	0.418±0.006	0.433±0.005
	C18:3n4	8,11,14-octadecatrienoic acid	0.098±0.001	0.101±0.010	0.096±0.001
	C18:3n3	α-Linolenic acid (ALA)	0.146±0.002	0.121±0.023	0.066±0.004
	C16:4n1	6,9,12,15-hexadecatetraenoic acid	1.257±0.021	1.223±0.005	1.260±0.014
	C18:4n3	Stearidonic acid	0.891±0.050	0.836±0.003	0.860±0.050
	C20:4n6	Arachidonic acid	0.598±0.008	0.571±0.002	0.474±0.076
	C20:4n3	Eicosatetraenoic acid (ETA)	0.297±0.008	0.278±0.010	0.192±0.006
	C20:5n3	Eicosapentaenoic acid (EPA)	21.735±0.254	22.486±0.043	20.142±0.355
	C21:5n3	Heneicosapentaenoic acid (HPA)	0.366±0.006	0.393±0.031	0.642±0.014
	C22:5n3	Docosapentaenoic acid (DPA)	0.284±0.006	0.278±0.004	0.213±0.008
	C22:6n3	Docosahexaenoic acid (DHA)	0.876±0.028	1.231±0.015	0.902±0.021
Total of Omega-3			24.594	25.623	23.018
Total of Omega-6			1.429	1.350	1.196
Total			29.302	30.112	27.451
Branched	4,8,12-Me-13:0	4,8,12-Trimethyltridecanoic acid	2.576±0.048	2.475±0.005	2.767±0.041
	Me-14:0 isomer 1	Methyltridecanoic acid	0.581±0.012	0.466±0.002	0.526±0.008
	Me-14:0 isomer 2		0.411±0.010	0.414±0.023	0.445±0.007
	Me-14:0 isomer 3		0.382±0.008	0.377±0.001	0.406±0.003
	i-16:0	14-methylpentadecanoic acid	4.399±0.071	4.349±0.010	4.783±0.035
	i-17:0	15-methylhexadecanoic acid	0.379±0.030	0.289±0.008	0.271±0.001
	ai-15:0	12-methyltetradecanoic acid	22.062±0.470	21.346±0.029	23.782±0.317
	ai-17:0	14-methylhexadecanoic acid	0.823±0.025	0.866±0.024	0.916±0.009
Total			31.614	30.582	33.896
Others *			5.062	4.980	3.220

**Note: Others refer to fatty acid isomers presenting in sample GC chromatograph other than external standards, which were suggested through GC-MS and NIST library.*

7.2. Conclusion

This chapter briefly compared the extraction yield and lipidic profiles of the Bligh & Dyer and $scCO_2$ extraction under the optimal condition. It revealed the conventional Bligh & Dyer method extracted one and a half times more of crude oils compared with $scCO_2$ extraction. However, $scCO_2$ extracted significantly more fatty acids at 14.400 g/100 g of the hot air-dried samples, while the Bligh & Dyer method yielded 12.718 g of fatty acids/100 g of the hot air-dried samples. The yields of selected Omega-3 PUFAs obtained from those two methods was comparable. Also, the fatty acids composition was not altered by different methods used.

Chapter 8: Investigation of The Effects of Pre-Treatments on Fatty Acids Yields

Sample matrix pre-treatment prior to extraction is regarded as an important factor for lipids extraction, as it can help the disruption of lipids-rich cells and then promotes the mass transfer of lipids through cell membranes (Crampon et al., 2013; Taher et al., 2014). In this chapter, effects of pre-treatments (hot air drying and freeze drying) on sample appearance, fatty acids yields and composition, moisture content, and morphology and microstructure, were investigated. In continuation to the previous chapter, the $scCO_2$ extraction of samples pre-treated by different drying methods was conducted in duplicate under the optimal condition suggested in Chapter 6: 75 ˚C of temperature, 45 MPa of pressure, 30 min of dynamic extraction time, and 2:1 of co-solvent to biomass mass ratio.

8.1. Moisture Content of Hot Air-Dried Samples and Freeze-Dried Samples

The moisture content of hot air-dried samples and freeze-dried samples was determined using AOAC method (AOAC International (AOAC), 2006). The moisture content of hot air-dried and freeze-dried samples were (6.445±0.027) % and (2.646±0.116) %, respectively. The results were comparable with those provided by Liu and her coworkers (Liu et al., 2021). Although freeze-dried method is more efficient for moisture removal, the cost associated with equipment purchase and energy consumption is a barrier for its commercial application. **Figure 8.1** shows the appearance differences of those samples. *Cucumaria frondosa* viscera dried under hot air appeared dark brown color and maintained relatively complete particles or formed clusters; whereas freeze-dried viscera demonstrated orange color and was broken into small pieces during drying process increasing the surface area. Therefore, freeze-dried samples containing less moisture might be due to the larger surface area accelerating water evaporation.

(a) Hot air-dried samples before grinding.

(b) Ground hot air-dried samples (left) and ground freeze-dried samples (right).

Figure 8.1 Pre-treated *Cucumaria frondosa* viscera: (a) hot air-dried samples before grinding, (b) ground hot air-dried samples and ground freeze-dried samples.

8.2. Effects of Pre-Treatments on Fatty Acids and Selected Omega-3 Fatty Acids Yields

Lipids of freeze-dried and air-dried samples were extracted under the optimal scCO2 extraction condition in duplicate; the extracts were transesterified using methanolic HCl method and analyzed by GC-FID. **Table 8.1** presents the yields of fatty acids and selected Omega-3 fatty acids. The table summarized the results of separate variance 2-sample t-test that freeze-dried samples yielded significantly more fatty acids (P-

value=0.035<0.05); however, there was not sufficient evidence that yields of selected Omega-3 fatty acids for each sample were different (P-value=0.054>0.05). Selected Omega-3 yields of samples with different pre-treatments was likely on the edge of significance due to the error result from multiple experimental treatments (i.e., $scCO_2$ extraction, transesterification, GC analysis). On the other hand, the $scCO_2$ extraction of freeze-dried samples produced more fatty acids compared with conventional extraction of hot air-dried samples ((17.427±0.201) g/100 g of samples on dry weight basis compared with (13.594±0.795) g/100 g of samples on dry weight basis). Because conventional organic solvents performed better on the recovery of fatty acids from hot air-dried samples (P-value of separate variance 2-sample t-test = 0.001 < 0.05), which means conventional solvents extraction may recover more fatty acids comparing with $scCO_2$ extraction in this study. As such, it might still suggest that freeze-dried samples are preferable to hot air-dried samples in the practice of fatty acids recovery. The lower moisture content of freeze-fried sample compared with the hot air-dried sample indicated that freeze-drying may have been more effective in cell wall disruption, resulting in enhanced scCO2 penetration within the biomass and subsequent improved fatty acid extraction from freeze-dried samples.

Crampon et al. (2013) found that $scCO_2$ extraction of neutral lipids (triacylglycerols and wax esters all contain fatty acids in their structures) from freeze-dried microalgae *Nannochloropsis oculate* was more efficient than conventional extraction. Different pre-treatments methods did not change the fatty acids profile of microalgae *Nannochloropsis oculate*; conversely, it was observed the $scCO_2$ extraction of the hot air-dried samples yielded more than 5 g of triglycerides and free fatty acid in every 100 g of the global extracts comparing with the $scCO_2$ extraction of freeze-dried samples (Crampon et al., 2013). Taher et al. (2014) reported that comparing with other lipid classes significantly more triglycerides were extracted from microalgae *Scenedesmus sp.* using $scCO_2$, but sun drying (70.4 g/100 of the global extracts) and freeze drying (73.0 g/100 of the global extracts) did not dramatically alter triglycerides yields (Taher et al., 2014).

Table 8.1 Experimental yields (g/100 g of samples on dry weight basis) of fatty acids and selected Omega-3 under the proposed optimal condition of $scCO_2$ extraction (n=2, Mean±SD).

Pre-treatment	Fatty acids yields	Selected Omega-3 yields
Hot air dry	15.392±0.113 [a]	3.145±0.078 [a]
Freeze dry	21.824±0.495 [b]	4.535±0.148 [a]

Note: Values in the same column with different letters are significantly different at $P < 0.05$. Dry weight basis means the moisture portion should be subtracted from the original sample weight.

8.3. Fatty Acids Composition of Hot Air-Dried Samples and Freeze-Dried Samples

It has been shown that *Cucumaria frondosa* viscera are rich sources of selected Omega-3 fatty acids (EPA and DHA). Therefore, the investigation of pre-treatment and extraction methods to retain the value-added compounds is of crucial importance.

Fatty acids profiles of hot air-dried samples and freeze-dried samples were detailed in **Table 8.2**. Branched fatty acids accounted for the highest portion (33.896 g/100 g of the total fatty acids and 34.536 g/100 g of the total fatty acids), followed by polyunsaturated fatty acids (27.451 g/100 g of the total fatty acids and 27.114 g/100 g of the total fatty acids), monounsaturated fatty acids (25.114 g/100 g of the total fatty acids and 27.077 g/100 g of the total fatty acids), and saturated fatty acids (10.319 g/100 g of the total fatty acids and 8.879 g/100 g of the total fatty acids). The content of Omega-3 fatty acids was around 23 g/100 g of the total fatty acids and Omega-6 fatty acids was about 1 g/100 g of the total fatty acids. Besides, 12-methyltetradecanoic acid (23.782 g/100 g of the total fatty acids & 25.985 g/100 g of the total fatty acids), EPA (20.142 g/100 g of the total fatty acids & 20.181 g/100 g of the total fatty acids), and palmitoleic acid (17.821 g/100 g of the total fatty acids & 20.275 g/100 g of the total fatty acids) were the predominant fatty acids in those two samples suffering different pre-treatments. EPA composition for both pre-treatments are almost the same, so pre-treatment methods have no influence on EPA profile. Although the overall fatty acids profile for those two scenarios was slightly different – samples dried under hot air yielded more saturated fatty acids and less monosaturated fatty

acids – generally they have very similar lipidic profile. Therefore, pre-treatment methods do not significantly affect the fatty acids composition. Comparable conclusion has been drawn by Crampon et al. (2013).

