Shrimp oil extracted from shrimp processing by-product is a rich source of omega-3 fatty acids and astaxanthin-esters, and reveals potential anti-adipogenic effects in 3T3-L1 adipocytes

By

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ABSTRACT

The province of Newfoundland and Labrador, Canada, generates tons of shrimp processing by-product every year. Shrimp processing by-product is a valuable source of shrimp oil and astaxanthin (Astx), a potent antioxidant that exist in either free or esterified form (Astx-E). As part of this thesis, extraction of shrimp oil was carried out from shrimp processing by-product using various extraction methods. The shrimp oil extracted from shrimp processing by-product using Soxhlet method (hexane: acetone 2:3) showed that shrimp oil is rich in omega (n)-3 polyunsaturated fatty acids (PUFA), phospholipids and Astx-E. Shrimp oil was also extracted from shrimp processing by-product using waste fish oil as a solvent, and also fish waste. The oil extracts were rich in triacylglycerols (TAG), monounsaturated fatty acids (MUFA), followed by PUFA, and contained low amount of total carotenoids. The next objective of this thesis was to investigate the effects of shrimp extract and shrimp oil on fat accumulation in 3T3-L1 preadipocytes, and the mRNA expression of genes involved in adipogenesis and lipogenesis. I also investigated the effects of fish oil, and a combination of fish oil plus Astx-E on fat accumulation, and the mRNA expression of genes involved in adipogenesis and lipogenesis in 3T3-L1 adipocytes. My findings demonstrated that shrimp oil extracted from shrimp processing by-product using the Soxhlet method decreased fat accumulation, and reduced the mRNA expression of adipogenic and lipogenic genes, compared to untreated cells. However, fish oil and fish oil plus Astx-E increased fat accumulation, and the mRNA expression of adipogenic and lipogenic genes, compared to untreated cells. Thus, shrimp oil and fish oil appear to regulate adipogenesis and lipogenesis via independent pathways. Shrimp oil extracts using waste fish oil revealed similar effects on adipogenesis and lipogenesis as that of fish oil. Overall, my findings demonstrate that shrimp oil extracted from shrimp processing by-product using the Soxhlet

method contains a significant amount of phospholipids, n-3 PUFA and Astx-E, and inhibits fat accumulation in 3T3-L1 cells. Thus, it has the potential to elicit anti-adipogenic effects.

CO-AUTHORSHIP STATEMENT

A part of the work presented in Chapter-4 has been published (Phadtare, I., Vaidya, H., Hawboldt, K., & Cheema, S. K. (2021). Shrimp Oil Extracted from Shrimp Processing By-Product Is a Rich Source of Omega-3 Fatty Acids and Astaxanthin-Esters, and Reveals Potential Anti-Adipogenic Effects in 3T3-L1 Adipocytes. Marine drugs, 19(5): 259-277). I, Indrayani Phadtare, was involved in the design of the study, conducted the cell culture experiments, analyzed, and interpreted the data. Shrimp oil extraction from shrimp processing by-product were carried out using various extraction methods, and extraction procedures were conducted in collaboration with Dr. Kelly Hawboldt's Research Group (Process Engineering, Faculty of Engineering and Applied Science, Memorial University). Sara Ahmadkelayeh, a PhD student from Dr. Hawboldt's laboratory established the optimum Soxhlet extraction procedure, which I used to generate shrimp oil for my study. I also studied the oil extracts received from their laboratory using waste fish oil as a solvent.

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ABBREVIATIONS

ACC1	Acetyl-CoA carboxylase
ADD1	Adipocyte determination and differentiation-dependent factor 1
Astx	Astaxanthin
Astx-E	Esterified astaxanthin
C/EBP	CCAAT enhancer-binding protein
ChREBP	Carbohydrate response element-binding protein
CPT1	Carnitine palmitoyltransferase 1
CREB	cAMP response element-binding protein
Dex	Dexamethasone
DGAT2	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethylsulphoxide
EPA	Eicosapentaenoic acid
FAS	Fatty acid synthase
FATP1	Fatty acid transport protein 1
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFA	Free fatty acids
FO	Fish oil
FO+Astx-E	Fish oil plus Astx-E

Free Astx	Free Astaxanthin
FOS	Fish oil extracted from waste fish that was then used as a solvent for shrimp oil extraction
Glut-4	Glucose transporter type 4
GRAS	Generally recognized as safe
H. pluvialis	Haematococcus pluvialis
HMG-CoA reductase	3-hydroxy-3-methylglutaryl CoA reductase
IBMX	Isobutylmethylxanthine
IGF	Insulin-like growth factor
LXR	Liver-X receptor
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
MUFA	Monounsaturated fatty acids
n-3	Omega-3
NCS	Newborn calf serum
NEFA	Non-esterified fatty acids
PBS	Phosphate-buffered saline
PC	L-α-phosphatidylcholine
PDI	Polydispersity index
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PPRE	Peroxisome proliferator response elements
PV	peroxidation value
RXR	Retinoid X receptor

SCD1	Stearoyl-CoA desaturase
SE	Shrimp extract
SFA	Saturated fatty acid
SO	Shrimp oil
SREBP	Sterol regulatory element binding protein
TAG	Triacylglycerol
TC	Total cholesterol
TLC	Thin layer chromatography
TLC-FID	TLC-flame ionization detection
VLDL	Very-low-density lipoprotein

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CHAPTER ONE

Introduction and overview

1.0 Introduction

1.1 Marine derived nutraceuticals in health

Obesity is a chronic medical condition and a major public health concern that is increasing in prevalence throughout most parts of the world. One in four adult Canadians, or about 6.3 million people, were obese in 2011-2012 (Statistics, Canada, 2015). In 2018, 26.8% of Canadians 18 and older (roughly 7.3 million adults) were reported as obese (Statistics, Canada, 2019). In fact, Newfoundland and Labrador has the highest rate of obesity in all of Canada (Statistics Canada, 2019). For example, the proportion of individuals aged 18 and older who were obese in Newfoundland and Labrador was determined to be 40.2%, considerably higher than the national average (26.8%) (Statistics Canada, 2019). Individuals with obesity are at a greater risk of chronic diseases such as cardiovascular diseases, dyslipidemia, hypertension, and diabetes (Hasani-Ranjbar et al., 2013; Fakhri et al., 2018; Manivasagan et al., 2018). Lifestyle patterns such as diet and physical activity play a major role in obesity through change of adiposity and insulin resistance. Alternatively, dietary modifications such as supplementation with nutraceuticals show great impact on the health of the population. Marine sources have gained a considerable interest from researchers for their health benefits. Marine-derived bioactive molecules are highly attractive as nutraceuticals in the food and supplement industries. Hippocrates, the "father of modern medicine", mentioned the importance and the therapeutic effects of marine invertebrates and their components on human health (Voultsiadou Eleni, 2010). Almost 40% of global fisheries are comprised of marine invertebrates. Seafoods acquired from these sources are highly credited for health benefits due to the presence of high amounts of polyunsaturated fatty acids, especially omega (n)-3 polyunsaturated fatty acids (n-3 PUFA), peptides, and other bioactive molecules such as carotenoids (Børresen, 2009). Fish is one of the most studied marine species for its various nutritive qualities and health benefits (Hosomi et al., 2012). Fish oil intake has been shown to improve insulin resistance (Lombardo et al., 2006; Albert et al., 2014), an effect that is attributed to its n-3 PUFA (Ramel et al., 2008; Larsen et al., 2011; Swanson et al., 2012). Another popular marine source

that is extensively harvested since the early 1960s is the cold-water shrimp found in the North Atlantic and Pacific areas, called Northern shrimp (*Pandalus borealis*). A recent study stated that Northern shrimp oil contains a high content of n-3 PUFA, and is rich in antioxidants (Subramanian et al., 2015), with a potential to provide health benefits (Vincent et al., 2009). Majority of the lipids in shrimp are present in the head and tail; however, these are commonly discarded as a waste material at shrimp processing units. The lack of information and data towards the nutritional value and health potential, along with the lack of regulation of ocean discharge for processing plant, leads to shrimp heads and tails be discarded as waste by processing units of the industry.

1.2 Shrimp processing by-product utilization for bioactives in health

Since 2010, the total global capture stated for the Northern shrimp species is between 315,511 - 446,909 tons (Jiao et al., 2015). Since the early 1960s, the Canadian fishery has been harvesting Northern shrimp (Dave and Routray, 2018). It is reported that a major concern in Newfoundland and Labrador has been the waste generated from seafood processing plants (Cull, 2000). In fact, shrimp accounted for the biggest part (30%) of approximately 240 thousand tons of total seafood that landed in 2014. Commercially, shrimp are also separated by size, and the small size that does not meet the selling scale gets discarded. More than 40% (w/w) of the shrimp is usually discarded and not used for commercial applications, and disposed of as solid shrimp processing by-product (Gildberg et al., 2001). Note that this shrimp-processing by-product mostly consists of head and tails. Usually, processing plants carry out the separation of flesh from the remaining body parts of shrimp. These body parts such as shrimp shells and heads represent around 45-60% of the whole shrimp, based on the species and processing method (Sachindra and Mahendrakar, 2005; Saini and Keum, 2018). These shrimp parts are generally dumped on ocean, thereby resulting in increased organic load on the marine environment, as well as a loss of value in the by-products. Although some of this waste can be utilized for extracting value-added products, there is still excess being discarded as processing effluents (Jamieson et al., 2013).

The discarded shrimp processing by-product is a rich source of value-added products, such as novel bioactives. For example, shrimp processing waste can be a significant source of high-value bioactive molecules that includes proteins, chitins, lipids, minerals, and different carotenoids such as astaxanthin, β-carotene, lutein, and canthaxanthin (De Holanda and Netto, 2006). Shrimp processing by-product usually comprises of 14-30 Wt.% chitin, 35 Wt.% minerals (mainly calcium carbonate), 33-40 Wt.% protein, 0.3-4% lipids (Kurita, 2006; Jiao et al., 2015; Ahmadkelayeh and Hawboldt, 2020; Ahmmed et al., 2020). Shrimp is also a good source of a carotenoid called astaxanthin that varies from 10 to 40 mg/Kg of wet shrimp processing by-product (Rødde et al., 2008; Sachindra and Mahendrakar, 2011; Parjikolaei et al., 2015). Evidently, the composition of shrimp varies with respect to the type of shrimp processing by-product, process of extraction, and season of capture/harvesting. These valuable components from shrimp processing by-product have been suggested to have an extensive application likely in the food, cosmetics, aquaculture, nutraceutical, and pharmaceutical industries.

1.2.1 Lipid composition of shrimp

The shrimp oil extracted as a byproduct from Northern shrimp processing waste was found to be rich in n-3 PUFA, and has a bright red color appearance due to the presence of astaxanthin, a carotenoid (Jiao et al., 2015; Nair et al., 2017). The majority of the lipids from marine oils are either composed of triacylglycerols (TAG) or phospholipids. For example, krill oil is primarily phospholipids, whereas fish oil is almost exclusively in the form of TAG (Vognild et al., 1998; Gigliotti et al., 2011; Hals et al., 2017). Previous studies have shown that Northern shrimp lipids are mainly in the form of phospholipids (60-80% of total lipids), followed by TAG (5%–15% of total lipids), and also contains other classes of lipids such as wax esters, sterols, hydrocarbons, alcohols (Ouellet et al., 1995; Vognild et al., 1998; Adeyeye, 2017). Phospholipids are principal components of all cell membranes, and play an important role in delivering n-3 PUFA to vital tissues. Phospholipid-bound n-3 PUFA such as eicosapentaenoic acid (EPA; C20:5n3) and docosahexaenoic acid (DHA; C22:6n3) are suggested to be better absorbed due to better bioavailability (Ramprasath et al., 2015).

1.2.2 Carotenoids in shrimp

Shrimp is a rich source of carotenoids, which are strong antioxidants. Shrimp consists of various carotenoids such as lutein, canthaxanthin, β-carotene, zeaxanthin and astaxanthin (Dalei et al., 2015). Amongst all other carotenoids astaxanthin, a xanthophyll carotenoid is predominant in shrimp (Ambati et al., 2014).

1.2.2.1 Astaxanthin

Astaxanthin (Astx) is primarily synthesized by microalgae; it accumulates in zooplankton and subsequently becomes a part of marine species such as shrimp, salmon, crab, and crustaceans (Hussein et al., 2006). Astx imparts a deep reddish coloration to shrimp, lobster, salmon, and trout. These marine species survive on the Astx-containing planktons and microalgae in marine environment (Hussein et al., 2006). The single-celled green alga called Haematococcus pluvialis (*H. pluvialis*) is a natural source of Astx and has the highest capacity to accumulate Astx (Boussiba, 2000).

1.2.2.1.1 Structure, chemistry, and antioxidant activity of astaxanthin

Astx (3,3'-dihydroxy-beta, beta-carotene-4,4'-dione) has a unique structure compared to other carotenoids due to the presence of hydroxyl and keto groups at both ends. This unique chemical structure brings distinctive features, such as the ability to be esterified, and a more polar configuration compared to other carotenoids (Higuera-Ciapara et al., 2006) (Fig. 1.1). Astx exists in either free form or conjugated with protein or esterified with one or two fatty acids i.e., monoester or diester form, which stabilizes the molecule (Hussein et al., 2006). Astx contains conjugated double bonds, hydroxy and keto moieties, attributing to both lipophilic and hydrophilic properties (Higuera-Ciapara et al., 2006). These conjugated double bond polyene chains give a strong antioxidant property, due to the ability to donate electrons and react with free radicals to convert into more stable products, hence terminating free radical chain reaction in a wide variety of living organisms (Guerin et al., 2003). Astx is a potent biological antioxidant, suggested to have 10 times higher antioxidant potential than other carotenoids such as zeaxanthin, lutein, canthaxanthin, β-carotene, and Astx is almost 100 times more potent

antioxidant than vitamin E and C (Ambati et al., 2014). Astx has the ability to incorporate effectively due to its unique structure in cell membrane in an *in vitro* membrane model (Pashkow et al., 2008; Ambati et al., 2014). Also, the polar ends of Astx can react with phospholipid head groups and scavenge radicals from the surface or in the interior of the cell membrane; this property contributes to a better biological activity of Astx compared to other carotenoids, such as lycopene and β-carotene (Goto et al., 2001). Moreover, it has been reported that Astx maintains cell membrane integrity and reduces lipid peroxidation, however lutein and β-carotene disturb the membrane structure and elevates the lipid hydroperoxides levels in an *in vitro* membrane model (McNulty et al., 2007).