The high level of EPA promotes *Cucumaria frondosa* viscera to be valorized as Omega-3 functional products. The ratio of Omega-6/Omega-3 is 0.0519 and 0.0447 for those two samples, respectively, which is significantly lower than recommended ratio in our daily diet (1-5:1). Therefore, those two pre-treatment methods are applicable in processing *Cucumaria frondosa* viscera into Omega-3 nutritional supplements to balance the excessive intake of Omega-6 fatty acids (15-20:1) (Calder, 2015; Simopoulos, 2008).

However, due to the high equipment acquisition cost and electricity fee as well as the relatively complex operation requirements of industry-scaled freeze dryer, current industrial practice prefers to use hot air drying in the pre-treatment stage. According to the comparison above, it is evident that hot air-drying works well in sea cucumber processing plants that mainly focus on the production of Omega-3 fatty acids enriched nutraceuticals.

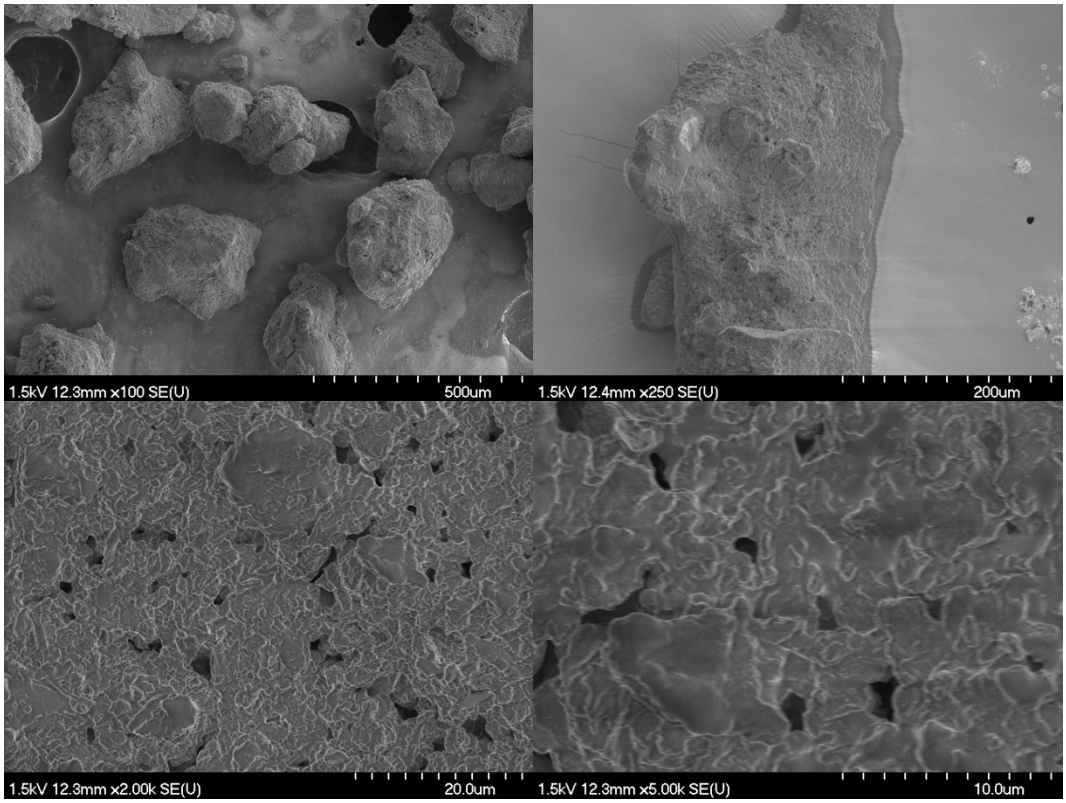
Table 8.2 Comparison of fatty acids composition (g/100 g of the total fatty acids) of *Cucumaria frondosa* viscera with different pre-treatments after supercritical fluid extraction.

Type	Isomer	Systematic name	Hot air dry	Freeze dry
Saturated	C14:0	Myristic acid	1.923±0.053	2.111±0.079
	C15:0	Pentadecylic acid	2.751±0.022	2.287±0.043
	C16:0	Palmitic acid	1.522±0.061	1.520±0.005
	C17:0	Heptadecanoic acid	0.283±0.008	0.148±0.004
	C18:0	Stearic acid	2.709±0.058	1.959±0.040
	C20:0	Arachidic acid	0.472±0.077	0.338±0.012
	C21:0	Henicosanoic acid	0.158±0.014	0.151±0.008
	C22:0	Behenic acid	0.500±0.014	0.361±0.029
Total			10.319	8.876
Monounsaturated	C16:1n7	Palmitoleic acid	17.821±0.046	20.275±0.267
	C17:1	10-Heptadecenoic acid	0.474±0.004	0.435±0.002
	C18:1n9 cis	Oleic acid	1.332±0.024	1.487±0.018
	C18:1n7	Vaccenic acid	2.898±0.045	2.711±0.042
	C20:1n9	11-Eicosenoic acid	0.495±0.009	0.519±0.031
	C22:1n9	Erucic acid	0.463±0.013	0.408±0.027
	C24:1n9	Nervonic acid	1.631±0.016	1.242±0.131
Total			25.114	27.077
Polyunsaturated	C16:2n4	Hexadecadienoic acid	0.981±0.020	0.778±0.003
	C18:2n6 trans	Linolelaidic acid	0.126±0.000	0.071±0.003
	C18:2n6 cis	Linoleic acid	0.276±0.005	0.278±0.008
	C18:2n4	11,14-Octadecadienoic acid	0.468±0.008	0.370±0.035
	C20:2n6	11,14-eicosadienoic acid	0.202±0.012	0.175±0.012
	C22:2n6	13,16-Docosadienoic acid	0.118±0.010	0.174±0.022
	C16:3n4	6,9,12-hexadecatrienoic acid	0.433±0.005	0.441±0.008
	C18:3n4	8,11,14-octadecatrienoic acid	0.096±0.001	0.099±0.006
	C18:3n3	α-Linolenic acid (ALA)	0.066±0.004	0.066±0.002
	C16:4n1	6,9,12,15-hexadecatetraenoic acid	1.260±0.014	1.450±0.028
	C18:4n3	Stearidonic acid	0.860±0.050	0.720±0.004
	C20:4n6	Arachidonic acid	0.474±0.076	0.328±0.029
	C20:4n3	Eicosatetraenoic acid (ETA)	0.192±0.006	0.187±0.010
	C20:5n3	Eicosapentaenoic acid (EPA)	20.142±0.355	20.181±0.553
	C21:5n3	Heneicosapentaenoic acid (HPA)	0.642±0.014	0.699±0.027
	C22:5n3	Docosapentaenoic acid (DPA)	0.213±0.008	0.165±0.020
	C22:6n3	Docosahexaenoic acid (DHA)	0.902±0.021	0.933±0.050
Total of Omega-3			23.018	22.951
Total of Omega-6			1.196	1.026
Total			27.451	27.114
Branched	4,8,12-Me-13:0	4,8,12-Trimethyltridecanoic acid	2.767±0.041	2.357±0.059
	Me-14:0 isomer 1		0.526±0.008	0.569±0.016
	Me-14:0 isomer 2	Methyltridecanoic acid	0.445±0.007	0.506±0.017
	Me-14:0 isomer 3		0.406±0.003	0.326±0.007
	i-16:0	14-methylpentadecanoic acid	4.783±0.035	3.786±0.051
	i-17:0	15-methylhexadecanoic acid	0.271±0.001	0.269±0.000
	ai-15:0	12-methyltetradecanoic acid	23.782±0.317	25.985±0.693
	ai-17:0	14-methylhexadecanoic acid	0.916±0.009	0.739±0.005
Total			33.896	34.536
Others *			3.220	2.391

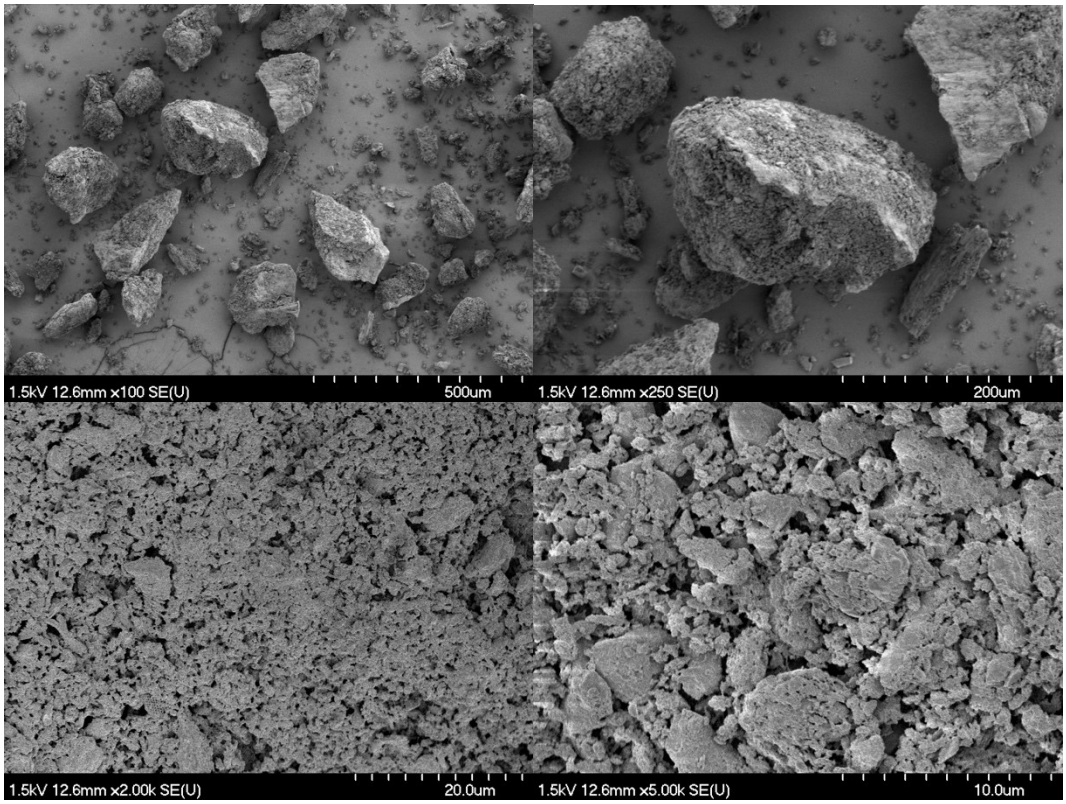
**Note: Others refer to fatty acid isomers presenting in sample GC chromatograph other than external standards, which were suggested through GC-MS and NIST library.*