Among the two forms of Astx, free and esterified (Astx-E); it has been reported that Astx-E show higher biological antioxidant activity compared to free Astx in human umbilical vein endothelial cells (HUVEC) (Régnier et al., 2015). In the context of multifunctional role of Astx, Rao et al. (Rao et al., 2013) compared the antitumor effect of Astx-E and free Astx in a rat model of skin cancer and stated higher antioxidant activity of the Astx-E compared to free Astx. In another related study, Aoi et al. (Aoi et al., 2018) studied the effects of the different forms of astaxanthin on endurance in mice, and the relationship of energy metabolism and oxidative damage with *in vivo* distribution of astaxanthin after administration to mice. They found that Astx-E promoted energy production, specifically 5'-adenosine monophosphate-activated protein kinase (AMPK) levels and protected mitochondria of skeletal muscle from oxidative damage during exercise, given that reactive oxygen species (ROS) are generated in the body during exercise and can cause oxidative stress. The intake of Astx-E significantly increased AMPK levels and exhibited a longer running time owing to the activation of energy metabolism. Authors suggested that this is likely attributed to the difference in the absorption between non-esterified and esterified forms of Astx. These studies show the importance of Astx-E and the potential to exert health beneficial effects.

Although Astx has been shown to induce health benefits, pure Astx is highly prone to oxidation. Several factors impact the oxidation of Astx, such as the presence of oxygen, light, and temperature (Takeungwongtrakul and Benjakul, 2016). On the other hand, Astx is more stable when extracted in oils, which also prevents the oxidation of oils. Moreover, Astx is highly lipophilic, thus it is not highly bioavailable when used alone. Similar to fat-soluble vitamins, Astx is maximally absorbed if consumed with dietary fat. The bioavailability of Astx is increased by more than three-fold when administered as a lipid-base formulation (Odeberg et al., 2003). In human subjects, it has been reported that bioavailability of Astx increases when made available in lipid-based formulation composed of lipophilic glycerol monooleate, dioleate and an emulsifier polysorbate 80, which caused an increase in Astx absorption (Odeberg et al., 2003). Astx is a fat-soluble compound, with enhanced absorption when delivered with dietary oils (Clark et al., 2000).

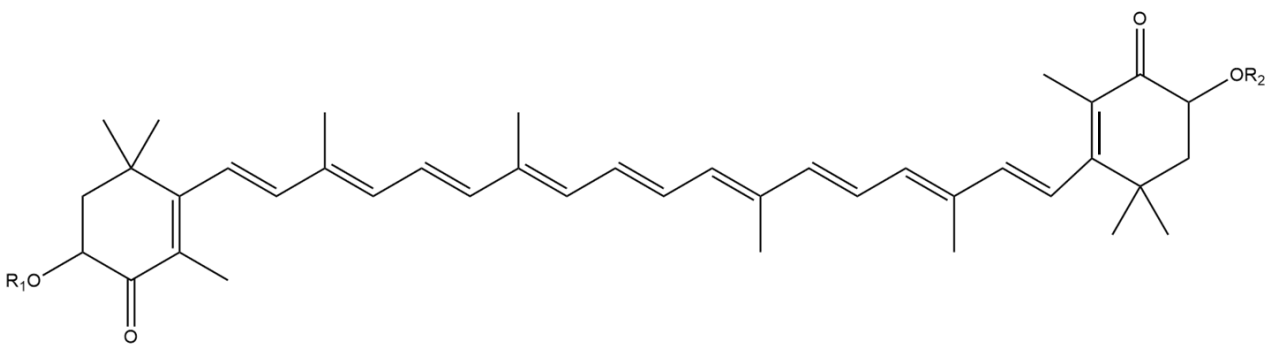


Figure 1.1. Planar structure of Astaxanthin

Astx, astaxanthin R1, R2 = H, represents free Astx; R1 or R2 = fatty acid, represents monoesterified Astx; R1 and R2 = fatty acid, represents diesterified Astx.

1.2.2.1.2 Astx as an antioxidant to prevent oxidation of oils highly prone to oxidation

Fish oil is a rich source of n-3 PUFA, which are associated with several health benefits (Ad Lordan et al., 2011; Abeywardena and Belobrajdic, 2016); however it has been suggested that the majority of fish oil in the market is oxidized to some extent (Jackowski et al., 2015). N-3 PUFAs are highly susceptible to oxidation (Benzie, 1996; Albert et al., 2013). The quality of the n-3 PUFA in oxidized oil is poor, and these oils are likely to elicit harmful health effects (Esterbauer et al., 1993; Bartsch and Nair, 2006; Albert et al., 2013). The rate of lipid peroxidation depends on several factors such as storage conditions, exposure to light, heat, and oxygen concentration even at room temperature (Bragadóttir, 2005). The oxidation of oil leads to the formation of lipid peroxides, which undergo further degradation to form potentially harmful secondary oxidation products (Esterbauer, 1993; Barden et al., 2011). Since the 1950s, the safety of oxidized fish oil has been the issue of concern (Matsuo, 1954). More recently, the concerns were rekindled when a study was conducted to evaluate the oxidation status and safety of commercially available North American n-3 PUFA supplements in Canada (Jackowski et al., 2015). This study revealed that majority of the tested fish oil supplements in the market were oxidized to some extent (Jackowski et al., 2015). Whereas addition of antioxidants has been shown to reduce oxidation of oil (Zuta et al., 2007).

A combination of Astx and fish oil has been shown to induce hypolipidemic and antioxidant effects in Wistar rats, compared to Astx or fish oil alone (Barros et al., 2012). However, most of the reported studies in literature do not discuss the effects of individual form of Astx (free or Astx-E) on preventing oxidation when combined with fish oil. There are still limited reports on the antioxidant potential of esterified Astx on fish oil, and the effects of a combination of esterified Astx and fish oil on the regulation of metabolic pathways. Moreover, a combination of Astx with flaxseed oil improved oxidative stress, reduced lipid levels and inflammation in rats fed a high fat diet (HFD), compared to HFD-fed animals (Xu et al., 2014); these effects were due to a decrease in lipid peroxidation, and an increase in the antioxidant capacity. Astx that has been used

in this study was extracted from microalga *H. pluvialis*. Furthermore, it has been stated that Astx (form of Astx is unclear) absorption increases when emulsified with oils (Clark et al., 2000).

1.2.2.1.3 Safety of astaxanthin

In 1987, the U.S. Food and Drug Administration (FDA) approved Astx for the aquaculture industry as a feed additive, and further in 1999 FDA authorized Astx extracted from *H. pluvialis* as a dietary supplement (Boussiba, 2000). In 2010, Astx extracted from *H. pluvialis* has received the "generally recognized as safe (GRAS)" status from (FDA) (Jia et al., 2012). Astx has been shown to be safe and well tolerated in animal studies (Fassett and Coombes, 2011) and in human clinical trials (Spiller and Dewell, 2003). The effects of Astx have been studied in healthy adults in a double-blind clinical trial where 6 mg/day was supplemented for 8 weeks; the subjects revealed no change in plasma metabolic profile, blood pressure and blood cell count, whereas there was a slight increase in the levels of calcium, total protein, and eosinophils within healthy ranges. Furthermore, human clinical studies have demonstrated that Astx (4 mg to 100 mg per day) induces no adverse side effects (Visioli and Artaria, 2017). It has been reported that a single dose of 100 mg Astx (Østerlie et al., 2000), or a daily dose of 40 mg Astx for 4 weeks in patients with functional dyspepsia (Kupcinskas et al., 2008), and even a 4 mg Astx dose for 12 months in human subjects with muscular degeneration (Parisi et al., 2008) causes no ill effects or toxicity. Therefore, there are no adverse effects reported till date regarding Astx supplementation in humans.

1.2.2.1.4. Anti-obesity effects of astaxanthin

In recent years, Astx gained significant amount of attention as a potential nutraceutical due to a number of suggested health benefits, such as anti-obesity effects (Maeda et al., 2007). Astx supplementation was found to reduce body weight gain, decrease hepatic and plasma total cholesterol and TAG levels, and improve insulin sensitivity in mice fed obesity-promoting diet (Bhuvaneswari et al., 2010); however, the authors did not specify the form of Astx (free/Astx-E). Astx (*H. pluvialis* extract) supplemented in a high-fat diet increased hepatic LDL-

receptor, decreased 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) and sterol-regulatory element binding protein (SREBP)-2 activity, which was associated with lowering plasma cholesterol levels in mice (Yang et al., 2011). The authors further reported a decrease in plasma TAG levels, which was due to a significant increase in the mRNA expression of carnitine palmitoyl transferase-1 (*Cpt1*), suggesting that the TAG lowering effect of Astx might be due to increased fatty acid β-oxidation in the liver (Yang et al., 2011). These authors suggested that enhanced β-oxidation of fatty acids could lower TAG synthesis, consequently limiting VLDL secretion from the liver. This notion is supported by the fact that a hypotriglyceridemic effect of fibrates, activators of PPARα, is primarily attributed to increased fatty acid β-oxidation. Jia et al. (Jia et al., 2012) suggested that the hypolipidemic effect of free Astx is by acting as a peroxisome proliferator-activated receptor-γ (PPARγ) antagonist, and PPARα agonist leading to lower plasma TAG levels by improving fatty acid β-oxidation. Authors reported that Astx induces PPARα-responsive gene expression, but reduces the expression of PPARγ-responsive genes. Moreover, Astx reduced lipid accumulation in HepG2 cells. In addition, Astx reduced the cellular cholesterol and TAG concentrations in HepG2 cells. Therefore, it was suggested that Astx produces hypolipidemic effects by regulating PPAR activity. Inoue et al. (Inoue et al., 2012) showed that free Astx acts as an antagonist for PPARγ to inhibit lipid accumulation and inhibited adipogenesis in 3T3-L1 adipocytes (Inoue et al., 2012). These findings indicate that Astx could be used as a functional food/nutraceutical to target obesity related lipid disorders. Although a number of studies have suggested hypolipidemic effects of Astx, the effects attributed to different forms of Astx, such as free Astx and Astx-E have not been clearly established in metabolic regulation.

1.2.3 Methods of extraction of shrimp oil and astaxanthin from shrimp processing by-product

There are several methods of extraction of shrimp oil and astaxanthin from shrimp processing by-product. Different extraction approaches include physical separation, fermentation, chemical extraction, enzymatic extraction, supercritical fluid extraction and solvent extraction (Ambati et al., 2014; Routray et al., 2019).

Recently, use of ultrasonic, pulsed electric field assisted extraction has also been reported (Saini and Keum, 2018). The efficiency of extraction depends on the nature of solid matrix, extraction method, extraction time, operating conditions (temperature and pressure), sample volume, solvents, and the ratio of solvent/s: solid (Pandey and Tripathi, 2014). All these factors play an important role in the extraction process due to the interaction between the compound of interest and the solvent used for extraction (Routray et al., 2019). Solvent based method includes atmospheric solvent extraction such as the Soxhlet extraction, accelerated solvent extraction and supercritical fluid extraction (Ahmadkelayeh and Hawboldt, 2020). A range of solvents are used in solvent extraction such as hexane, acetone, isopropanol, ethanol, methanol, and/or a combination of solvents for optimum recovery of oil and Astx from shrimp processing by-product (Sachindra et al., 2006). Carotenoids in shrimp processing by-product can vary in polarity, thus it is difficult for one solvent to extract all carotenoids, as well as optimum amounts of lipids. Therefore, the synergistic effect of a combination of polar and non-polar solvents have been suggested for the extraction of Astx and lipids (Sachindra et al., 2006; Saini and Keum, 2018). Many conventional solvent extraction methods involving organic solvents can take several hours to complete extraction, and may present safety and environmental hazards (Pandey and Tripathi, 2014; Saini and Keum, 2018); thus "Green" alternatives with higher extraction efficiency and low environmental impact have also been suggested (Chemat et al., 2012). Some of those approaches are supercritical extraction using CO_2 and co-solvents (Sánchez-Camargo et al., 2011; Ahmadkelayeh and Hawboldt, 2020), and the use of edible oils to extract carotenoids from shrimp processing by-product. It has been reported that use of edible oils to extract astaxanthin from shrimp processing by-product increases the stability of carotenoids despite the low carotenoids content, compared to the conventional methods (Handayani et al., 2008). Moreover, it has been stated that the use of edible oils as a "Green" solvent for extraction, and as a "Green" co-solvent in supercritical extraction can serve as an alternative to organic extractions (Ahmadkelayeh and Hawboldt, 2020). However, which method provides the best yield for oil and astaxanthin extraction from shrimp processing by-product is yet to be clearly established.

Furthermore, the effects of these extracts on adipose tissue metabolism and its relationship to obesity is yet to be fully understood.

1.3 Adipose tissue and metabolic disorders

Obesity considerably increases the prevalence of other metabolic diseases such as diabetes, hypertension, hypertriglyceridemia, coronary heart disease, stroke, and fatty liver disease (Blüher, 2013). Obesity is linked to changes in the function of adipocytes, thereby increasing the adipose tissue mass and size (Spalding et al., 2008). Obesity is primarily associated with marked alterations in the secretory function of adipocytes that lead to chronic low-grade inflammation, a risk to develop insulin resistance, and/or vascular disease (Iacobellis et al., 2005). Inflammation of the adipose tissue leads to an increase in lipolysis, thereby elevating the levels of FFA in circulation and causing dyslipidemia (Julius, 2003). Adipose tissue dysfunction is caused mainly due to the interaction of genetic, behavioral, and environmental factors that causes adipocyte hypertrophy, ectopic fat accumulation, hypoxia, impaired mitochondrial function, and inflammatory processes within adipose tissue (Blüher, 2013).