8.4. Morphology of Hot Air-Dried Samples and Freeze-Dried Samples

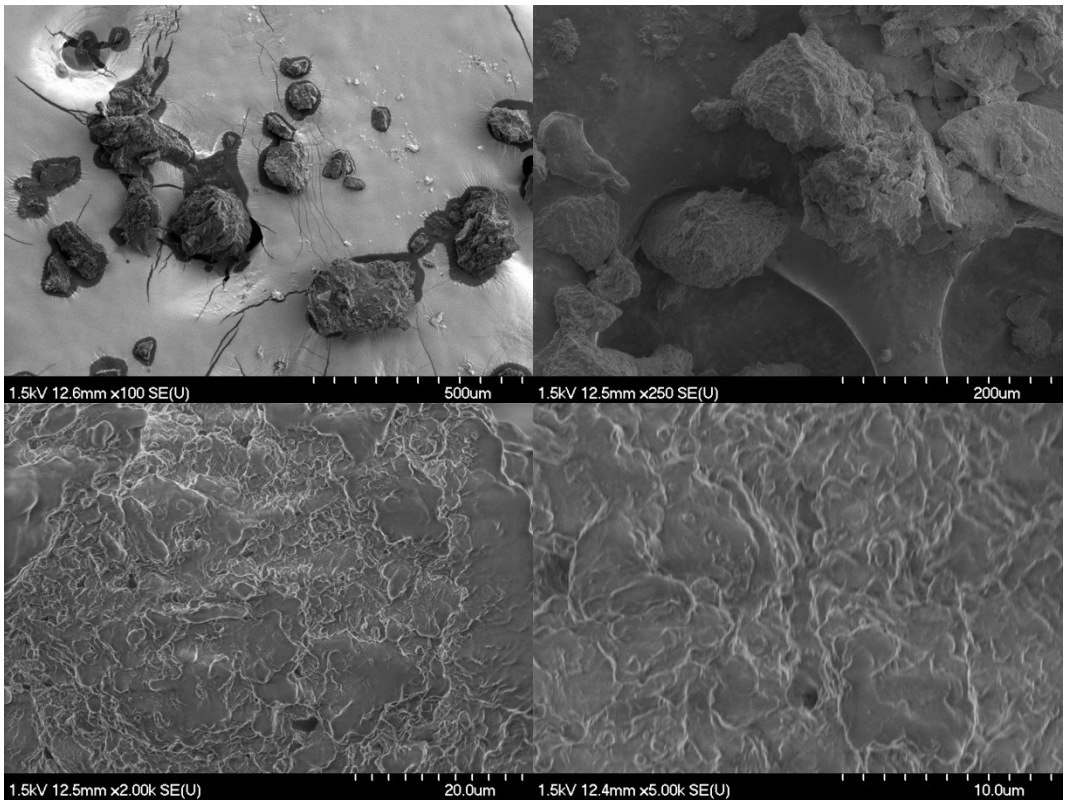
In order to obtain better understanding on the effects of the pre-treatment methods, namely hot-air dried samples and freeze-dried samples before and after supercritical fluid extraction were analyzed using SEM. Morphological images of samples dried under hot air before extraction, samples dried under hot air after extraction, freeze-dried samples before extraction, and freeze-dried samples after extraction are depicted in **Figure 8.2**. The comparison between structures of samples before extraction and those after extraction showed samples were smoother before extraction while cracks and visible pores appeared after extraction. This may be attributed to revealing apart from lipids within particles significant amount of free lipids attached in the surface of particles. The structure change was mainly due to the mechanical disruption caused by $scCO_2$. 500 um-scaled images of samples after extraction exhibited smaller particles – which was not found in the same scaled images of samples before extraction. Also, moisture inside samples, vaporized as a result of pressurization and depressurization, may have made the sample more susceptible for disruption.



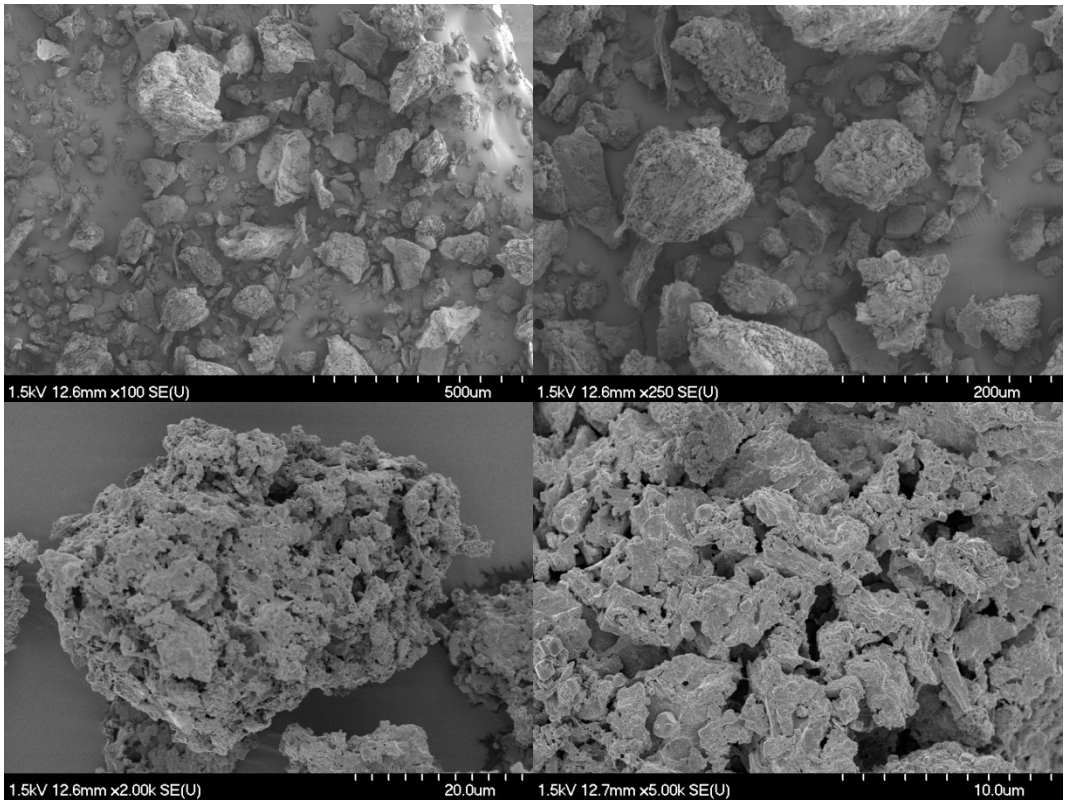
(a) Hot air-dried samples before $scCO_2$ extraction



(b) Hot air-dried samples after $scCO_2$ extraction



(c) Freeze-dried samples before $scCO_2$ extraction



(d) Freeze-dried samples after $scCO_2$ extraction

Figure 8.2 Morphological images of (a) hot air-dried samples before supercritical fluid extraction, (b) hot air-dried samples after supercritical fluid extraction, (c) freeze-dried samples before supercritical fluid extraction, and (d) freeze-dried samples after supercritical fluid extraction.

On the other hand, the contrast between hot air-dried samples and freeze-dried samples, especially in **Figure 8.2** (a) vs. (c) and (b) vs. (d) at 20 and 10 um, presented more obvious dents/concavities and wrinkles on the surface of freeze-dried samples before extraction and more apparent fractures and pores on freeze-dried samples after extraction. This indicates that freeze drying led to more alteration of biomass surface compared with hot air-drying method. As such, sample rigidity was stronger for hot air-dried samples but weaker for freeze-dried samples because of the low drying temperature of hot air drying and the freeze-thaw cycle of freeze drying (Moayyedi et al., 2018). Taher et al. (2014) also found the surface structure of samples were highly cracked and altered by freeze drying pre-treatment in comparison with sun-dried samples (Taher et al., 2014).

Additionally, lower moisture content achieved in freeze drying may be an indication of better performance of freeze drying compared with hot air drying in biomass disruption. The appearance of freeze-dried sample and air-dried samples is different as shown in **Figure 8.1**. It was also observed that freeze-dried samples were easier to be broken than hot air-dried samples in the grinding process. Furthermore, the more obvious microstructures on freeze-dried samples increased the surface area. Taher et al. (2014) discovered the surface are of freeze-dried samples was larger (Taher et al., 2014). Besides, their lower rigidity made them simpler to be broken into smaller particles under the stream of $scCO_2$, leading to larger surface area and then further increasing the contact with extraction solvents. Therefore, freeze-drying improved the mass transfer within samples while simultaneously $scCO_2$ could penetrate freeze-dried sample matrix more sufficiently, enhancing lipid dissolution, and hence, the fatty acids yields.

8.5. Conclusion

In this chapter, two different pre-treatments (hot air drying and freeze drying) was explored to evaluate whether such a difference could alter fatty acids and selected Omegra-3 fatty acids yields as well as their composition. It was found that freeze-dried samples contained lower moisture and appeared more attractive orange color. Comparing with hot air-dried samples, significantly different yields of fatty acids but insignificant difference on selected Omega-3 fatty acid yields were detected in freeze-dried samples. Also, different pre-treatments only had marginal effects on fatty acids profile. Through comparing the SEM images of those two types of samples before and after extraction, some microstructure differences, like dents/concavities, fractures, wrinkles, and pores, were observed, indicating that the larger surface area and the weaker rigidity of freeze-dried samples help the penetration of $scCO_2$ recovering more fatty acids. However, the insignificantly different yields of selected Omega-3 fatty acids, the similar Omega-6/Omega-3 ratio, and the cost of freeze drying, still suggest that it is not necessary to switch to freeze drying for fatty acids extraction at the industrial level.