1.4 Adipose tissue metabolism

The key role of adipose tissue is to regulate energy balance. The primary function of adipose tissue is to store energy by accumulating TAG (Ali et al., 2013), and it also functions as an endocrine organ (Coelho et al., 2013). Adipose tissue is involved in regulating lipid metabolism, insulin-regulated glucose uptake, and inflammatory response (Lefterova and Lazar, 2009; Sarjeant and Stephens, 2012). Adipose tissue has substantial capacity to expand that is evident by the storage of TAG in adipocytes resulting in the expansion of the adipose tissue (hypertrophy) (Rosen et al., 2000; Tan, 2008; Lafontan et al., 2009); or increased fat storage is due to adipocyte hyperplasia (increase in number). At cellular level, the proliferation and subsequent differentiation of adipocyte precursor cells leads to the expansion of preadipocytes to mature fat cells. The precursor cells are located in the stromal vascular fraction of adipose tissue and can be stimulated to proliferate, and then

differentiate *in vitro* using chemically defined media to study the process of adipogenesis. There are intricate sequences of events that regulate TAG pool within the adipocyte such as *de novo* synthesis from carbon precursors called lipogenesis, and the process of TAG hydrolysis called lipolysis (Louveau and Gondret, 2004). Lipolysis releases glycerol and free fatty acids (FFA) that is transported to other tissues such as liver and muscle via blood circulation (Frayn, 2002).

1.4.1 Regulation of adipogenesis

The process of differentiation starts from adipoblast to preadipocytes; preadipocytes get converted to an immature adipose cell and then subsequently to a mature adipose cell, called an adipocyte. The process of differentiation of committed fibroblast-like preadipocytes into the lipid-laden adipocytes defines the process of adipogenesis (Lefterova and Lazar, 2009; Ali et al., 2013). The formation of adipocytes from preadipocytes comprises a complicated and highly organized program of gene regulations (Lowe et al., 2011) that regulate the specific adipocyte phenotype (Niemelä et al., 2008). Adipocyte differentiation comprises of a broad network of transcription factors that are involved in regulating the gene expression of key proteins (Farmer, 2006). The G1 phase of the cell cycle is the stage where the proliferation of preadipocytes is arrested upon reaching confluency (Niemelä et al., 2008). The growth-arrested preadipocytes re-enter the cell cycle after hormonal induction, and undertake two rounds of cell division, called mitotic clonal expansion (Fajas Coll, 2003). The cell then stops proliferating with the cell cycle exit and drops its fibroblast morphology. This leads to the stimulation of specific genes that are involved in regulating fat accumulation to alter adipocyte phenotype (Tang and Lane, 2012). One of the crucial steps towards a differentiated adipocyte phenotype includes, activation of regulatory genes such as, CCAAT enhancer-binding protein (C/EBP)-β which is necessary for activation of PPARγ and C/EBPα that regulate adipocyte differentiation (Wu et al., 1999). The accumulation of TAG signifies the full differentiation of a cell into an adipocyte.

1.4.1.1 The role of PPARγ and SREBP1c in adipogenesis

The differentiation of preadipocytes into mature adipocytes is induced mainly by members of two families of transcription factors called C/EBP and PPARγ (Rosen et al., 2000). PPARγ is an important regulator of adipogenesis that belongs to the nuclear receptor superfamily (Wang et al., 2015). PPARs function by establishing a heterodimer with the retinoid X receptor, which binds to a specific sequence known as the peroxisome proliferator response elements (PPRE) in target genes (Tontonoz and Spiegelman, 2008). C/EBPs are stimulated during adipogenesis and function with PPARγ towards adipocyte differentiation; PPARγ and C/EBPα induce each other's expression in a positive feedback loop and maintains the differentiated cell state (El-Jack et al., 1999; Zuo et al., 2006). PPAR*γ* and C/EBP*α* not only coordinate each other's expression, they also regulate insulin sensitivity in adipocytes (Wu et al., 1999). The regulation of lipogenesis in adipocytes involves another important transcription factor called SREBP1c (Eberlé et al., 2004). SREBP1c belongs to the helix-loop-helix leucine zipper family, and promotes adipocyte differentiation by regulating the expression of genes linked to fatty acid and cholesterol synthesis (Kim and Spiegelman, 1996).

1.4.2 Regulation of lipogenesis

Carbohydrate response element-binding protein (ChREBP), SREBP1c and liver-X receptor (LXR) regulate the number of enzymes involved in *de novo* lipogenesis at the transcriptional level (Wang et al., 2015). SREBP1c gets proteolytically cleaved once activated, and gets translocated to the nucleus where it binds to sterol response elements (Eberlé et al., 2004; Dong et al., 2020) to regulate genes associated with lipid metabolism, such as, acetyl-CoA carboxylase (ACC1), fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD1) and diacylglycerol acyltransferase (DGAT2) (Wang et al., 2015). LXR are members of the nuclear receptor superfamily that heterodimerize with retinoid X receptor (RXR) (Chawta et al., 2001). LXRα, one of the two isomers, is expressed in lipogenic tissues and plays crucial role in transcriptional regulation of lipogenic genes by activating SREBP1c (Wong and Sul, 2010).

FAS is a multi-subunit protein that synthesizes palmitate from malonyl-CoA and acetyl-CoA (Menendez et al., 2009). FAS gene expression in adipose tissue is associated to fat accumulation, and inhibition of its activity reduces the adipocyte size and number (Liu et al., 2004). SCD1 is the rate-limiting enzyme for the synthesis of monounsaturated fatty acids (MUFA) from saturated fatty acid (SFA) (Peck and Schulze, 2016). SCD1 plays role in regulating lipid and glucose metabolism, and maintaining the insulin sensitivity (ALJohani et al., 2017). SCD1 deficiency upregulates insulin-sensitizing components and affects glycogen metabolism (Dobrzyn et al., 2010). Both SCD1 and DGAT2 are co-localized in the endoplasmic reticulum and have a similar pattern of expression (Weng et al., 2006). DGAT2 is the key enzyme that catalyzes the final step in TAG synthesis for fat storage in the adipocyte. Overexpression of DGAT2 promotes lipid deposition and affects insulin signaling (Zhang et al., 2014).

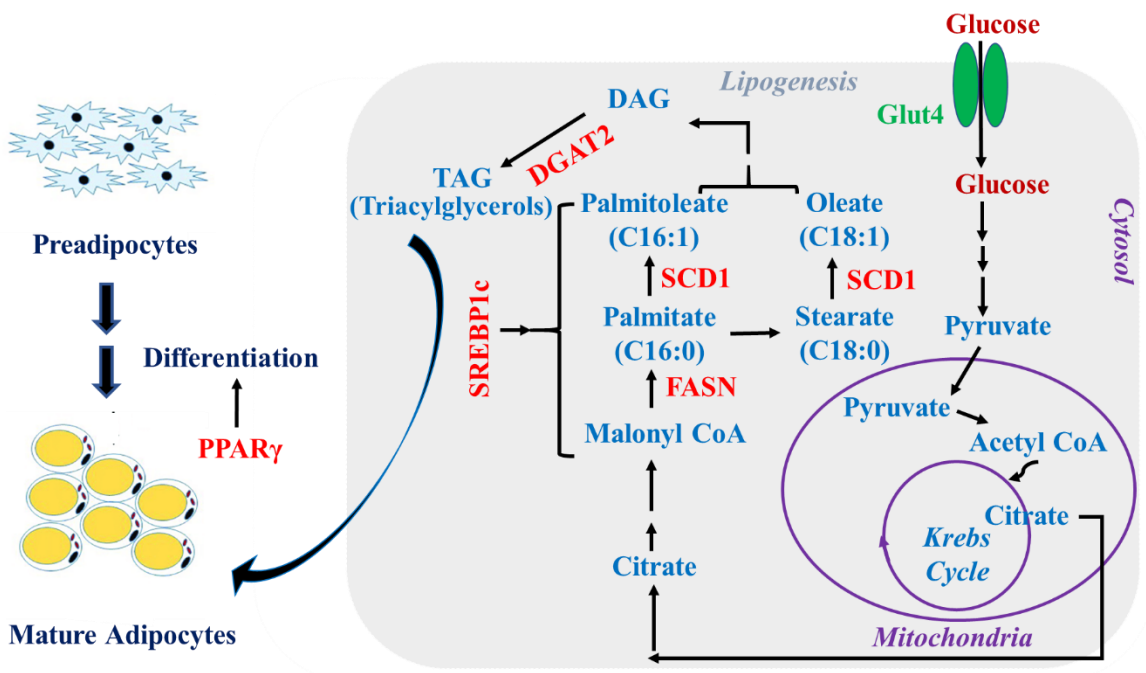


Figure 1.2. The pathway involved in the regulation of adipogenesis and lipogenesis in adipocytes

Confluent preadipocytes are differentiated into mature adipocytes upon induction with a differentiation media. FASN, fatty acid synthase; PPARγ, peroxisome proliferator-activated receptor-gamma; SCD1, stearoyl-CoA desaturase; SREBP1c, sterol regulatory element-binding protein 1; DAG, diacylglycerol; DGAT2, Diacylglycerolacyl transferase; TAG, triacylglycerol.

1.4.3 Hormonal regulation of adipogenesis using *in vitro* cell culture models

Preadipocytes differentiation is initiated using a combination of inducers that comprise of insulin, dexamethasone (Dex), and isobutylmethylxanthine (IBMX) in a fetal bovine serum-containing medium (Kawai et al., 2007). Once insulin binds to the receptor, it activates the insulin-like growth factor (IGF)-1 signaling pathway by recruiting and phosphorylating insulin receptor substrate proteins. This activates the phosphatidylinositol-3-kinase pathway (Hemati et al., 1997). IBMX is a cyclic nucleotide phosphodiesterase inhibitor that elevates intracellular cAMP levels (Essayan, 2001). Then the cAMP signal activates C/EBPβ gene expression which is needed for PPARγ and C/EBPα activation via the cAMP response element-binding protein (CREB) transcription factor (Tang and Lane, 2012). Differentiation of preadipocytes is induced by glucocorticoids, and Dex, is a synthetic glucocorticoid that activates glucocorticoid receptor, thus, activates C/EBP-δ, and induces adipogenesis (Vishwanath et al., 2013).

1.4.4 Marine oils in regulating adipose tissue metabolism

Our laboratory has previously shown that marine species, such as blue mussels and sea cucumber that are rich in n-3 PUFA and other nutrients, affect adipose tissue function to elicit potential anti-obesity effects (Vaidya et al., 2017). N-3 PUFA, specifically EPA and DHA, play a crucial role in maintaining lipid metabolism (Buckley and Howe, 2009), and adipogenesis (Todorčević and Hodson, 2015). EPA and DHA, and their metabolites are natural ligands for PPARγ (Magee et al., 2012), and have been shown to act as antagonists. N-3 PUFA specifically target *Pparγ* gene expression to decrease lipogenesis, thereby inducing beneficial effects on adipose tissue metabolism and function (Todorčević and Hodson, 2015). The anti-adipogenic effects of DHA

were shown to be due to a decrease in TAG accumulation (Kim et al., 2006). Studies have shown that EPA and DHA reduce fat accumulation in 3T3-L1 cells (Kim et al., 2006; Manickam et al., 2010). Other studies have also stated that EPA and DHA reduced the mRNA expression of *Pparγ* (Lorente-Cebrián et al., 2006; Manickam et al., 2010). Studies have also demonstrated that EPA and DHA reduces the mRNA expression of both adipogenic and lipogenic genes, such as *Srebp1c, Fasn* and *Pparγ*. N-3 PUFA have been shown to reduce the mRNA expression of *Srebp1c*, causing a decrease in the mRNA expression of genes involved in lipogenesis, such as *Fasn, Scd1*, and *Acc* (Dentin et al., 2005; Kaur et al., 2011). Animal based studies have also confirmed that n-3 PUFA supplementation reduces fat accumulation by downregulating lipogenesis, and stimulating lipid oxidation (Azain, 2004; Lapillonne et al., 2004; Ruzickova et al., 2004). Marine sources of n-3 PUFA, such as fish oil, improve dyslipidemia (Skulas-Ray et al., 2019) and insulin resistance (Lombardo et al., 2006; Albert et al., 2014), and mediate some of its beneficial effects by modulating the TAG storage and/or secretory functions of adipose tissue; and these beneficial effects of fish oil have been mainly attributed to the n-3 PUFA content of fish oil (Puglisi et al., 2011; Todorčević and Hodson, 2015). Although fish oil is rich in n-3 PUFA such as EPA and DHA, it also contains other fatty acids that may exert a combined effect on adipogenesis. Previously, our laboratory has shown that arachidonic acid, an n-6 PUFA, has a dominant effect on the regulation of lipogenic genes when given along with EPA and DHA in 3T3-L1 adipocytes (Vaidya and Cheema, 2015). Unfortunately, there are no studies reported in the literature to date that have investigated the effect of fish oil on adipogenesis using *in vitro* model such as 3T3-L1 adipocytes. Thus, it will be important to investigate the regulation of adipogenesis and lipogenesis in 3T3-L1 adipocytes upon treatment with fish oil.

Shrimp oil is also rich in n-3 PUFA but it also contains significant amounts of Astx, which has been shown to reduce body weight gain, and improved insulin sensitivity in diet-induced obese rats (Nair et al., 2017). Studies have shown that besides n-3 PUFA, free Astx found in marine species also acts as an antagonist for PPARγ to inhibit lipid accumulation and decrease mRNA expression of *Pparγ* target genes in 3T3-L1 adipocytes

(Inoue et al., 2012). Shrimp oil is rich in both n-3 PUFA and Astx; however, there are no reports to date to investigate the effects of shrimp oil extracted from shrimp processing by-product on the process of adipogenesis and lipogenesis in 3T3-L1 cells. Moreover, Astx is a potent antioxidant and acts like a novel selective PPARγ antagonist (Inoue et al., 2012). Interestingly, among the two forms of Astx, free and Astx-E; Astx-E show higher antioxidant activity compared to free Astx (Régnier et al., 2015). However, most of the reports in literature does not clarify the effect of Astx-E on preventing oxidation when combined with fish oil, and the effect of this combination on adipogenesis compared to fish oil alone. Given that the majority of fish oil in the market is measurably oxidized (Jackowski et al., 2015), it will be important to investigate the effects of a combination of fish oil with Astx-E on preventing fish oil oxidation. Since shrimp processing by-product is rich in both n-3 PUFA and Astx, it is of interest to establish a method to extract these valuable bioactives from shrimp processing by-product, and to investigate their effects on the regulation of adipose tissue metabolism. The findings will establish whether shrimp extracts from shrimp processing by-product have potential anti-obesity effects, and can be developed as nutraceuticals or functional foods.