Chapter 9: Conclusions and Recommendations for Future Work

9.1. Overall Conclusion

In this study, extraction of lipids from *Cucumaria frondosa* viscera using $scCO_2$ was presented and discussed, in which, the promising extraction technique was examined to be technically feasible. The effects of process variables on product yields and the optimal conditions for maximum yields was investigated. Moreover, comparison studies between $scCO_2$ and conventional methods and on different pre-treatments were conducted. Specific conclusions include:

1. The ultrasonic-assisted *in-situ* transesterification resulted in fatty acid content of 16.304 g/100 g of the hot air-dried samples and selected Omega-3 fatty acid content of 3.686 g/100 g of the hot air-dried samples, which was higher compared with two-step extraction followed by transesterification and *in-situ* transesterification. In addition, EPA was reported to be the predominant fatty acids in *Cucumaria frondosa* viscera, and lower Omega-6/Omega-3 ratio was obtained, proving *Cucumaria frondosa* viscera are potential natural sources for the development of Omega-3 enriched nutraceuticals. Based on the application as nutrition supplement materials, we analyzed the fatty acids content other than the crude oil content to describe the lipidic profile of *Cucumaria frondosa* viscera.
2. The effects of process variables including temperature (35-75 ˚C), pressure (20-50 MPa), dynamic extraction time (30-70 min), and mass ratio of co-solvent to biomass (0-2:1) were investigated. A response surface design was used with the purpose of optimizing the process of $scCO_2$ extraction of fatty acids from *Cucumaria frondosa* viscera. Experimental extraction condition including temperature of 55 ˚C, pressure of 35 MPa, dynamic time of 50 min, and co-solvent to biomass ratio of 2:1 yielded a maximum amount of fatty acids with recovery efficiency of 97.67 %, while the highest selected Omega-3 fatty acids yields with a recovery efficiency of 82.404% was obtained when the processing variables were set as 75 ˚C of temperature, 35 MPa of pressure, 50 min of dynamic extraction time, and 1:1 of mass ratio of co-solvent to biomass.

3. In the optimization, 75 ˚C of temperature, 45 MPa of pressure, 30 min of dynamic extraction time and 2:1 of co-solvent to biomass mass ratio was suggested by software, with experimental fatty acids and selected Omega-3 yields at 14.400 g/100 g of the hot air-dried samples and 2.939 g/100 g of the hot air-dried samples, respectively. The experimental yields all fell in the 95% of predict interval, indicating the generated regression models were applicable. Moreover, an optimum condition while setting the co-solvent ratio as 0 (75 ˚C of temperature, 44 MPa of pressure, 41 min of dynamic extraction time) was also assessed, but its experimental results were significantly lower. Therefore, $scCO_2$ extraction under the co-solvent involved optimal condition is suggested regarding the product yields.
4. Through the comparison between conventional and $scCO_2$ methods, it was reported the Bligh & Dyer method extracted significantly more crude oils due to the co-extraction of some untargeted compounds, but $scCO_2$ extraction showed higher selectivity to fatty acids. Those two methods yielded comparable selected Omega PUFAs and fatty acids composition.
5. Freeze drying was examined to be the most effective pre-treatment method with higher fatty acids yields and lower moisture content, but different pre-treatments did not alter yields of selected Omega-3 fatty acids significantly and only had marginal impacts on fatty acids profiles. Also, comparable Omega-6/Omega-3 ratios were obtained from samples with different pre-treatments. Thus, it is not necessary to use industrial freeze dryers in sea cucumber processing plants for fatty acids extraction.

9.2. Study Contribution

This study aims to use the green technology, $scCO_2$ extraction, to recover fatty acids from *Cucumaria frondosa* viscera. The current state of knowledge in the extraction of value-added compounds from marine sources mainly focuses on using conventional methods which involves the use of organic solvents. Hence, the developed process is a greener alternative to conventional solvent extraction methods, which helped in eliminating the use of toxic organic solvents and addressed the concern over the presence of residual organic solvents in pharmaceutical products.

On the other hand, the current practice of preparing sea cucumber for the food market often relies on cutting/trimming and removing internal organs during processing, resulting in a significant amount of sea cucumber by-products containing valuable compounds that ends up in the landfill. This project aiming at "turning waste to treasure" enhances the sustainability of sea cucumber processing procedures.

9.3. Future Work

Apart from fatty acids, *Cucumaria frondosa* viscera also contain various valuable compounds that have yet to be explored. Therefore, the following aspects will be the focus of future work:

1. $ScCO_2$ extraction has been proven a promising technique to recover value-added compounds from biomass in recent decades. This study employed this technique to extract liposoluble constituents from *Cucumaria frondosa* viscera, but there was no further action being taken for the biomass residues. Hossain et al. (2020) reviewed the bioactive compounds in *Cucumaria frondosa*, pointing out the presence of polysaccharides and saponins, which could be incorporated with fatty acids to develop potential pharmaceuticals. Also, polysaccharides have attracted increasing interests as nature polymers to design hydrogel materials (Bhattarai et al., 2010; S.-C. Chen et al., 2004; Essawy et al., 2016). Therefore, it is highly recommended that to apply supercritical fluid technology to sequentially extract pharmaceutically important compounds from *Cucumaria frondosa* viscera through changing $scCO_2$ density, adding co-solvents, or using pre-treatments. The proposed integrated process for sequential extraction and fractionation of *Cucumaria frondosa* viscera can make comprehensive utilization of biomass.
2. The lipids-extracted biomass can be further processed to analyze its nutritional profiles so that we can know its sustainability as a source of other value-added compounds. That will be done as a foundation prior to integrated sequential extraction.
3. As mentioned before, the application of fatty acids, polysaccharides, and saponins from *Cucumaria frondosa* viscera brings more opportunities and vast prospects. The innovative materials, hydrogels, designed from polysaccharides,

are also regarded as a potential candidate in drug delivery due to the biocompatibility, low toxicity, and biodegradability of these natural polymers. Thus, the future work can extend to use the extracted polysaccharide to develop hydrogel-based controlled drug delivery platform for novel therapeutic compounds, like a combination of fatty acids and saponins derived from sea cucumbers which has been proven a synergistic effect in alleviating obesity-related insulin resistance and orotic acid-induced symptoms (Guo et al., 2018b). Notably, that will be the first attempt in pharmaceutical industries to use sulphated polysaccharides to design hydrogel for controlled drug delivery.

4. Although this study is regarded as innovative and forward-looking, it still suffers some limitations that should be noticed in future study. Firstly, this study used the ratio of internal standard methyl C19:0 concentration and its peak area to quantify the amount of select fatty acids. It was not an accurate measurement, as such, it is necessary to prepare solutions with different concentration gradients using use individual EPA and DHA to quantify our target compounds more accurately with established calibration curves. Secondly, it will be better to incorporate a test for curvature using 2k factorial design following the single factor test in the preliminary screening, because single factor experiments ignore the interactive effects between variables, which may lead to incorrect results.
5. Regarding of using SFE at the industrial scale to extract fatty acids from *Cucumaria frondosa* viscera, although this study evaluated the advantages of this technique over conventional methods from the perspective of extraction yields, it still requires a further evaluation based on life cycle assessment and techno-economic assessment for deeper insights.

References

Abbas, K., Mohamed, A., Abdulamir, A., & Abas, H. (2008). A review on supercritical fluid extraction as new analytical method. *American Journal of Biochemistry and Biotechnology*, *4*(4), 345–353.

Abuzaytoun, R. (2017). *Seasonal variation of lipid and carotenoid content of Cucumaria frondosa viscera oil and the development of methods for its quality control* [Dissertation for PhD]. Dalhousie University.

Anisuzzaman, M., Jin, F., Kabery, K., Jeong, U., Jung, H.-C., Lee, S.-R., & Kang, S.-J. (2019). Lipid Class and Fatty Acid Compositions of Dried Sea Cucumber Apostichopus japonicus. *The Open Food Science Journal*, *11*(1).

AOAC International (AOAC). (2006). *Official methods of analysis*.

Attoub, S., Arafat, K., Gélaude, A., Al Sultan, M. A., Bracke, M., Collin, P., Takahashi, T., Adrian, T. E., & De Wever, O. (2013). Frondoside a suppressive effects on lung cancer survival, tumor growth, angiogenesis, invasion, and metastasis. *PLoS One*, *8*(1).

Bahrami, Y., Zhang, W., & Franco, C. (2014). Discovery of novel saponins from the viscera of the sea cucumber Holothuria lessoni. *Marine Drugs*, *12*(5), 2633–2667.

Barzkar, N., Attaran Fariman, G., & Taheri, A. (2017). Proximate composition and mineral contents in the body wall of two species of sea cucumber from Oman Sea. *Environmental Science and Pollution Research*, *24*(23), 18907–18911. https://doi.org/10.1007/s11356-017-9379-5

Bhattarai, N., Gunn, J., & Zhang, M. (2010). Chitosan-based hydrogels for controlled, localized drug delivery. *Advanced Drug Delivery Reviews*, *62*(1), 83–99.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917.

Bonilla-Méndez, J. R., Hoyos-Concha, J. L., Bonilla-Méndez, J. R., & Hoyos-Concha, J. L. (2018). Methods of extraction refining and concentration of fish oil as a source of omega-3 fatty acids. *Ciencia y Tecnología Agropecuaria*, *19*(3), 645–668. https://doi.org/10.21930/rcta.vol19_num2_art:684

Bordbar, S., Anwar, F., & Saari, N. (2011). High-value components and bioactives from sea cucumbers for functional foods—A review. *Marine Drugs*, *9*(10), 1761–1805.

Breil, C., Abert Vian, M., Zemb, T., Kunz, W., & Chemat, F. (2017). “Bligh and Dyer” and Folch methods for solid–liquid–liquid extraction of lipids from microorganisms. Comprehension of solvatation mechanisms and towards substitution with alternative solvents. *International Journal of Molecular Sciences*, *18*(4), 708.

Budisa, N., & Schulze-Makuch, D. (2014). Supercritical carbon dioxide and its potential as a life-sustaining solvent in a planetary environment. *Life*, *4*(3), 331–340.

Budzinski, P., Maimeun, M., Mutrakulcharoen, P., Wonganu, B., & Sriariyanun, M. (2020). *Profiling Analysis of Fatty Acids and Collagens Obtained from Sea Cucumbers*. *141*, 03006.

Calder, P. C. (2015). Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1851*(4), 469–484.

Caudell, T. (1999). *Practical supercritical fluid chromatography and extraction* (Vol. 2). CRC Press.

Cavonius, L. R., Carlsson, N.-G., & Undeland, I. (2014). Quantification of total fatty acids in microalgae: Comparison of extraction and transesterification methods. *Analytical and Bioanalytical Chemistry*, *406*(28), 7313–7322. https://doi.org/10.1007/s00216-014-8155-3

Charest, D., Balaban, M., Marshall, M., & Cornell, J. (2001). Astaxanthin extraction from crawfish shells by supercritical CO2 with ethanol as cosolvent. *Journal of Aquatic Food Product Technology*, *10*(3), 81–96.

Chemat, F., Abert-Vian, M., Fabiano-Tixier, A. S., Strube, J., Uhlenbrock, L., Gunjevic, V., & Cravotto, G. (2019). Green extraction of natural products. Origins, current status, and future challenges. *TrAC Trends in Analytical Chemistry*, *118*, 248–263. https://doi.org/10.1016/j.trac.2019.05.037

Chen, J., Lovatelli, A., Conand, C., Purcell, S., Uthicke, S., Hamel, J., & Mercier, A. (2005). Present status and prospects of sea cucumber industry in China. *Ile*, *1*, 25–38.

Chen, S.-C., Wu, Y.-C., Mi, F.-L., Lin, Y.-H., Yu, L.-C., & Sung, H.-W. (2004). A novel pH-sensitive hydrogel composed of N, O-carboxymethyl chitosan and alginate

cross-linked by genipin for protein drug delivery. *Journal of Controlled Release*, *96*(2), 285–300.

Cheng, C.-H., Du, T.-B., Pi, H.-C., Jang, S.-M., Lin, Y.-H., & Lee, H.-T. (2011). Comparative study of lipid extraction from microalgae by organic solvent and supercritical CO2. *Bioresource Technology*, *102*(21), 10151–10153. https://doi.org/10.1016/j.biortech.2011.08.064

Cheung, P. C. K., Leung, A. Y. H., & Ang, P. O. (1998). Comparison of Supercritical Carbon Dioxide and Soxhlet Extraction of Lipids from a Brown Seaweed, Sargassum hemiphyllum (Turn.) C. Ag. *Journal of Agricultural and Food Chemistry*, *46*(10), 4228–4232. https://doi.org/10.1021/jf980346h

Christian, J. R., Grant, C., Meade, J. D., & Noble, L. (2010). *Habitat requirements and life history characteristics of selected marine invertebrate species occurring in the Newfoundland and Labrador region*. Fisheries and Oceans Canada Ottawa.

Christie, W. W. (1982). *Lipid analysis: Isolation, separation, identification, and structural analysis of lipids* (2nd ed..). Oxford, Oxfordshire, England.

Collin, P. D., Yang, P., & Newman, R. (2003). *Methods and compositions for treating lipoxygenase-mediated disease states* (Patent No. 6541519). https://www.freepatentsonline.com/6541519.html

Conand, C., & Byrne, M. (1993). A Review of Recent Developments in the World Sea Cucumber Fisheries. *Marine Fisheries Review*, *55*(4), 1.

Crampon, C., Mouahid, A., Toudji, S.-A. A., Lépine, O., & Badens, E. (2013). Influence of pretreatment on supercritical CO2 extraction from Nannochloropsis oculata. *The Journal of Supercritical Fluids*, *79*, 337–344.

Dave, D., Ramakrishnan, V. V., Trenholm, S., Manuel, H., Pohling, J., & Murphy, W. (2014). Marine oils as potential feedstock for biodiesel production: Physicochemical characterization. *Journal of Bioprocessing & Biotechniques*, *4*(5), 1.

Dickey, L., Teter, B., Sampugna, J., & III, L. (2002). Comparison of a Direct Transesterification Method and the Bligh and Dyer Method to Determine Fatty Acid Content in Striped Bass Tissues and Diet. *North American Journal of*

Aquaculture, *64*, 158–163. https://doi.org/10.1577/1548-8454(2002)064<0158:COADTM>2.0.CO;2

Duan, X., Zhang, M., Mujumdar, A. S., & Wang, S. (2010). Microwave freeze drying of sea cucumber (Stichopus japonicus). *Journal of Food Engineering*, *96*(4), 491–497.

Egerrs, R. (1996). *Supercritical Fluid Extraction of Oilseeds/Lipids in Natural Products in Supercritical Fluid Technology in Oil and Lipid Chemistry*.

Eller, F. J., & King, J. W. (1996). *Determination of fat content in foods by analytical SFE*. *1*(14.5-I), 62.

Essawy, H. A., Ghazy, M. B., El-Hai, F. A., & Mohamed, M. F. (2016). Superabsorbent hydrogels via graft polymerization of acrylic acid from chitosan-cellulose hybrid and their potential in controlled release of soil nutrients. *International Journal of Biological Macromolecules*, *89*, 144–151.

Ewald, G., Bremle, G., & Karlsson, A. (1998). Differences between Bligh and Dyer and Soxhlet extractions of PCBs and lipids from fat and lean fish muscle: Implications for data evaluation. *Marine Pollution Bulletin*, *36*(3), 222–230. https://doi.org/10.1016/S0025-326X(97)00176-8

Félix-Valenzuela, L., Higuera-Ciapara, I., Goycoolea-Valencia, F., & Argüelles-Monal, W. (2001). Supercritical co2/ethanol extraction of astaxanthin from blue crab (Callinectes sapidus) shell waste. *Journal of Food Process Engineering*, *24*(2), 101–112. https://doi.org/10.1111/j.1745-4530.2001.tb00534.x

Ferdosh, S., Sarker, M. Z. I., Rahman, N. N. N. A., Akand, M. J. H., Ghafoor, K., Awang, M. B., & Kadir, M. O. A. (2013). Supercritical carbon dioxide extraction of oil from Thunnus tonggol head by optimization of process parameters using response surface methodology. *Korean Journal of Chemical Engineering*, *30*(7), 1466–1472. https://doi.org/10.1007/s11814-013-0070-3

Fiori, L., Solana, M., Tosi, P., Manfrini, M., Strim, C., & Guella, G. (2012). Lipid profiles of oil from trout (Oncorhynchus mykiss) heads, spines and viscera: Trout by-products as a possible source of omega-3 lipids? *Food Chemistry*, *134*(2), 1088–1095.

Folch, J., Lees, M., & Stanley, G. S. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry*, *226*(1), 497–509.

Food and Agriculture Organization of the United Stations. (2005). *FAO yearbook. Fishery statistics: Capture production, 2003*. FAO. http://www.fao.org/3/a-a1165t.pdf

Food and Agriculture Organization of the United Stations. (2017). *FAO Yearbook. Fishery and Aquaculture Statistics, 2015*. FAO. http://www.fao.org/3/a-i7989t.pdf

Gedi, M. A., Bakar, J., & Mariod, A. A. (2015). Optimization of supercritical carbon dioxide (CO 2) extraction of sardine (Sardinella lemuru Bleeker) oil using response surface methodology (RSM). *Grasas y Aceites*, *66*(2), 074. https://doi.org/10.3989/gya.0824142

Gianasi, B. L., Parrish, C. C., Hamel, J.-F., & Mercier, A. (2017). Influence of diet on growth, reproduction and lipid and fatty acid composition in the sea cucumber Cucumaria frondosa. *Aquaculture Research*, *48*(7), 3413–3432. https://doi.org/10.1111/are.13168

Gomes, A. R., Freitas, A. C., Rocha-Santos, T. A., & Duarte, A. C. (2014). Bioactive compounds derived from echinoderms. *Rsc Advances*, *4*(56), 29365–29382.

Grosso, C., Valentão, P., Ferreres, F., & Andrade, P. B. (2015). Alternative and efficient extraction methods for marine-derived compounds. *Marine Drugs*, *13*(5), 3182–3230.

Gudimova, E., Gudimov, A., & Collin, P. (2004). *A study of the biology for fishery in two populations of Cucumaria frondosa: In the Barents Sea (Russia) and in the Gulf of Maine (USA)*. 269–275.

Guo, Y., Han, X., Che, H., Li, Z., Dong, P., Xue, C., Zhang, T., & Wang, Y. (2018a). Synergistic effect of eicosapentaenoic acid-enriched phospholipids and sea cucumber saponin on orotic acid-induced non-alcoholic fatty liver disease in rats. *Royal Society Open Science*, *5*(7), 172182.

Guo, Y., Han, X., Che, H., Li, Z., Dong, P., Xue, C., Zhang, T., & Wang, Y. (2018b). Synergistic effect of eicosapentaenoic acid-enriched phospholipids and sea

cucumber saponin on orotic acid-induced non-alcoholic fatty liver disease in rats. *Royal Society Open Science*, *5*(7), 172182.

Hădărugă, D. I., Ünlüsayin, M., Gruia, A. T., Birău Mitroi, C., Rusu, G., & Hădărugă, N. G. (2016). Thermal and oxidative stability of Atlantic salmon oil (Salmo salar L.) and complexation with β-cyclodextrin. *Beilstein Journal of Organic Chemistry*, *12*, 179–191. PubMed. https://doi.org/10.3762/bjoc.12.20

Hajeb, P., Selamat, J., Afsah-Hejri, L., Mahyudin, N. A., Shakibazadeh, S., & Sarker, M. Z. I. (2015). Effect of Supercritical Fluid Extraction on the Reduction of Toxic Elements in Fish Oil Compared with Other Extraction Methods. *Journal of Food Protection*, *78*(1), 172–179. https://doi.org/10.4315/0362-028X.JFP-14-248

Hamel, J., & Mercier, A. (1996). Gonad morphology and gametogenesis of the sea cucumber Cucumaria frondosa. *SPC Beche-de-Mer Inf Bull*, *8*, 22–33.