CHAPTER TWO

Rational, Objectives and Hypotheses

2.1 Murine 3T3-L1 cells as an *in vitro* model to study adipogenesis

The murine 3T3-L1 cell line is a well-established cell line, and a suitable model of adipocyte differentiation; it has been widely used to study the mechanisms involved in adipocyte differentiation (Armani et al., 2010). The 3T3-L1 cells were originated from the Swiss 3T3 mouse embryo, based on their potential to accumulate lipids (Green and Kehinde, 1975). Murine 3T3-L1 preadipocytes have a fibroblast like morphology and can be differentiated into lipid laden mature adipocytes under chemical induction using insulin, 3-isobutyl-1-methylxanthine and dexamethasone (Lefterova and Lazar, 2009). Our laboratory (Vaidya and Cheema, 2015, 2018), and others (Manickam et al., 2010; Wójcik et al., 2014) have previously studied adipogenic differentiation using 3T3-L1 cells, as a cell culture model. Since the mechanisms of adipogenesis are well understood and characterized in 3T3-L1 adipocytes (Armani et al., 2010; Li et al., 2017), I selected this cell culture model to investigate the effects of shrimp extract, shrimp oil, fish oil, and astaxanthin on adipogenesis.

2.2 Rationale and hypotheses

The shrimp processing plants usually discard shrimp parts (shells and heads accounting for 45-60% of the shrimp body mass) as a waste (Sachindra and Mahendrakar, 2005; Saini and Keum, 2018), primarily impacting the environment. Commercially, shrimp is separated by size, and the small size that does not meet selling scale also get discarded. Reportedly, over 40% (w/w) of the shrimp is usually rejected for commercial applications and disposed of as solid shrimp processing by-product (Gildberg et al., 2001). However, shrimp waste is a valuable source of shrimp oil rich in n-3 PUFA and Astx, a highly potent antioxidant that exists in either free form or Astx-E (Hussein et al., 2006b). Our laboratory has previously shown that other marine species, such as blue mussel and sea cucumber that are rich in n-3 PUFA and other nutrients, affect adipose tissue function to elicit anti-obesity effects (Vaidya et al., 2017). It has been studied that n-3 PUFA, specifically EPA and DHA, play an important role in maintaining lipid metabolism (Buckley and Howe, 2009), and adipogenesis (Todorčević and Hodson, 2015). Besides n-3 PUFA, free Astx found in marine species has also been shown to reduce fat

accumulation by inhibiting adipogenesis in 3T3-L1 adipocytes (Inoue et al., 2012). However, there are no reports to date to establish whether shrimp oil extracted from shrimp processing by-product affects adipogenesis and lipogenesis in 3T3-L1 cells. Furthermore, the majority of the tested fish oil supplements (rich in n-3 PUFA) in the market have been measurably oxidized (Jackowski et al., 2015). Astx is a highly potent antioxidant; however, it is not known whether a combination of fish oil and Astx prevents the oxidation of fish oil, and inhibits adipogenesis to a greater extent, compared to fish oil alone.

2.2 Overall Aim and specific objectives

The overall aim of my thesis was to extract shrimp oil from shrimp processing by-product using different extraction methods (in collaboration with Dr. Hawboldt, Process Engineering, Faculty of Engineering and Applied Science, Memorial University), and to test the effects of the extracted oils on adipogenesis and lipogenesis in 3T3-L1 cells. The specific objectives and hypotheses of the study were as follows:

Objectives:

1. To extract shrimp oil from shrimp processing by-product using various extraction methods, and to measure the lipids, fatty acids composition, and Astx content.
2. To investigate whether shrimp oil extracted from shrimp processing by-product using various extraction methods affects fat accumulation and adipogenesis in 3T3-L1 cells.
3. To investigate whether Astx-E prevents the oxidation of fish oil, compared to fish oil alone.

Hypotheses:

1. Treatment of 3T3-L1 adipocytes with shrimp oil rich in n-3 PUFA and Astx-E will reduce fat accumulation and inhibit adipogenesis, compared to control (untreated) cells.

2. Treatment of 3T3-L1 adipocytes with a combination of fish oil plus Astx-E will reduce fat accumulation and inhibit adipogenesis to a greater extent, compared to fish oil alone.
3. Addition of Astx-E to fish oil will prevent the oxidation of oil, compared to fish oil alone.

2.3 Covid-19 impacts

Due to COVID-19 induced pandemic, I encountered some logistical problems that prevented me from conducting some planned experiments:

1. I measured mRNA expression of *Glut-4*, which is not the sequestered and active versions of *Glut-4*. I had planned to investigate whether treatments will improve insulin sensitivity in 3T3-L1 cells using radioisotope assays for glucose uptake. However, glucose uptake assays could not be conducted due to Covid-19 lockdown.
2. I had planned to perform extractions using larger sample size to generate a larger amount of extracts from shrimp processing by-product to conduct additional cell culture experiments, such as lipid extraction from treated 3T3-L1 adipocytes cells to determine the fatty acids composition and total TAG analysis on extracted lipids. However, these experiments could not be conducted due to the limited amount of shrimp oil to repeat experiments in larger cell culture dishes to extract lipids. Furthermore, attempts to scale up the extraction process could not be carried out due to Covid-19 associated restrictions.

CHAPTER THREE

Materials and Methods

3.1 Shrimp oil extraction from shrimp processing by-product

3.1.1 Soxhlet method

Shrimp extract was prepared from Northern shrimp (*Pandalus borealis*) processing by-product (provided by St. Anthony Basin Resources Inc. (SABRI), St. Anthony, NL, Canada). The Soxhlet method of extraction was used, in collaboration with Dr. Kelly Hawboldt, Process Engineering, Memorial University of Newfoundland, Canada. The extraction process was optimized in Dr. Hawboldt's laboratory using a range of organic solvents [(Hexane, ethanol, acetone: hexane (60:40; v/v), isopropanol: hexane (50:50; v/v)], and the types of shrimp processing by-product (wet, dry, freeze dried) in order to determine the maximum extraction potential for carotenoids and lipids from Northern shrimp processing by-product. The optimum extraction procedure established in Dr. Hawboldt's research group to generate shrimp extracts was used in this study. In this study, 20 g of Northern shrimp processing by-product was used, which was ground to fine particles and placed in the thimble of a Soxhlet apparatus (Fig. 3.1), to which 200 mL of hexane: acetone (2:3, v/v) was added and refluxed for 6 hours. Four independent batches of extraction were performed to make sufficient amount of shrimp extracts; the extracts were combined and concentrated in a rotatory evaporator at a temperature of 40ºC under vacuum and stored at 4ºC until further use. Shrimp oil was then extracted from shrimp extract using the Folch method (Folch et al., 1957). Chloroform: methanol (2:1 v/v) and 600 µL of 50 mM NaCl was added to 1000 mg of shrimp extract, vortexed and stored overnight at 4ºC. Samples were then centrifuged at 2,000 x g for 4 minutes, the lower layer was collected, evaporated under nitrogen and shrimp oil was stored at 4ºC for future studies. Both the shrimp extract and shrimp oil were used for cell culture experiments.

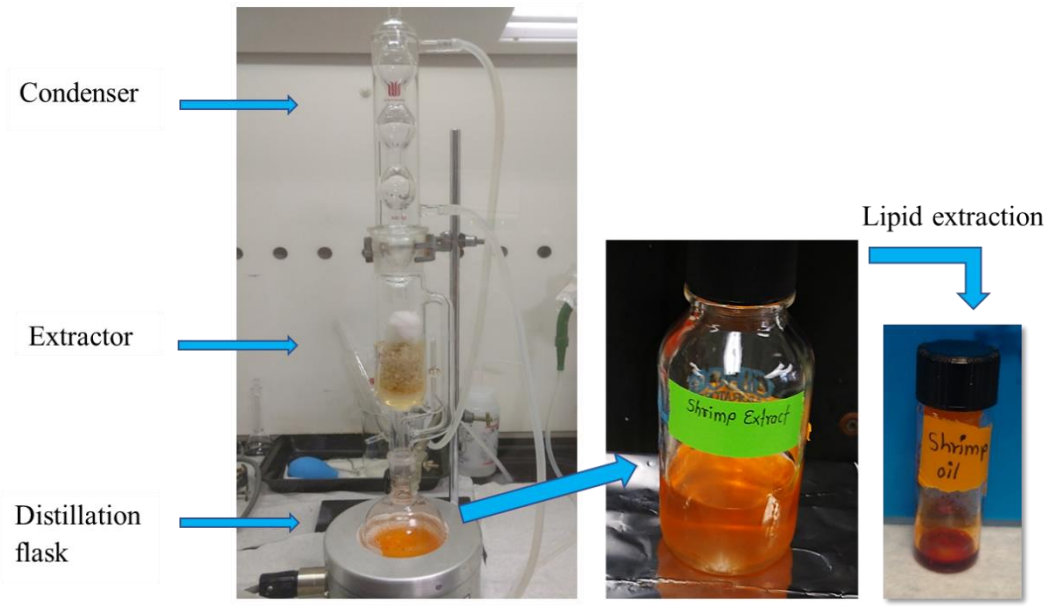


Figure 3.1. The Soxhlet setup and extraction of shrimp extract from shrimp processing by-product

The Soxhlet setup comprised of condenser, extractor, and distillation flask. Shrimp processing by-product was placed in thimble, situated inside the extractor and continuous extraction was carried out for 6 hours using the solvent hexane: acetone (2:3 v/v) as explained in the methods.

3.1.2 Extraction of shrimp oil from shrimp processing by-product using waste fish oil as a solvent

Dr. Hawboldt's laboratory used fish oil that was extracted from waste fish (FOS) to extract lipids and carotenoids from shrimp processing by-product. Fish oil was first extracted from fish waste using a previously established extraction method in their laboratory (Jayasinghe et al., 2013); fish oil was then mixed with shrimp processing by-product in various ratios (3, 6, 9 v/w). The mixture was heated for various times (1, 2 and 3 hours), and at various temperatures (50, 60 and 70ºC) for optimum recovery of lipids and carotenoids from shrimp processing by-product (Fig 3.2). Oil extractions were performed using different combinations of the above-mentioned operating conditions (ratio, time, and temperature); the oil layer was separated from the solid phase by centrifugation. I received four samples: FOS, fish oil extracted from waste fish (used as a solvent for extraction); FS1 (ratio of fish oil to shrimp processing by-product: 3; time: 2 hours; temperature: 50ºC); FS2 (ratio of fish oil to shrimp processing by-product: 3; time: 3 hours; temperature: 60ºC), and FS3 (ratio of fish oil to shrimp processing by-product: 3; time: 3 hours; temperature: 70ºC). Total lipids, fatty acids composition, and total carotenoids data were received from Dr. Hawboldt's group and is given in appendix IV.

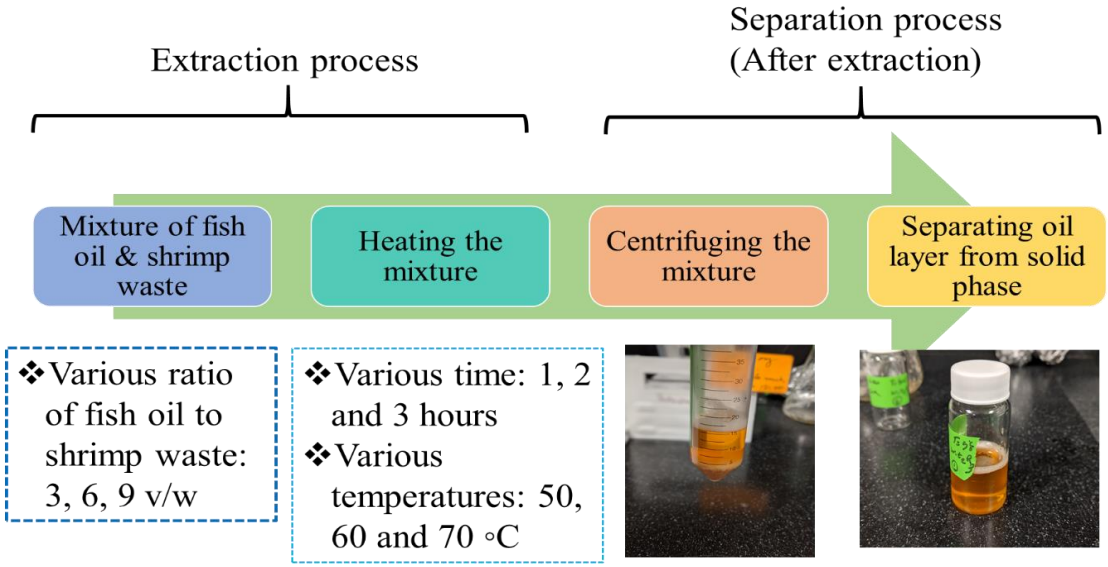


Figure 3.2. Experimental design of oil extract preparation from shrimp processing by-product using waste fish oil as a solvent

3.1.3 Shrimp processing by-product extraction using fish waste

I extracted oil from a mixture of shrimp processing by-product and fish waste (Fig. 3.3). I performed extractions using various extraction ratios: 2:1 (fish waste + shrimp processing by-product) [2:1 (F+S)], 2.5:1 (fish waste + shrimp processing by-product) [2.5:1 (F+S)], 3:1 (fish waste + shrimp processing by-product) [3:1 (F+S)], and from only fish waste (salmon gut), using a previously established method in Dr. Hawboldt's laboratory (Jayasinghe et al., 2013). However, this method was previously established in Dr. Hawboldt's laboratory to extract fish oil from fish waste. A mixture of fish waste (salmon gut) and shrimp processing by-product was put through a grinder and then heated at 80ºC in water bath for 20 mins. Samples were centrifuged 450 x g for 10 minutes twice; distilled water (at 90ºC) was then added in 2:1 ratio, and another centrifugation was carried out at 2906 x g for 5 mins. The oil layer was collected, filtered through a 0.2 µm PTFE membrane filter under vacuum, and stored at 4ºC (Fig. 3.3). This pilot experiment did not provide meaningful data due to issues with extractions, discrepancies in oil recovery and carotenoid yield (Appendix I); thus, no further experiments were undertaken using these extracts.