Hamel, J., & Mercier, A. (2008). *Sea Cucumbers: A Global Review of Fisheries and Trade*.

Hamel, J.-F., & Mercier, A. (1998). Diet and feeding behaviour of the sea cucumber Cucumaria frondosa in the St. Lawrence estuary, eastern Canada. *Canadian Journal of Zoology*, *76*(6), 1194–1198.

Hao, S., Wei, Y., Li, L., Yang, X., Cen, J., Huang, H., Lin, W., & Yuan, X. (2015). The effects of different extraction methods on composition and storage stability of sturgeon oil. *Food Chemistry*, *173*, 274–282. https://doi.org/10.1016/j.foodchem.2014.09.154

Haq, M., Ahmed, R., Cho, Y.-J., & Chun, B.-S. (2017). Quality Properties and Bio-potentiality of Edible Oils from Atlantic Salmon By-products Extracted by Supercritial Carbon Dioxide and Conventional Methods. *Waste and Biomass Valorization*, *8*(6), 1953–1967. https://doi.org/10.1007/s12649-016-9710-2

Hara, A., & Radin, N. S. (1978). Lipid extraction of tissues with a low-toxicity solvent. *Analytical Biochemistry*, *90*(1), 420–426. https://doi.org/10.1016/0003-2697(78)90046-5

Hardardottir, I., & Kinsella, J. E. (1988). Extraction of Lipid and Cholesterol from Fish Muscle with Supercritical Fluids. *Journal of Food Science*, *53*(6), 1656–1658. https://doi.org/10.1111/j.1365-2621.1988.tb07808.x

Heckert, N. A., Filliben, J. J., Croarkin, C. M., Hembree, B., Guthrie, W. F., Tobias, P., & Prinz, J. (2012). *NIST/SEMATECH e-Handbook of Statistical Methods*. http://www.itl.nist.gov/div898/handbook/

Holtz, E. H., & MacDonald, B. A. (2009). Feeding behaviour of the sea cucumber Cucumaria frondosa (Echinodermata: Holothuroidea) in the laboratory and the field: Relationships between tentacle insertion rate, flow speed, and ingestion. *Marine Biology*, *156*(7), 1389–1398. https://doi.org/10.1007/s00227-009-1179-0

Hossain, A., Dave, D., & Shahidi, F. (2020). Northern Sea Cucumber (Cucumaria frondosa): A Potential Candidate for Functional Food, Nutraceutical, and Pharmaceutical Sector. *Marine Drugs*, *18*(5), 274. https://doi.org/10.3390/md18050274

Iverson, S. J., Lang, S. L. C., & Cooper, M. H. (2001). Comparison of the bligh and dyer and folch methods for total lipid determination in a broad range of marine tissue. *Lipids*, *36*(11), 1283–1287. https://doi.org/10.1007/s11745-001-0843-0

Janakiram, N. B., Mohammed, A., & Rao, C. V. (2015). Sea cucumbers metabolites as potent anti-cancer agents. *Marine Drugs*, *13*(5), 2909–2923.

Jiao, G., & Kermanshahi pour, A. (2018). Extraction of anthocyanins from haskap berry pulp using supercritical carbon dioxide: Influence of co-solvent composition and pretreatment. *LWT*, *98*, 237–244. https://doi.org/10.1016/j.lwt.2018.08.042

Lee, M.-H., Kim, Y.-K., Moon, H. S., Kim, K.-D., Kim, G.-G., Cho, H.-A., Yoon, N. Y., Sim, K. B., Park, H.-Y., Lee, D.-S., Lim, C.-W., Yoon, H.-D., & Han, S.-K. (2012). Comparison on proximate composition and nutritional profile of red and black sea cucumbers (Apostichopus japonicus) from Ulleungdo(Island) and Dokdo(Island), Korea. *Food Science and Biotechnology*, *21*(5), 1285–1291. https://doi.org/10.1007/s10068-012-0169-z

Létisse, M., Rozières, M., Hiol, A., Sergent, M., & Comeau, L. (2006). Enrichment of EPA and DHA from sardine by supercritical fluid extraction without organic modifier: I. Optimization of extraction conditions. *The Journal of Supercritical Fluids*, *38*(1), 27–36. https://doi.org/10.1016/j.supflu.2005.11.013

Li, H., Xu, Q., & Li, W. (2016). *A extraction process for sea cucumber oil* (Patent No. CN106479663A). https://patents.google.com/patent/CN106479663A/en

Li, Z., Fu, P., Yang, W., & Xu, Z. (2004). Study on the freeze-dry processing technology of Stichopus japonicus [J]. *Marine Tieheries*, *1*.

Liu, J., Lin, S., Wang, Z., Wang, C., Wang, E., Zhang, Y., & Liu, J. (2011). Supercritical fluid extraction of flavonoids from Maydis stigma and its nitrite-scavenging ability. *Food and Bioproducts Processing*, *89*(4), 333–339. https://doi.org/10.1016/j.fbp.2010.08.004

Liu, Y., Dave, D., Trenholm, S., Ramakrishnan, V. V., & Murphy, W. (2021). Effect of Drying on Nutritional Composition of Atlantic Sea Cucumber (Cucumaria frondosa) Viscera Derived from Newfoundland Fisheries. *Processes*, *9*(4), 703. https://doi.org/10.3390/pr9040703

Lopes, B. L., Sánchez-Camargo, A. P., Ferreira, A. L., Grimaldi, R., Paviani, L. C., & Cabral, F. A. (2012). Selectivity of supercritical carbon dioxide in the fractionation of fish oil with a lower content of EPA+ DHA. *The Journal of Supercritical Fluids*, *61*, 78–85.

Mamelona, J., Saint-Louis, R., & Pelletier, É. (2010). Proximate composition and nutritional profile of by-products from green urchin and Atlantic sea cucumber processing plants. *International Journal of Food Science & Technology*, *45*(10), 2119–2126. https://doi.org/10.1111/j.1365-2621.2010.02381.x

Manirakiza, P., Covaci, A., & Schepens, P. (2001). Comparative Study on Total Lipid Determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh & Dyer Extraction Methods. *Journal of Food Composition and Analysis*, *14*(1), 93–100. https://doi.org/10.1006/jfca.2000.0972

Moayyedi, M., Eskandari, M. H., Rad, A. H. E., Ziaee, E., Khodaparast, M. H. H., & Golmakani, M.-T. (2018). Effect of drying methods (electrospraying, freeze drying and spray drying) on survival and viability of microencapsulated Lactobacillus rhamnosus ATCC 7469. *Journal of Functional Foods*, *40*, 391–399. https://doi.org/10.1016/j.jff.2017.11.016

Montgomery, D. C. (2017). *Design and analysis of experiments*. John wiley & sons.

Nelson, E. J., MacDonald, B. A., & Robinson, S. M. C. (2012). A Review of the Northern Sea Cucumber Cucumaria frondosa (Gunnerus, 1767) as a Potential Aquaculture

Species. *Reviews in Fisheries Science*, *20*(4), 212–219. https://doi.org/10.1080/10641262.2012.719043

Nishanthan, G., Kumara, P. A. D. A., de Croos, M. D. S. T., Prasada, D. V. P., & Dissanayake, D. C. T. (2018). Effects of processing on proximate and fatty acid compositions of six commercial sea cucumber species of Sri Lanka. *Journal of Food Science and Technology*, *55*(5), 1933–1941. PubMed. https://doi.org/10.1007/s13197-018-3111-4

Parrish, C. C. (1999). Determination of Total Lipid, Lipid Classes, and Fatty Acids in Aquatic Samples. In M. T. Arts & B. C. Wainman (Eds.), *Lipids in Freshwater Ecosystems* (pp. 4–20). Springer. https://doi.org/10.1007/978-1-4612-0547-0_2

Patil, P. D., Dandamudi, K. P. R., Wang, J., Deng, Q., & Deng, S. (2018). Extraction of bio-oils from algae with supercritical carbon dioxide and co-solvents. *The Journal of Supercritical Fluids*, *135*, 60–68. https://doi.org/10.1016/j.supflu.2017.12.019

Ramalhosa, M. J., Paíga, P., Morais, S., Rui Alves, M., Delerue-Matos, C., & Oliveira, M. B. P. P. (2012). Lipid content of frozen fish: Comparison of different extraction methods and variability during freezing storage. *Food Chemistry*, *131*(1), 328–336. https://doi.org/10.1016/j.foodchem.2011.07.123

Ratti, C. (2001). Hot air and freeze-drying of high-value foods: A review. *Journal of Food Engineering*, *49*(4), 311–319.

Ren, X., Zhao, X., Turcotte, F., Deschênes, J.-S., Tremblay, R., & Jolicoeur, M. (2017). Current lipid extraction methods are significantly enhanced adding a water treatment step in Chlorella protothecoides. *Microbial Cell Factories*, *16*(1), 26–26. PubMed. https://doi.org/10.1186/s12934-017-0633-9

Rincón-Cervera, M. Á., Villarreal-Rubio, M. B., Valenzuela, R., & Valenzuela, A. (2017). Comparison of fatty acid profiles of dried and raw by-products from cultured and wild fishes. *European Journal of Lipid Science and Technology*, *119*(9), 1600516. https://doi.org/10.1002/ejlt.201600516

Rubio-Rodríguez, N., de Diego, S. M., Beltrán, S., Jaime, I., Sanz, M. T., & Rovira, J. (2012). Supercritical fluid extraction of fish oil from fish by-products: A comparison with other extraction methods. *Journal of Food Engineering*, *109*(2), 238–248. https://doi.org/10.1016/j.jfoodeng.2011.10.011

Rubio-Rodríguez, N., Sara, M., Beltrán, S., Jaime, I., Sanz, M. T., & Rovira, J. (2008). Supercritical fluid extraction of the omega-3 rich oil contained in hake (Merluccius capensis–Merluccius paradoxus) by-products: Study of the influence of process parameters on the extraction yield and oil quality. *The Journal of Supercritical Fluids*, *47*(2), 215–226.