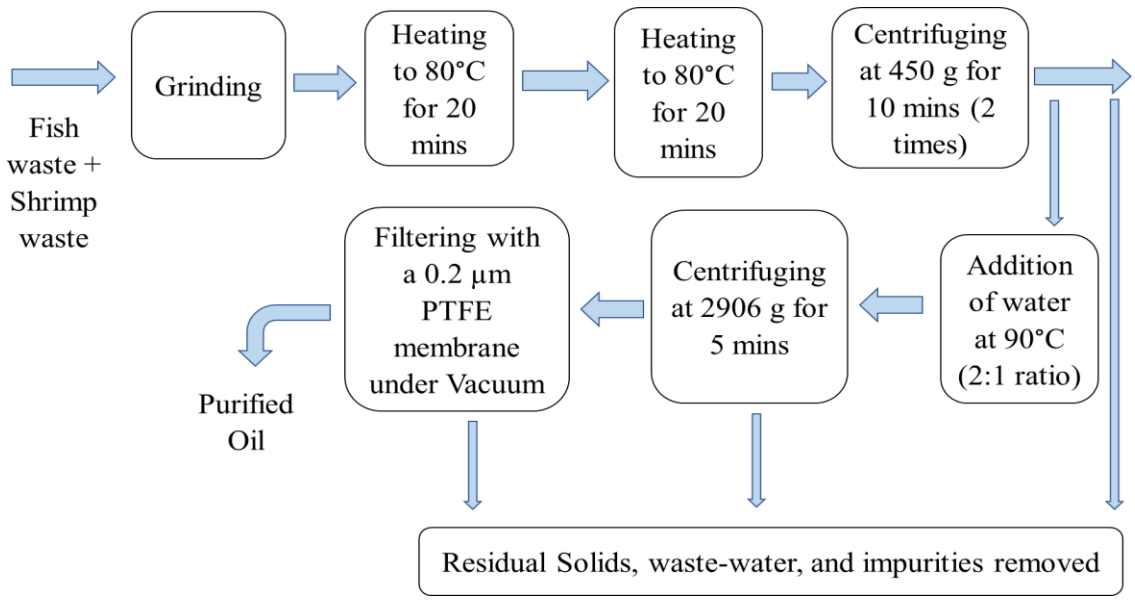


Figure 3.3. Experimental design of oil extraction from a mixture of fish and shrimp processing by-product

3.2 Total lipids analysis of shrimp oil

Total lipids were extracted from shrimp extract solution using the Folch method (Folch et al., 1957), and the lipid composition of shrimp oil was analyzed using TLC-flame ionization detection (TLC-FID) (Mark VI Iatroscan, NTS, USA). The calibration was performed using a nine component standard (#1787-1AMP, Sigma Chemicals, St.Louis, Mo., USA); the composition of the standard is given in Table 3.1. The samples were spotted on silica coated chromarods, which were developed in respective solvent systems and scanned in three respective sections (Parrish, 1999). First scan was performed for separation of hydrocarbon, wax ester/steryl ester and ketone classes of lipids using first development system as hexane: anhydrous diethyl ether: formic acid with 98.95: 1: 0.05 ratios. The second scan was performed for separation of TAG, free fatty acids, alcohol, and sterol classes of lipids using developing system of hexane: diethyl ether: formic acid with a 79: 20: 1 ratio. Third scan was performed for acetone mobile polar lipids and phospholipids using the solvent chloroform: methanol: chloroform extracted water with a 50: 40: 10 ratio. A hydrogen flow between 195 - 200 mL/min and an air flow of 20 mL/min were used for scanning all the rods. The calibration was performed by spotting two consecutive rods with 0.5, 1, 1.5, 2 and 3 μL of the standard. The PeakSimple software (version 4.54, 6 channel, SRI Instruments, Torrance, CA, USA) was used to obtain the results for each rod for peak areas, and correlation coefficients were attained by considering the amount spotted and obtained peak area for each rod, while maintaining R^2 values above 0.95 (correlation above 95%).

Shrimp oil samples were developed and scanned to identify lipids classes using similar procedure. Shrimp oil lipid extract was resuspended in 250 μL of chloroform and spotted in amounts varying from 1-3 μL on rods for further analysis. The standard was spotted on the tenth rod of each rack to confirm and identify the lipid classes qualitatively in the sample. Total lipids and lipid classes were obtained for each chromarod using the regression equations that were derived during calibration. These values were used for determining the percentage lipid composition (Wt. %) along with the total lipid content.

3.3 Fatty acids analysis of shrimp oil

Total lipids extracted from shrimp extracts were transferred to a transmethylation vial (5 mL V-Vial CLR) and 10 µL of internal standard (heptadecanoate, C17:0; 10 mg/mL in methanol) was added. Transmethylation was performed as per our previously published methods (Chechi et al., 2010), by adding 2 mL of transmethylation reagent (94 % methanol and 6 % of 98 % concentrated sulfuric acid) and approximately 20 mg of hydroquinone to prevent oxidation. Samples were incubated at 65ºC for 2 hours (Arvidson and Olivecrona, 1962) followed by extraction of the organic fatty acid methyl esters (FAME) using 1.5 mL of hexane. The extract was washed with 1.5 mL of water, and samples were placed at -20ºC overnight. The hexane layer was carefully transferred into a new tube, then evaporated under nitrogen gas (N_2), and resuspended in 50 µL carbon disulfide.

The fatty acids composition was analyzed using gas chromatography (GC)-FID as per our previously published methods (Chechi et al., 2010). An Omegawax X 320 (30 m X 0.32 mm) Supelco column (Sigma-Aldrich, Canada) was used, and the samples were run at the following conditions: 200ºC for oven temperature; 240ºC for injector; and 260ºC for detector. The fatty acids peaks were identified using the retention time of PUFA-2 and PUFA-3 standards (Sigma-Aldrich, Canada); the concentration of each fatty acid was determined using the internal standard and expressed as the percentage nmol of the total extracted fatty acids.

Table 3.1 The composition of the standard used for Iatroscan calibration

	Lipid class	Concentration (g/L)	Abbreviation
Nanodecane	Hydrocarbon	1.360	HC
Cholesteryl Palmitate	Wax ester/steryl ester	1.060	WE/SE
3-Hexdecanone	Ketone	2.410	KET
Tripalmitin	Triacylglycerol	2.110	TAG
Palmitic Acid	Free fatty acid	1.030	FFA
Cetyl Alcohol	Alcohol	2.020	ALC
Cholesterol	Sterol	1.480	ST
Monopalmitoyl	Acetone mobile polar lipids	2.070	AMPL
Phosphatidylcholine Dipalmitoyl	PL	2.330	PL

3.4 Astaxanthin analysis of shrimp oil

Astaxanthin from shrimp extract was analyzed using thin layer chromatography (TLC) method (Núñez-Gastélum et al., 2016). Shrimp extract was spotted on pre-coated Silica gel-G plates (# 805012; Macherey-Nagel, Düren, NRW, Germany), along with free Astx and Astx-E standards (Sigma-Aldrich, Oakville, ON, Canada; # SML0982 and # 1044210, respectively) and eluted using a mobile phase of acetone: hexane (25:75, v/v). The spots corresponding to each fraction were scraped, collected in 250 μL of ethanol, vortexed (1 min), and the solution was decanted into a vial. Since astaxanthin is prone to oxidation, the procedure was undertaken with minimal exposure to light and samples were covered using aluminum foil. Astx content of each fraction was measured spectrophotometrically after separation using respective standard curves of free and Astx-E standards (Meyers and Bligh, 1981; Núñez-Gastélum et al., 2016). Ethanol was used as a blank. The absorbance was recorded at 472 nm using a spectrophotometer by following the method of Meyers and Bligh (Meyers and Bligh, 1981; Núñez-Gastélum et al., 2016).

3.5 3T3-L1 cell culture

3.5.1 Materials

The 3T3-L1 preadipocytes were obtained from American Type Culture Collection (ATCC, # CL-173, Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM; # 41965039), sodium pyruvate (100 mM; # 11360070), newborn calf serum (NBCS; # 26010074), and fetal bovine serum (FBS; # 12484028) were purchased from Gibco Life Technologies, Burlington, ON, Canada. Insulin solution (10 mg/mL in 25 mM HEPES, pH 8.2; # I0516), 3-isobutyl-1-methylxanthine (IBMX; # I5879), dexamethasone (Dex; # D4902), L-α-phosphatidylcholine (PC; # P3556), dimethyl sulfoxide (DMSO; # D2650), Menhaden fish oil (FO; # F8020), and glycerol (# G2025) were purchased from Sigma-Aldrich, Canada.

3.5.2 Culturing 3T3-L1 cells

3T3-L1 preadipocytes were maintained in culture medium (DMEM) containing 10% calf serum (NBCS) in a 5% CO_2, humidified environment at 37ºC. Once preadipocytes reached 70-80 % confluency, differentiation was induced using fresh medium containing DMEM + 10 % FBS, insulin (10 µg/mL), IBMX (0.5 mM), Dex (1 µM) and designated as Day 0 (Vaidya et al., 2013; Vaidya and Cheema, 2015), and cells were differentiated over an 8-day period. The differentiation medium was replaced with DMEM + 10% FBS and 10 µL/mL insulin after 48 hours (day 2). Then, culture media were changed to fresh DMEM + 10% FBS on day 4 and day 6 until day 8 of differentiation, with day 8 representing fully differentiated mature adipocytes, as determined by fat accumulation.

3.5.3 Lipid emulsions preparation for treating 3T3-L1 cells

Lipid emulsions were prepared (Anez-Bustillos et al., 2016) to treat 3T3-L1 cells because stable oil emulsions are necessary for efficient and effective delivery of oils. Lipid emulsions were prepared by using L-α-phosphatidylcholine (1.2%, w/v), glycerol (2.5%, w/v), fish oil or shrimp oil (10%, w/v), and autoclaved water, followed by ultrasonication using modification to the method of Meisel et. al (Meisel et al., 2011). To prepare the oil emulsions, autoclaved water was slowly added to a mixture of L-α-phosphatidylcholine and glycerol, followed by dropwise addition of the oil sample. The mixture was sonicated for 3 mins with 60 second intervals using a 22.5 KHz sonicator (MicrosonTM, Model XL-2000, Ultrasonic liquid processor, Newtown, CT, USA); the samples were kept on ice during the entire process. Fresh lipid emulsions were prepared for all experiments.

Size distribution of emulsion particles was characterized using dynamic light scattering (DLS) method (Cai et al., 2019) to confirm uniform distribution of the lipid emulsion. DLS measurements were carried out using a Zetasizer 1000Hs/3000Hs (Malvern Instruments, Malvern, WR, UK), which uses a helium neon laser light and integrated analysis software. The temperature was adjusted to 25°C, and the scattering angle was set to 90° before measurements. The data were expressed as the z-average (d. nm) and polydispersity index (PDI). The

size of the particles was in the nano-range with Z-average of 0.61 nm (Fig. 3.4). PDI value of 0.0 is considered as a perfectly uniform sample, whereas a value of 1.0 value is considered as a highly polydispersed sample with respect to the particle size (Danaei et al., 2018). PDI of the emulsion particles was 0.16 (Table 3.2), indicating a relatively uniform (monodisperse) distribution of the particles.

3.5.4 Treatment of 3T3-L1 cells with various concentrations of fish oil to establish the effect on cell differentiation

There is limited literature to show the effect of fish oil emulsions on differentiation of 3T3-L1 preadipocytes. I undertook a pilot experiment to establish the effect of various concentrations of fish oil on adipogenesis (differentiation) in 3T3-L1 adipocytes. Preadipocytes were differentiated to mature adipocytes in the presence or absence of fish oil emulsions for 8 days. The 3T3-L1 preadipocytes were grown in culture medium (DMEM + 10 % NBCS) in a 5 % CO2, humidified environment at 37ºC. Upon reaching 70-80 % confluency, differentiation was induced using fresh medium containing DMEM + 10 % FBS, insulin (10 µg/mL), IBMX (0.5 mM) and Dex (1 µM) (Day 0), along with fish oil emulsions as prepared in section 3.5.3 (0.25, 0.5, 0.75, 1 mg/mL culture medium). The differentiation medium was replaced with DMEM + 10% FBS and 10 µL/mL insulin, along with treatments after 48 hours (day 2). Culture media were changed to fresh DMEM + 10% FBS on day 4 and 6, along with treatments until day 8 of differentiation. The 3T3-L1 preadipocytes were also treated with various doses of PC (30, 60, 90, 120 µg/mL culture medium) that were used to prepare lipid emulsions. Untreated cells received culture media only. On day 8, the cells were viewed using a Leica DMIL LED Microscope (Leica Microsystems, Concord, ON, Canada) at 40x magnification, and Infinity Camera Analyze Software (version 6.5.5, Lumenera Corporation, Ottawa, ON, Canada) was used for capturing the images (Appendix II). Fish oil at a concentration of 0.75 and 1 mg/mL was toxic to the cells as cells started to detach by day 3. Fish oil at a concentration of 0.5 mg/mL showed cell detachment by day 8. However, fish oil at

a concentration of 0.25 mg/mL showed no adverse effects on 3T3-L1 cells till day 8 of differentiation (Appendix II). Thus, for my future cell culture experiments, I used a concentration of 0.25 and 0.5 mg/mL of oil emulsions.