Sánchez-Camargo, A. P., Martinez-Correa, H. A., Paviani, L. C., & Cabral, F. A. (2011). Supercritical CO2 extraction of lipids and astaxanthin from Brazilian redspotted shrimp waste (Farfantepenaeus paulensis). *The Journal of Supercritical Fluids*, *56*(2), 164–173.

Sánchez-Camargo, A. P., Meireles, M. Â. A., Ferreira, A. L. K., Saito, E., & Cabral, F. A. (2012). Extraction of ω-3 fatty acids and astaxanthin from Brazilian redspotted shrimp waste using supercritical CO2+ethanol mixtures. *The Journal of Supercritical Fluids*, *61*, 71–77. https://doi.org/10.1016/j.supflu.2011.09.017

Shao, J., & Jiao, J. (2009). *Sea cucumber oil product and method for preparing same* (Patent No. CN101530127A). https://patents.google.com/patent/CN101530127A/en

Sharif, K. M., Rahman, M. M., Azmir, J., Mohamed, A., Jahurul, M. H. A., Sahena, F., & Zaidul, I. S. M. (2014). Experimental design of supercritical fluid extraction – A review. *Journal of Food Engineering*, *124*, 105–116. https://doi.org/10.1016/j.jfoodeng.2013.10.003

Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine & Pharmacotherapy*, *56*(8), 365–379.

Simopoulos, A. P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Experimental Biology and Medicine*, *233*(6), 674–688.

Singh, R., MacDonald, B. A., Lawton, P., & Thomas, M. L. (1998). Feeding response of the dendrochirote sea cucumber Cucumaria frondosa (Echinodermata: Holothuroidea) to changing food concentrations in the laboratory. *Canadian Journal of Zoology*, *76*(10), 1842–1849.

Singh, R., MacDonald, B. A., Thomas, M. L., & Lawton, P. (1999). Patterns of seasonal and tidal feeding activity in the dendrochirote sea cucumber Cucumaria frondosa

(Echinodermata: Holothuroidea) in the Bay of Fundy, Canada. *Marine Ecology Progress Series*, *187*, 133–145.

Smedes, F. (1999). Determination of total lipid using non-chlorinated solvents. *Analyst*, *124*(11), 1711–1718. https://doi.org/10.1039/A905904K

Sun, J., Xu, Z., Dai, X., Sun, Z., Mou, J., liu, Y., & Ma, Y. (2017). *A supercritical fluid extraction method for a group of Glycosphingolipids (Cerebrosides) in sea cucumbers* (Patent No. CN106977562A). https://patents.google.com/patent/CN106977562A/en

Sun, Y., Ju, W., Tang, W., & Tang, X. (2017). *A method for lipids extraction from the internal organs of sea cucumbers* (Patent No. CN107418708A). https://patents.google.com/patent/CN107418708A/en

Taher, H., Al-Zuhair, S., Al-Marzouqi, A. H., Haik, Y., Farid, M., & Tariq, S. (2014). Supercritical carbon dioxide extraction of microalgae lipid: Process optimization and laboratory scale-up. *The Journal of Supercritical Fluids*, *86*, 57–66. https://doi.org/10.1016/j.supflu.2013.11.020

Therkildsen, N. O., & Petersen, C. W. (2006). A review of the emerging fishery for the sea cucumber Cucumaria frondosa: Biology, policy, and future prospects. *Beche-de-Mer*, 200616.

Treyvaud Amiguet, V., Kramp, K. L., Mao, J., McRae, C., Goulah, A., Kimpe, L. E., Blais, J. M., & Arnason, J. T. (2012). Supercritical carbon dioxide extraction of polyunsaturated fatty acids from Northern shrimp (Pandalus borealis Kreyer) processing by-products. *Food Chemistry*, *130*(4), 853–858. https://doi.org/10.1016/j.foodchem.2011.07.098

Wall, R., Ross, R. P., Fitzgerald, G. F., & Stanton, C. (2010). Fatty acids from fish: The anti-inflammatory potential of long-chain omega-3 fatty acids. *Nutrition Reviews*, *68*(5), 280–289.

Woźniak, Ł., Marszałek, K., Skąpska, S., & Jędrzejczak, R. (2017). The Application of Supercritical Carbon Dioxide and Ethanol for the Extraction of Phenolic Compounds from Chokeberry Pomace. *Applied Sciences*, *7*(4). https://doi.org/10.3390/app7040322

Yahyavi, M., Afkhami, M., Javadi, A., Ehsanpour, M., Khazaali, A., & Mokhlesi, A. (2012). Fatty acid composition in two sea cucumber species, Holothuria scabra and Holothuria leucospilata from Qeshm Island (Persian Gulf). *African Journal of Biotechnology*, *11*(12), 2862.

Yamaguchi, K., Murakami, M., Nakano, H., Konosu, S., Kokura, T., Yamamoto, H., Kosaka, M., & Hata, K. (1986). Supercritical carbon dioxide extraction of oils from Antarctic krill. *Journal of Agricultural and Food Chemistry*, *34*(5), 904–907.

Yun, X., Han, X., Nong, S., & Yang, H. (2006). Technological condition of vacuum freeze-drying in Stichopus japonicas. *Journal of Fishery Sciences of China*, *13*(4), 662–665.

Zhao, Y.-C., Xue, C.-H., Zhang, T.-T., & Wang, Y.-M. (2018). Saponins from Sea Cucumber and Their Biological Activities. *Journal of Agricultural and Food Chemistry*, *66*(28), 7222–7237. https://doi.org/10.1021/acs.jafc.8b01770

Zhong, Y., Khan, M. A., & Shahidi, F. (2007). Compositional Characteristics and Antioxidant Properties of Fresh and Processed Sea Cucumber (Cucumaria frondosa). *Journal of Agricultural and Food Chemistry*, *55*(4), 1188–1192. https://doi.org/10.1021/jf063085h

Zhu, B.-W., Qin, L., Zhou, D.-Y., Wu, H.-T., Wu, J., Yang, J.-F., Li, D.-M., Dong, X.-P., & Murata, Y. (2010). Extraction of lipid from sea urchin (Strongylocentrotus nudus) gonad by enzyme-assisted aqueous and supercritical carbon dioxide methods. *European Food Research and Technology*, *230*(5), 737–743. https://doi.org/10.1007/s00217-010-1216-8

Appendix

1. Identification of Peaks in External Standards

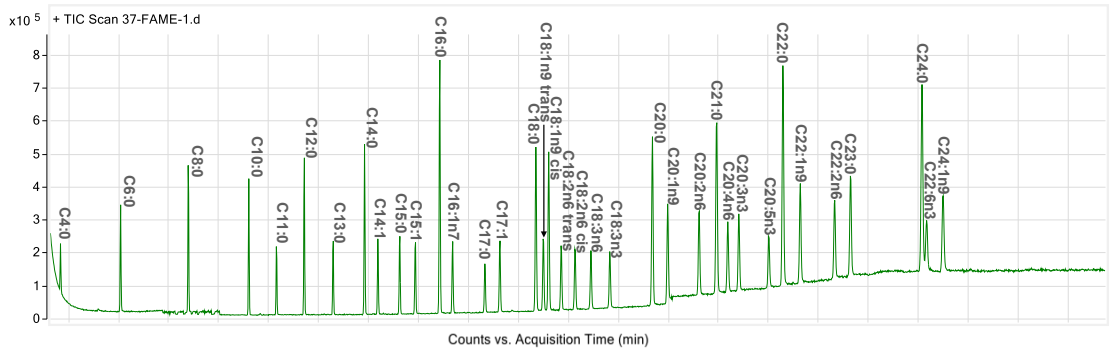


Figure A. 1 Order of peaks in 37 FAME mix.

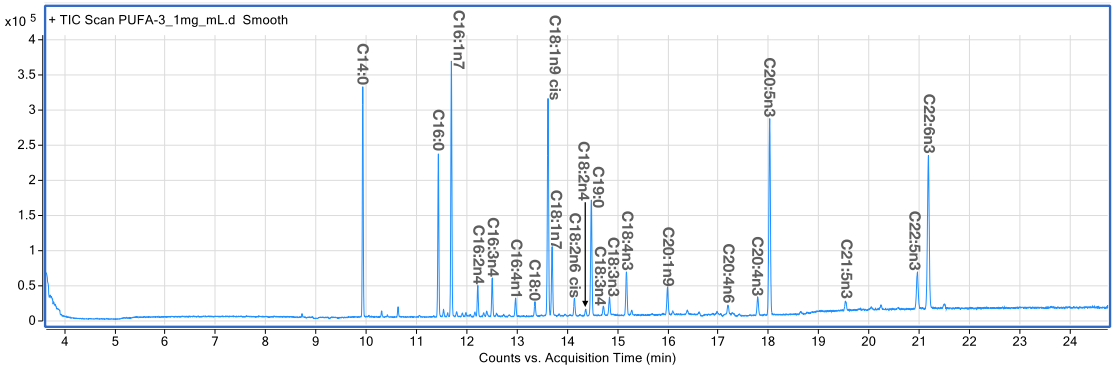
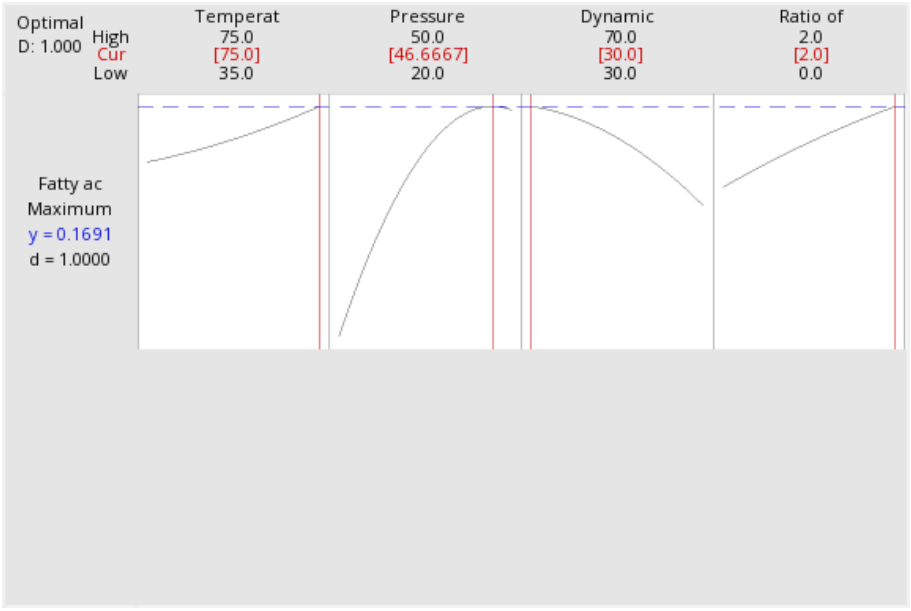
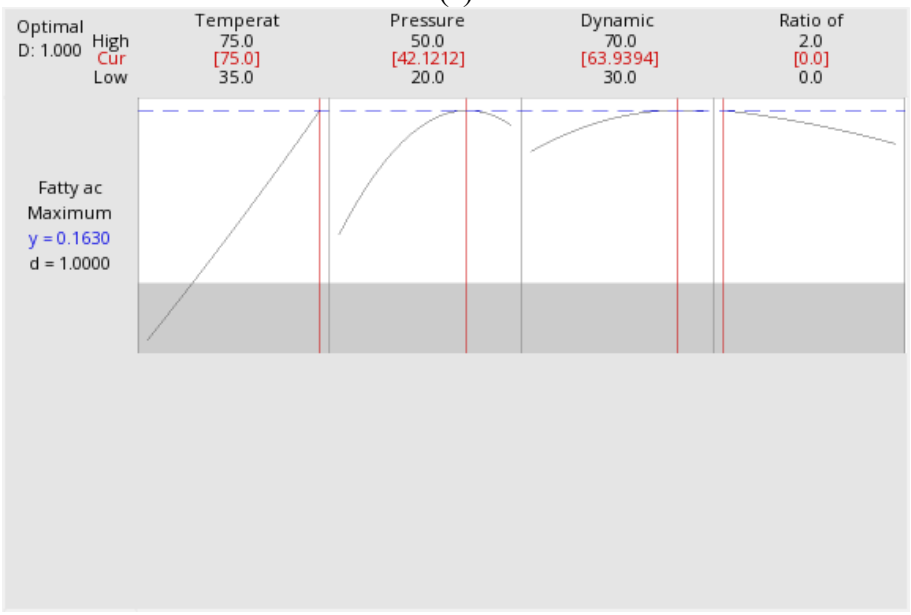


Figure A. 2 Order of peaks in PUFA NO.3 (including C19:0).

2. Optimization plots

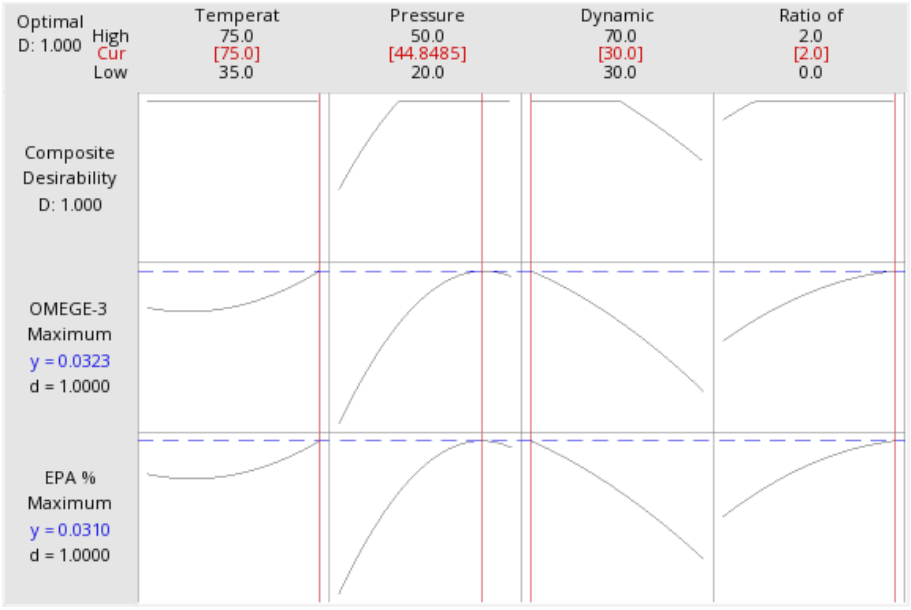


(a)

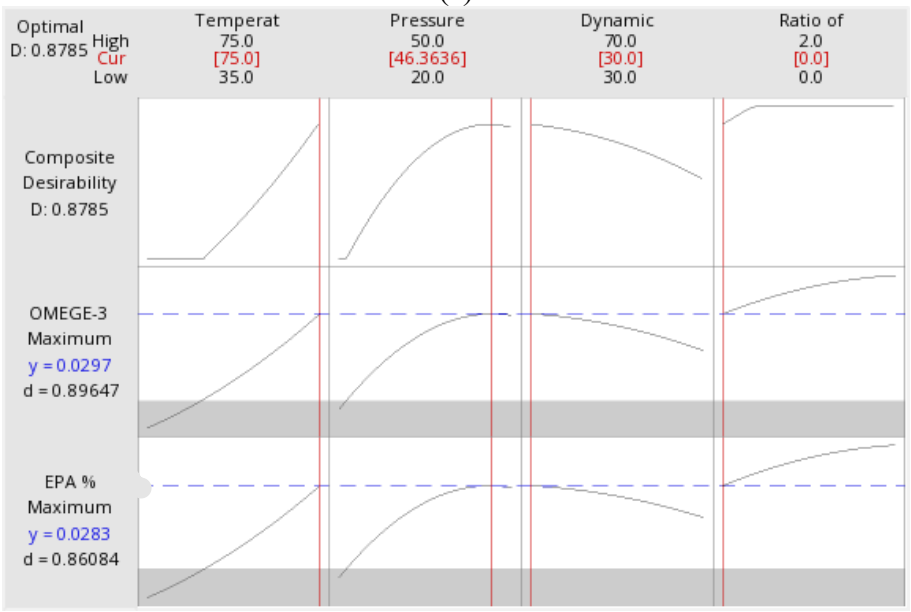


(b)

Figure A.3 Optimization plot for maximum fatty acid yields (g/g of the hot air-dried samples).

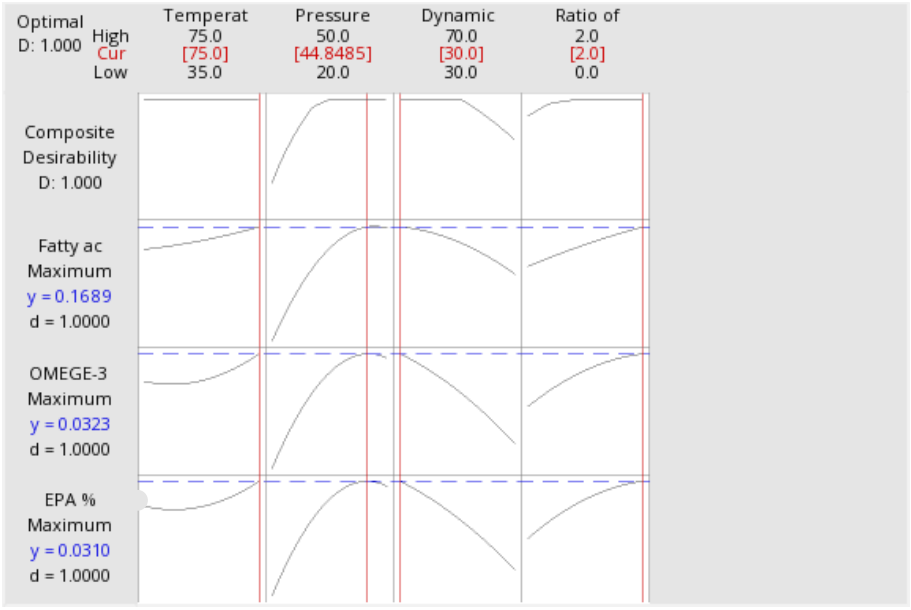


(a)

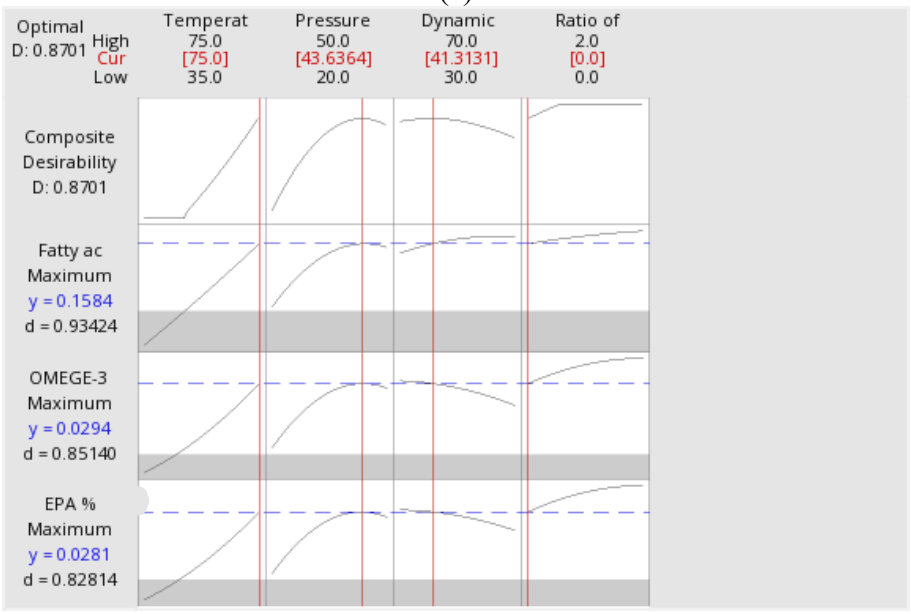


(b)

Figure A.4 Optimization plot for maximum EPA and selected Omega-3 fatty acids yields (g/g of the hot air-dried samples).



(a)



(b)

Figure A.5 Optimization plot for maximum all three responses: yields of FA, EPA, and DHA (g/g of the hot air-dried samples).