Table 3.2 Polydispersity index of oil emulsion

Sample	T (°C)	Z-Ave (d.nm)	Polydispersity Index (PDI)	Scattering Angle (°)
Oil emulsion	25	0.61	0.16	90

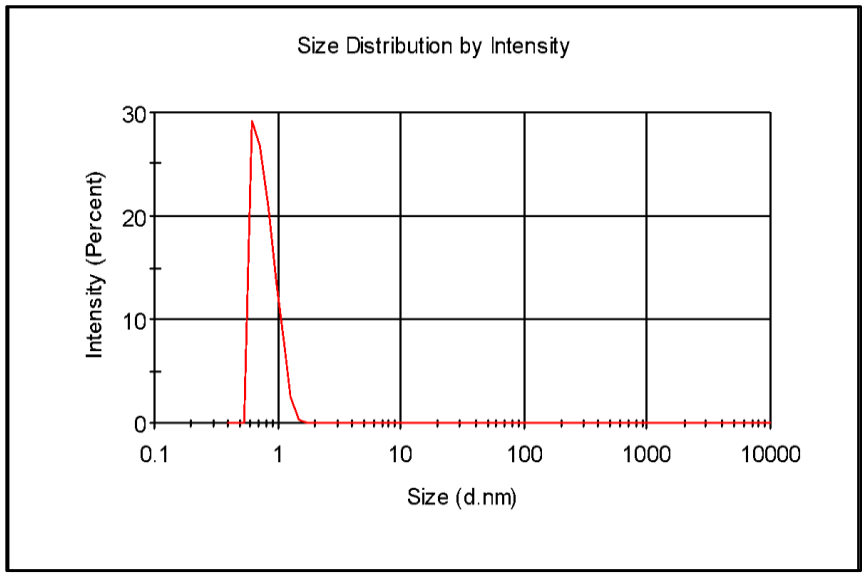


Figure 3.4. Analysis of particle size distribution of oil emulsion using dynamic light scattering (DLS)

DLS measurements were carried out using a Zetasizer 1000Hs/3000Hs (Malvern Instruments, Malvern, WR, UK) as described in the methods section. The data were expressed as the z-average (d. nm) and polydispersity index (PDI).

3.5.5 Cell metabolic activity

The cell metabolic activity was monitored to establish the concentration of oil emulsion treatments at which there is no adverse effect of the treatments on the cell metabolic activity of 3T3-L1 preadipocytes (Fig. 3.5). Preadipocytes with a density of 1 x 10^4 cells per well were seeded into 96 well microplates and incubated for 24 hours. The culture medium was then replaced with 200 µL of fresh culture media (DMEM + 10 % NBCS), along with different concentrations of the oil emulsions: SO, SE, and FO (0.125, 0.25, and 0.5 mg/mL culture medium). These concentrations of SO and SE contained 7.95, 15.9, and 31.8 ng Astx-E, respectively. Thus, cells were also treated with Astx-E (7.95, 15.9, and 31.8 ng/mL culture medium) and FO + Astx-E (0.125 mg + 7.95 ng, 0.25 mg + 15.9 ng, and 0.5 mg + 31.8 ng)/mL culture medium). The 3T3-L1 preadipocytes were also treated with various doses of the appropriate vehicles that were used to prepare lipid emulsions and/ or to dissolve astaxanthin: PC (15 µg, 30 µg, 60 µg/mL culture medium); DMSO (0.03%, 0.06%, 0.1%), and PC + DMSO [(15 µg + 0.03%, 30 µg + 0.06%, 60 µg + 0.1%)/mL culture medium]. In another set of experiments, cells were treated with different concentrations of oil extracts: FOS, FS1, FS2, FS3 (0.125 mg, 0.25 mg, 0.5 mg/mL culture medium) (Fig. 3.6), and also various doses of the appropriate vehicle (PC at 15, 30, and 60 µg/mL culture medium). Untreated cells received cell culture media only.

The cells were incubated for 48 hours, and then the MTT colorimetric assay was performed to measure the cell metabolic activity (Hadrich and Sayadi, 2018). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) was dissolved in 1X phosphate-buffered saline (PBS) at a concentration of 5 mg/mL. MTT media was prepared by adding 10 % MTT solution in culture media (DMEM + 10 % NBCS); then 200µL of MTT media was added to each well, and the plates were incubated for 6 hours at 37 ºC in a 5 % CO2 incubator. The media were removed, and 200 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, with further incubation for 30 minutes at 37ºC. The absorbance was recorded

at 570 nm using a spectrophotometer. The cell metabolic activity was calculated as percentage fold change with respect to the untreated control cells as detailed below:

Cell metabolic activity (%) = [Absorbance 570 (sample)/Absorbance 570 (control)] x 100 %

Vehicles used in the treatments (PC, DMSO, PC+DMSO) revealed no effect on the cell metabolic activity, compared to the untreated cells (Fig. 4.2A). Furthermore, various doses of individual treatments (SE, SO, Astx-E, FO, and FO + Astx-E) had no effect on the cell metabolic activity, compared to untreated cells (Fig. 4.2B). Similarly, various doses of FOS, FS1, FS2 and FS3 showed no effect on the cell metabolic activity, compared to untreated cells (Fig. 4.7B). However, considering the effects of fish oil emulsions on cell differentiation in the above section 3.5.4, I used a concentration of 0.25 mg/mL culture medium of the oil emulsions for my future experiments knowing that this concentration had no effect on the cell metabolic activity.

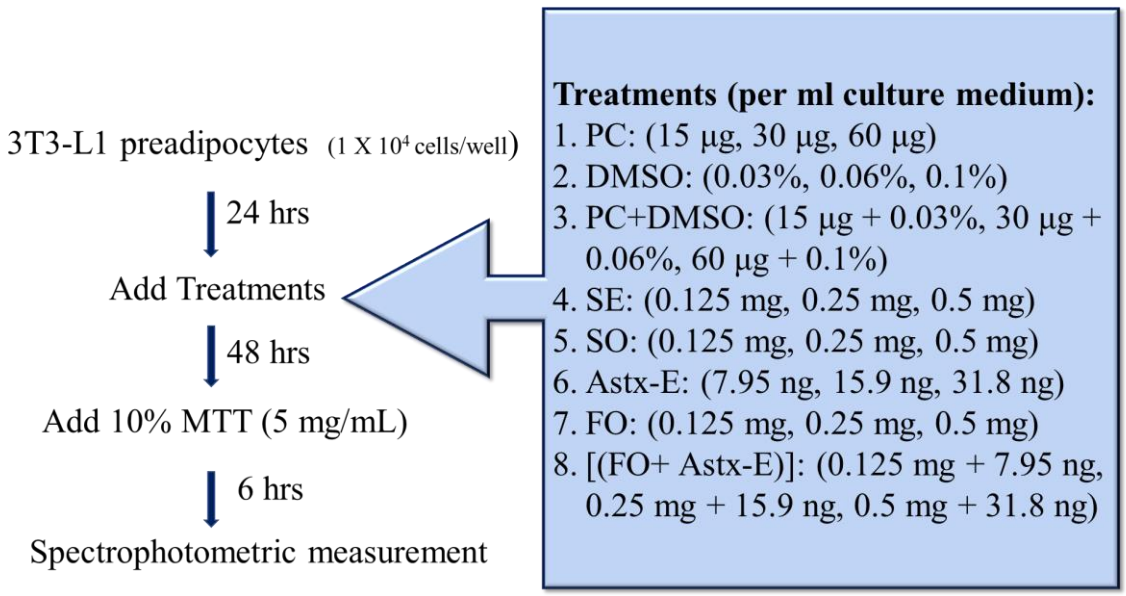


Figure 3.5. Experimental design to investigate the effect of shrimp oil and fish oil with or without Astx-E on cell metabolic activity of 3T3-L1 preadipocytes. *PC: l-α-phosphatidylcholine; DMSO: Dimethyl sulfoxide; PC + DMSO: L-α-phosphatidylcholine + dimethyl sulfoxide; SE: Shrimp extract; SO: Shrimp oil; Astx-E: Esterified astaxanthin; FO: Fish oil; FOA: FO + Astx-E.*

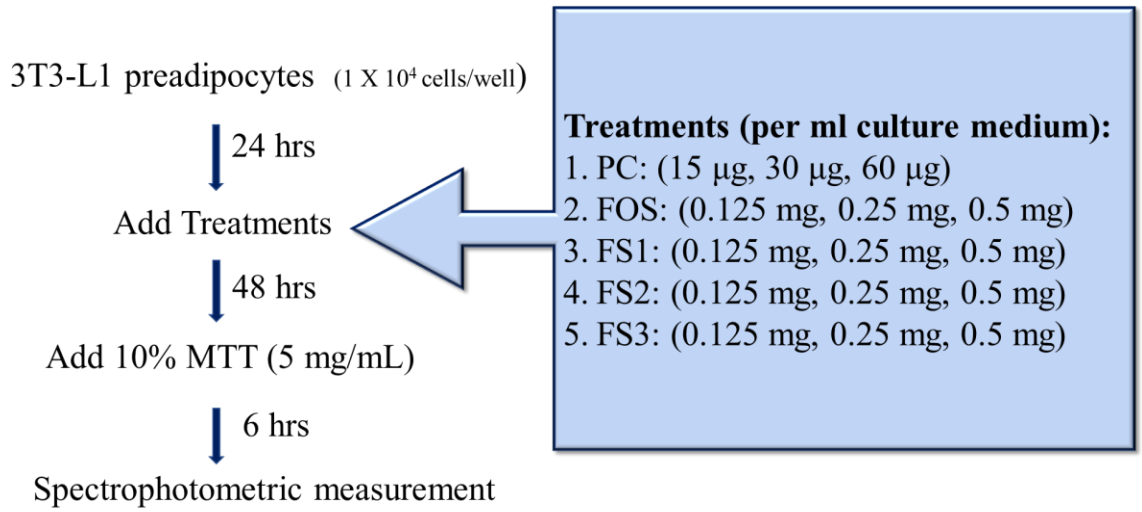


Figure 3.6. Experimental design to investigate the effect of oil extracts on cell metabolic activity of 3T3-L1 preadipocytes. *PC: l-α-phosphatidylcholine; FOS: fish waste oil, FS1: 50ºC oil extract sample 1, FS2: 60ºC oil extract sample 2, FS3: 70ºC oil extract sample 3.*

3.5.6 Treatments of 3T3-L1 preadipocytes with shrimp oil and fish oil emulsions to measure fat accumulation and the expression of adipogenic and lipogenic genes

The 3T3-L1 preadipocytes were grown in culture medium (DMEM + 10 % NBCS) in a 5 % CO2, humidified environment at 37ºC. Once preadipocytes reached 70-80 % confluency, differentiation was induced using fresh media containing DMEM + 10 % FBS, insulin (10 µg/mL), IBMX (0.5 mM) and Dex (1 µM) (Day 0), along with the treatments. The differentiation media was replaced with DMEM + 10% FBS and 10 µL/mL insulin, along with each treatment after 48 hours (day 2). Then, culture media were changed to fresh DMEM + 10% FBS on day 4 and day 6 with each treatment until day 8 of differentiation. Preadipocytes were differentiated to mature adipocytes in the presence or absence of shrimp extract, shrimp oil or fish oil emulsions at a final concentration of 0.25 mg/mL, for 8 days (Fig. 3.7). This concentration of shrimp oil contained 15.9 ng Astx-E; thus, cells were also treated with 15.9 ng/mL of Astx-E, and fish oil plus 15.9 ng/mL of Astx-E. Cells also received the appropriate vehicles (30 µg/mL PC, 0.06 % DMSO, and 30 µg/mL + 0.06 % PC+DMSO). Untreated cells received cell culture media only. Fully differentiated cells were washed with 1X PBS and harvested to perform Oil Red O staining to study fat accumulation, and total RNA extraction to measure gene expression.

In an independent set of experiments, preadipocytes were differentiated to mature adipocytes in the presence or absence of FOS, FS1, FS2 and FS3 oil emulsions at a final concentration of 0.25 mg/mL, for 8 days (Fig. 3.8). Cells also received appropriate vehicle (30 µg/mL PC), and untreated cells received cell culture media only. Fully differentiated cells were washed with 1X PBS and harvested to perform Oil Red O staining to study fat accumulation, and total RNA was extracted to measure gene expression.

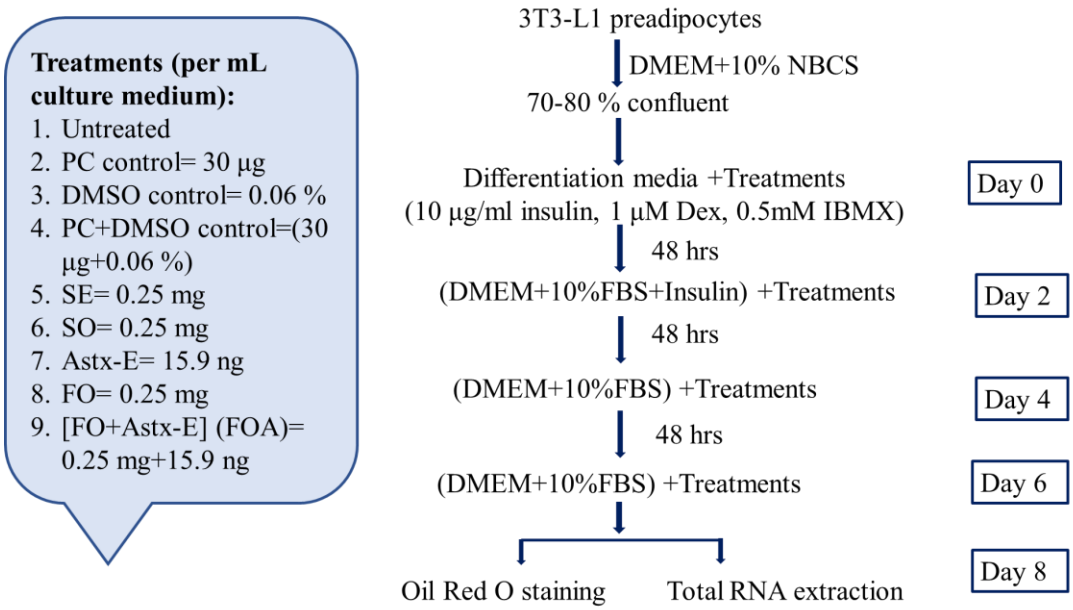


Figure 3.7. Experimental design to investigate the effects of shrimp oil and fish oil with or without Astx-E on adipogenesis in 3T3-L1 adipocytes. *The 3T3-L1 preadipocytes were differentiated to mature adipocytes in the presence or absence of various treatments for 8 days, fat accumulation and the mRNA expression of genes involved in adipogenesis, and lipogenesis were studied. Untreated: Untreated cells received cell culture media only; PC: l-α-phosphatidylcholine; DMSO: Dimethyl sulfoxide; PC + DMSO: L-α-phosphatidylcholine + dimethyl sulfoxide; SE: Shrimp extract; SO: Shrimp oil; Astx-E: Esterified astaxanthin; FO: Fish oil; FOA: FO + Astx-E; DMEM: Dulbecco's modified eagle medium; NBCS: Newborn calf serum; Dex: Dexamethasone; IBMX: 3-isobutyl-1-methylxanthine; FBS: Fetal bovine serum.*

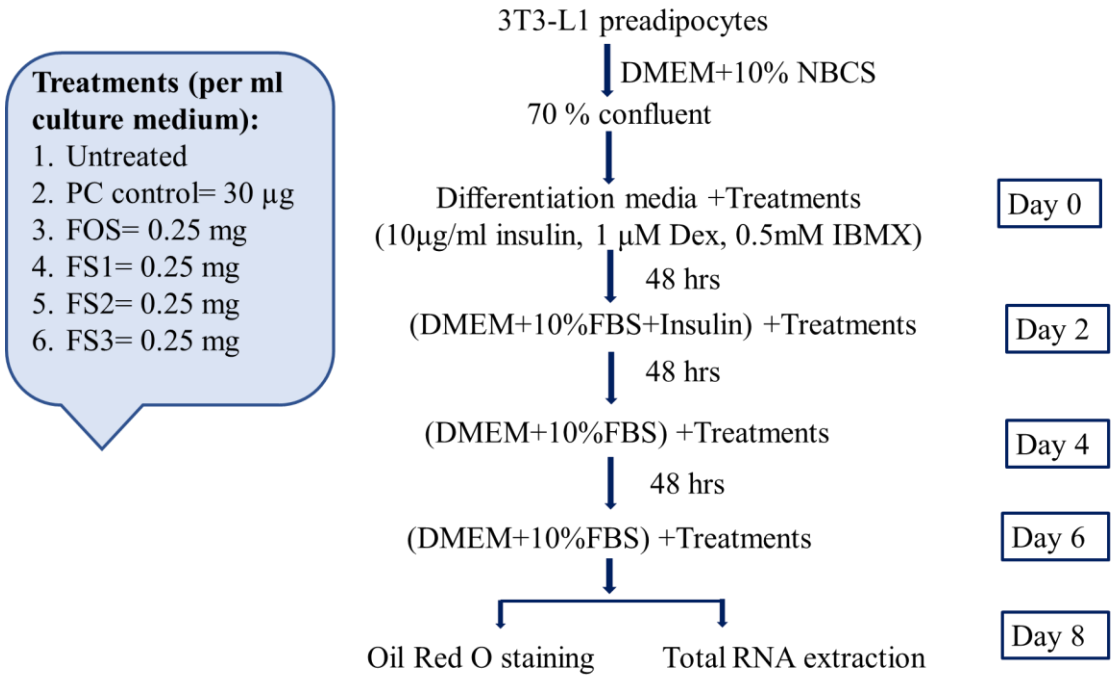


Figure 3.8. Experimental design to investigate the effects of oil extracts on adipogenesis in 3T3-L1 adipocytes. *The 3T3-L1 preadipocytes were differentiated to mature adipocytes in the presence or absence of various treatments for 8 days, fat accumulation and the mRNA expression of genes involved in adipogenesis, and lipogenesis were studied. Untreated: Untreated cells received cell culture media only; PC: l-α-phosphatidylcholine; FOS: fish oil extracted from waste fish, FS1: 50ºC oil extract sample 1, FS2: 60ºC oil extract sample 2, FS3: 70ºC oil extract sample 3; DMEM: Dulbecco's modified eagle medium; NBCS: Newborn calf serum; Dex: Dexamethasone; IBMX: 3-isobutyl-1-methylxanthine; FBS: Fetal bovine serum.*

3.6 Oil Red O staining of mature 3T3-L1 cells

Oil Red-O staining and quantification was performed on day 8 of differentiation. Lipid accumulation in 3T3-L1 adipocytes was investigated using an Oil Red O solution (0.5 % in isopropanol; # O1391, Sigma-Aldrich, Canada). The working standard Oil Red O solution was prepared by mixing 6 parts of the stock solution with 4 parts of distilled water, and staining was performed as per the manufacturer instructions (Maeda et al., 2006). On day 8 of differentiation, cells were washed thrice with 1X PBS, and 1 mL of Oil Red O working solution was added to each well and incubated for 15 min at room temperature. The dye was then removed, and cells were washed with 2 mL of distilled water to remove any non-binding dye. Oil Red O-stained cells were viewed using a Leica DMIL LED Microscope at 400x magnification, and Infinity Camera Analyze Software (version 6.5.5) was used for capturing the images of stained cells. Extraction of Oil Red O-stained lipids was carried out by adding 500 µL of 100 % isopropanol to each well, and the plate was placed on a shaker for 30 min. The absorbance of extracted dye was determined using a spectrophotometer at 520 nm; 100 % isopropanol was used as a blank.

3.7 Total RNA extraction

Total RNA was extracted from mature, fully differentiated 3T3-L1 adipocytes on day 8 using TRIzol (Invitrogen, Carlsbad, CA, USA) method (Chomczynski and Sacchi, 2006). The removal of genomic DNA contamination was carried out using the DNase treatment (Promega, Wisconsin, USA). Nanodrop 2000 (Thermo Scientific, USA) was used to determine the concentration and purity (260/280) of extracted RNA, and RNA integrity was confirmed using 1.2 % agarose gel. Reverse transcription kit (#A3500, Promega, Wisconsin, USA) was used to synthesize cDNA, from 1 µg of total RNA. Preparation of the Master mix was carried out by mixing respective volumes of 25 mM $MgCl_2$, 10X reverse transcription buffer, 10 mM dNTP mixture, 0.5 µL Recombinant RNasin ribonuclease inhibitor, 15 u AMV reverse transcriptase, and random primers (0.5 µg/mL). Master Mix was added to the DNase free RNA and incubated at room temperature for 10 min. Samples were

placed in Eppendorf PCR machine for cDNA synthesis with appropriate parameters set as: 45ºC for 15 minutes, 95ºC for 5 minutes and 5ºC for 5 minutes. The synthesized cDNA was stored at -20ºC until further analysis.

3.8 Real-time quantitative polymerase chain reaction

Gene expression analysis was performed using the Bio-Rad CFX96^{TM} Real-Time System. Primers used in real-time quantitative polymerase chain reaction (qPCR) were designed using NCBI primer blast. All primers were purchased from IDT Technologies (Coralville, IA, USA), and the efficiency of all primers was within the acceptable range of 90-110 %. Primer sequences are presented in Table 3.3. Amplification was carried out using iQ SYBR Green Supermix (# 1708880, Bio-Rad, Hercules, CA, USA), and a reaction volume was 10 µL with 50 ng of cDNA per reaction. Each reaction was run for 40 cycles and appropriate parameters were set as: denaturation at 95ºC for 15 mins, annealing at 58-60ºC for 15 seconds and extension at 72ºC for 15 seconds. Data analysis were carried out using the CFX Manager TM Software Version 3.0 (Bio-Rad, Hercules, CA, USA). The delta Ct values for each gene of interest were obtained. Expression of target genes was normalized to RPLP0 as the reference gene, a large ribosomal protein. Expression of target genes were calculated using ΔΔCt method (Livak and Schmittgen, 2001).

3.9 Effect of astaxanthin on oxidation of fish oil

Menhaden fish oil and Astx-E from *H. pluvialis* were purchased from Sigma-Aldrich (Ontario, Canada, # F8020 and # 1044210, respectively). The oxidation of fish oil, alone or in combination with different concentrations of Ast-E, was measured using peroxidation value (PV) index. All procedures were performed in the dark to protect Astx-E from oxidation. Fish oil alone (10 g), or in combination with Astx-E (50, 75, 100 µg/g of oil), were aliquoted in glass test tubes and incubated at 37ºC in a forced air incubator. Samples were collected at 0, 4, 8, 12, 24 and 48 hrs., and stored at -20ºC until further analysis. Oxidation of the samples was measured using PV assay according to the European pharmacopoeia (Pharmacopoeia, 2005) with modifications. Oil (400 mg) was taken in a conical flask and 5 ml of 3:2 (glacial acetic acid: trimethylpentane) was added with 1 to 2

drops of Tween-40 and swirled it till a clear solution was obtained. Then, saturated potassium iodide solution (100 μL) was added to the mixture, and the flask was covered with aluminum foil and vigorously stirred for 60 seconds followed by adding 3 ml distilled water. Starch solution (1% in distilled water; 200 μL) was added as an indicator. The blueish-yellow solution was titrated against 0.01 N sodium thiosulphate solution until it becomes almost colorless. The volume of the sodium thiosulphate solution was used to estimate the PV in Milliequivalents/kg (mEq/kg) as follows,

$$PV = [1000 \times (V - V_{control})] \times C/m$$

Where, C is the concentration of titrant, V is the volume of titrant, and m is the mass of the oil. All samples were tested in triplicates.

3.10 Statistical analysis

The GraphPad Prism Software, Version 8 (GraphPad Software, San Diego, CA, USA), was used for analyzing all data. Gene expression data were analyzed using one-way ANOVA, followed by Tukey's post-hoc test. The mRNA expression data were expressed in both fold change and Log2 fold change. The data was transformed to Log2 fold change to clearly observe the change in terms of higher or lower mRNA expression with the treatments. Results were expressed as mean ± standard deviation (SD), n=3 (each experiment was repeated three times with three independent wells); $p<0.05$ was considered significant. Superscripts (a, b, c) represent significant differences. PV assay data were assessed using two tailed paired student's t-test, n=3; $P<0.05$ was considered significant. *Represent significant differences at a given time point.

Table 3.3 Primer sequences for Real-time quantitative polymerase chain reaction

Gene	Sequence		Ascension No
Pparγ	5'-GAGCTGACCCAATGGTTGCTG-3'	Forward	XM_017321456.1
	5'-GCTTCAATCGGATGGTTCTTC-3'	Reverse	
Srebp1c	5'-CGGCTCTGGAACAGACACTG-3'	Forward	NM_001313979.1
	5'-TGAGCTGGAGCATGTCTTCG-3'	Reverse	
Scd1	5'-CACCTGCCTCTTCGGGATTT-3'	Forward	NM_009127.4
	5'-CTTGACAGCCGGGTGTTTG-3'	Reverse	
Dgat2	5'-CTGCTGTTGGCTGGTTTCAC-3'	Forward	NM_026384.3
	5'-CAGGAGGATATGCGCCAGAG-3'	Reverse	
Fasn	5'-CTGCGGAAACTTCAGGAAATG-3'	Forward	NM_007988.3
	5'-GGTTCGGAATGCTATCCAGG-3'	Reverse	
Glut-4	5'-GATTCTGCTGCCCTTCTGTC-3'	Forward	AB_008453.1
	5'-ATTGGACGCTCTCTCTCCAA-3'	Reverse	
Rplp0	5'-AATTTCAATGGTGCCTCTGG-3'	Forward	NM_007475.5
	5'-TCACTGTGCCAGCTCAGAAC-3'	Reverse	

Primers used in qPCR were designed using NCBI primer blast and purchased from IDT Technologies. Pparγ: peroxisome proliferator-activated receptor-gamma; Srebp1c: sterol regulatory element-binding protein 1; Scd1: stearoyl-CoA desaturase 1; Dgat2: diacylglycerol O-acyltransferase 2; Fasn: fatty acid synthase; Glut-4: Glucose transporter type 4; Rplp0: ribosomal protein large.

CHAPTER FOUR

Results

4.1 The composition of shrimp oil extracted from shrimp processing by-product using the Soxhlet method

4.1.1 Lipid composition of shrimp oil

Shrimp oil extracted from shrimp processing by-product was analyzed for total lipids and lipid class composition. The total lipids content was 3.92 mg/g wet shrimp processing by-product. Lipid composition showed that shrimp oil is rich in phospholipids (64.20 Wt. %), as given in Table 4.1. Other lipid components included TAG (13.66 Wt. %), alcohols (4.46 Wt. %) and sterols (21.18 Wt. %). Shrimp oil also contained a small amount of wax/steryl esters (0.67 Wt. %), methyl esters (0.56 Wt. %), ethyl esters (1.32 Wt. %); and free fatty acids (0.33 Wt. %).

4.1.2 Fatty acids composition of oils

Shrimp oil contained 18.33 % SFA, while MUFA was 40.58 %, and PUFA was 41.08 % (Table 4.2). SFA comprised mainly of C16:0 (15.73 %) and C18:0 (2.42 %), while MUFA comprised mainly of C16:1n7 (9.58 %), C18:1n9 (21.33 %) and C18:1n7 (6.49 %).

Shrimp oil was rich in n-3 PUFA (37.09 %) (Table 4.2), of which 21% was EPA (C20:5n3) and 13.89 % was DHA (C22:6n3). Shrimp oil also contained a small amount of total n-6 PUFA 3.99 % (Table 4.2), mainly comprising of C18:2n6 (1.96 %), and C20:4n6 (1.69 %).

FO contained 23.85% SFA, 32.82% MUFA, and 43.32% PUFA (Table 4.2). SFA comprised of C16:0 (21.03%) and C18:0 (2.73%), while MUFA comprised mainly of C16:1n7 (18.30%) and C18:1 (14.26%). FO was high in n-3 PUFA (38.88%), of which 20.14% was EPA (C20:5n3) and 13.77% was DHA (C22:6n3). FO contained a small amount of total n-6 PUFA (4.44%), mainly comprising of C18:2n6 (1.95%) and C20:4n6 (1.62%).

4.1.3 Astaxanthin content of shrimp oil

TLC analysis confirmed the presence of Astx in shrimp oil, along with other unknown carotenoids (Fig. 4.1). Soxhlet extraction recovered 4.38 mL shrimp extract/g of shrimp processing by-product. Yields of free Astx and Astx-E were 24.03 μg and 187.76 μg/g of shrimp processing by-product, respectively (Table. 4.3). Astx-E content was almost 8 times higher than free Astx, indicating that shrimp oil is rich in Astx-E; thus, the following experiments focused on Astx-E. I also performed preliminary analysis of Astx in shrimp oil and shrimp extract using mass spectrometry (MS) (Appendix VI). My preliminary investigation confirmed the presence of Astx-E; however, free Astx product fragments could not be detected in my preliminary shrimp extract. I had planned to perform detailed MS characterization of the respective Astx fractions from TLC analysis; however further analysis could not be conducted due to Covid-19 lockdown.

Table 4.1 Lipid composition of shrimp oil extracted from shrimp processing by-product using Soxhlet method

Lipid Composition	**Shrimp oil Wt. (%)**
Wax/Steryl esters	0.67±0.09
Methyl esters	0.56±0.06
Ethyl esters	1.32±0.11
Triacylglycerols	13.66±5.35
Free fatty acids	0.33±0.47
Alcohols	4.46±0.36
Sterols	21.18±1.69
Phospholipids	64.20±4.68

Total lipids were extracted and analyzed as explained in the methods section. Data are expressed as the percentage weight of the total extracted lipids, values are expressed as mean ± SD, n = 2.

Table 4.2 Fatty acids composition of shrimp oil extracted from shrimp processing by-product using the Soxhlet method, and fish oil

Fatty Acids (%)	**Shrimp Oil**	**Fish Oil**
C14:0	0.17 ± 0.00	0.09 ± 0.00
C16:0	15.73 ± 0.33	21.03 ± 0.06
C16:1n7	9.58 ± 0.65	18.30 ± 0.36
C18:0	2.42 ± 0.07	2.73 ± 1.13
C18:1n9	21.33 ± 1.03	5.95 ± 3.51
C18:1n7	6.49 ± 1.56	8.30 ± 5.78
C18:2n6	1.96 ± 0.12	1.95 ± 0.04
C18:3n6	0.30 ± 0.09	0.55 ± 0.01
C18:3n3	0.61 ± 0.08	1.69 ±0. 03
C20:1n9	0.45 ± 0.20	0.27 ±0. 00
C20:4n6	1.69 ± 0.14	1.62 ±0. 03
C20:5n3	21.10 ± 0.11	20.14 ± 0.4
C22:4n6	0.03 ± 0.05	0.33 ±0. 00
C22:5n3	1.48 ± 0.11	3.28 ±0. 07
C22:6n3	13.89 ± 0.13	13.77 ± 0.28
∑SFA	18.34 ± 0.25	23.85 ± 1.06
∑MUFA	40.58 ± 0.09	32.82 ± 1.89
∑n-3 PUFA	37.09 ± 0.04	38.88 ± 0.8
∑n-6 PUFA	3.99 ± 0.11	4.44 ± 0.03

The fatty acids composition of shrimp oil and fish oil was measured as described in the Methods section. Data are expressed as the percentage nmol of the total extracted fatty acids; values are expressed as mean ± SD, n = 2. ΣSFA: Sum of saturated fatty acids, ΣMUFA: Sum of monounsaturated fatty acids, ΣPUFA: Sum of polyunsaturated fatty acids, Σn-3 PUFA: Sum of omega-3 PUFA, Σn-6 PUFA: Sum of omega-6 PUFA.

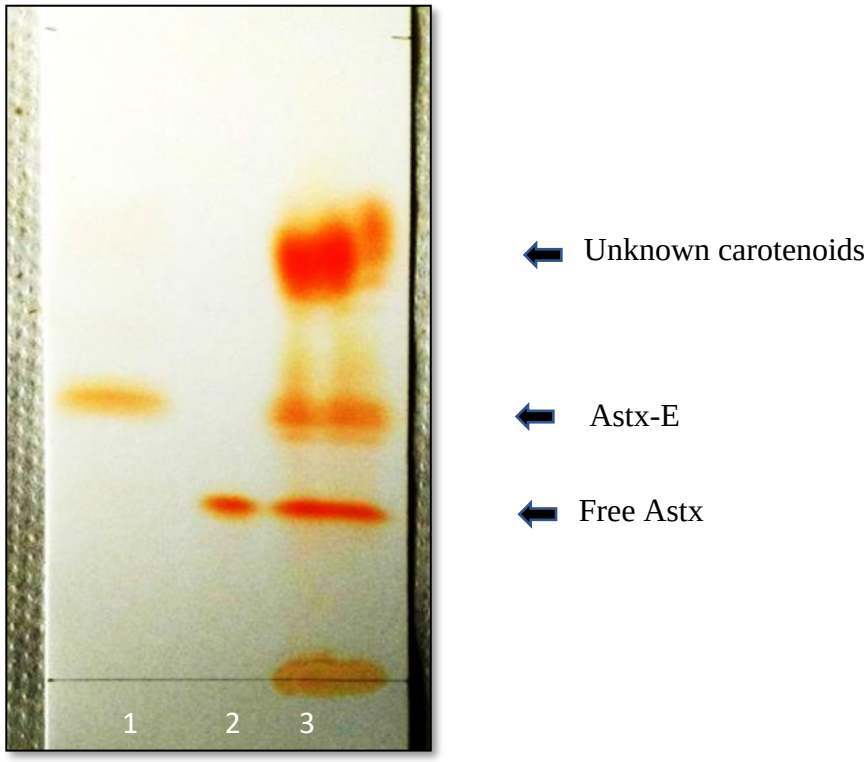


Figure 4.1. Analysis of astaxanthin from shrimp oil using thin layer chromatography (TLC)

Astx-E standard (400 PPM) (lane 1), free Astx standard (40 PPM) (lane 2), and shrimp extract (lane 3) were spotted on pre-coated Silica gel-G plates and separated using details provided in the methods section. Free Astx= free astaxanthin, Astx-E=esterified astaxanthin

Table 4.3 Astaxanthin content of shrimp extract

Fraction	Concentration (µg/mL Shrimp Extract)	Astaxanthin yield (µg/g Shrimp Processing By-Product)
Free Astx	8.24	24.03
Astx-E	64.37	187.76

The astaxanthin spots corresponding to each fraction were scrapped from TLC plates, and analyzed spectrophotometrically as mentioned in the methods section. Free Astx: Free astaxanthin, Astx-E: Esterified astaxanthin.

4.2 Effect of shrimp oil extracted from shrimp processing by-product using the Soxhlet method on fat accumulation and adipogenesis in 3T3-L1 adipocytes

4.2.1 Effect of lipid emulsions on the cell metabolic activity of 3T3-L1 preadipocytes

The 3T3-L1 preadipocytes treated with various doses of respective vehicles used in the treatments (PC, DMSO, PC+DMSO) revealed no statistical difference on the cell metabolic activity, compared to the untreated cells (Fig. 4.2A). Furthermore, there was no difference in the cell metabolic activity of 3T3-L1 preadipocytes treated with various doses of individual treatments (SE, SO, Astx-E, FO, and FO+ Astx-E), compared to untreated cells (Fig. 4.2B).

4.2.2 Shrimp extract decreased, while fish oil increased fat accumulation in 3T3-L1 mature adipocytes

Preadipocytes were differentiated to mature adipocytes in the absence or presence of various treatments for 8 days. Oil Red O staining of mature 3T3-L1 adipocytes treated with SO, SE and Astx-E revealed a lower fat accumulation, compared to untreated cells; while FO and FOA showed greater fat accumulation, compared to untreated cells (Fig. 4.3A). Quantification of extracted Oil Red O confirmed that SE revealed significantly lower ($p=0.01$) fat accumulation, while FO and FOA had significantly greater ($p=0.01$) fat accumulation, compared to untreated cells (Fig. 4.3B). Interestingly, quantification of extracted Oil Red O showed no significant effect on fat accumulation after treatment with SO and Astx-E, compared to control cells, even though Oil Red O staining revealed lower fat accumulation.

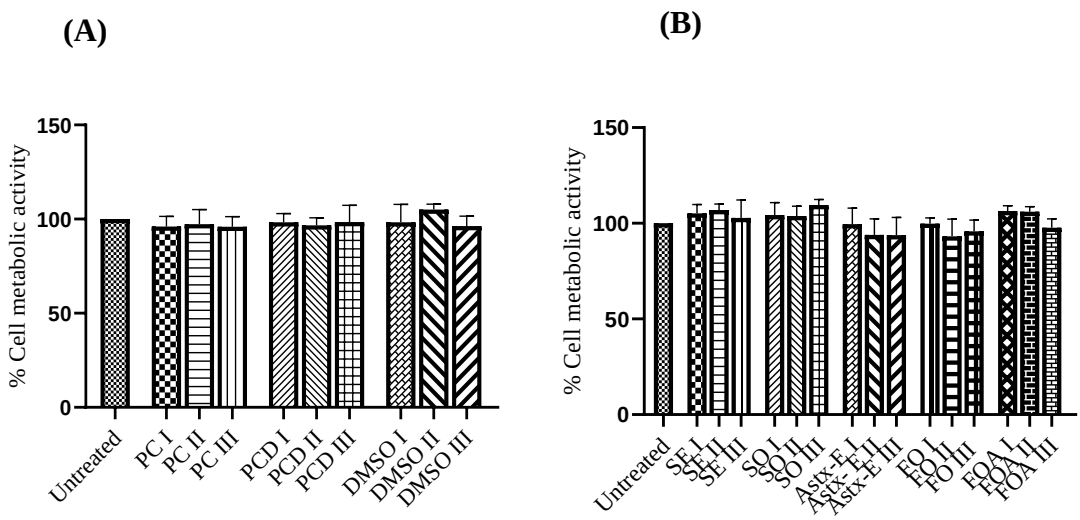
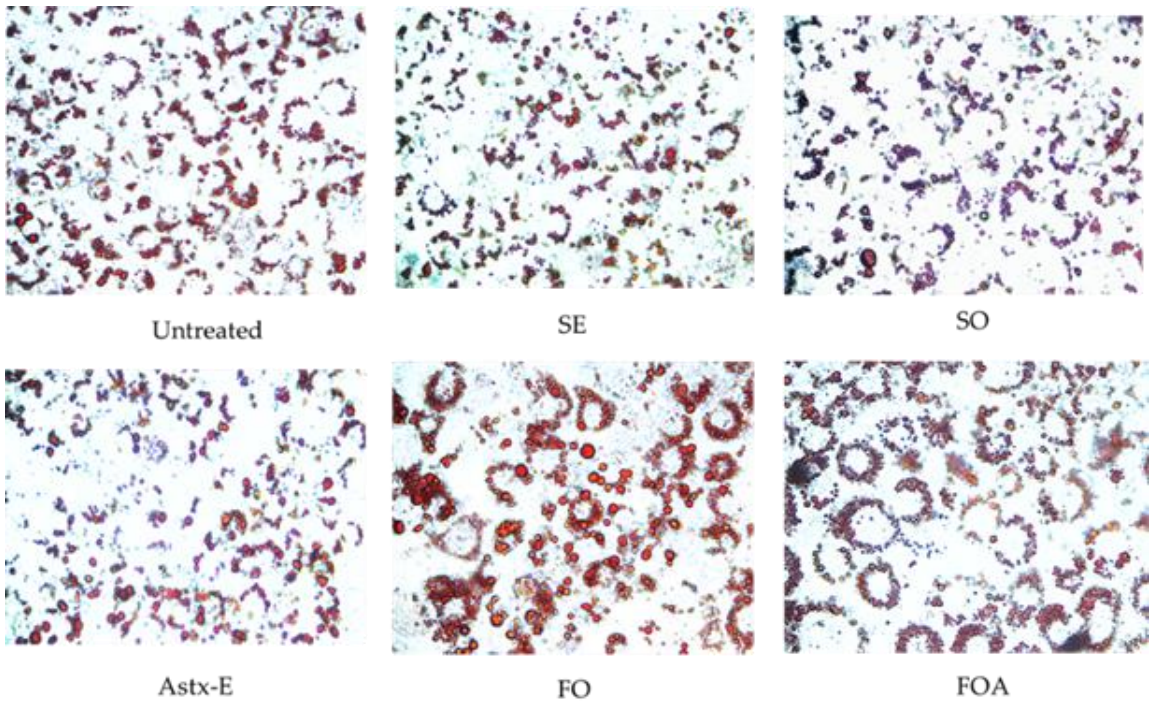


Figure 4.2. Cell metabolic activity of 3T3-L1 preadipocytes treated with various concentrations of vehicles and treatments

The cell metabolic activity of 3T3-L1 preadipocytes was measured using MTT assay after treatment with various concentrations of the vehicles and treatments, as explained in the methods section. (A) Vehicles: PC, DMSO, PCD; (B) Treatments: SE, SO, Astx-E, FO, and FO+Astx-E. Data were analyzed using one-way ANOVA to determine significance ($P<0.05$). Values are expressed as means ± SD, n=3. Untreated: Untreated cells Untreated cells received cell culture media only, PC: L-α-phosphatidylcholine (15, 30, and 60 μg/mL culture medium), PCD: PC+DMSO [(15 μg + 0.03%, 30 μg + 0.06%, 60 μg + 0.1%)/mL culture medium], DMSO: dimethyl sulfoxide (0.03, 0.06, and 0.1%), SE: shrimp extract (0.125, 0.25, and 0.5 mg/mL culture medium), SO: shrimp oil (0.125, 0.25, 0.5 mg/mL culture medium), Astx-E: esterified astaxanthin (7.95, 15.9, and 31.8 ng/mL culture medium), FO: fish oil (0.125, 0.25, and 0.5 mg/mL culture medium), FOA: FO+Astx-E [(0.125 mg + 7.95 ng, 0.25 mg + 15.9 ng, 0.5 mg + 31.8 ng)/mL culture medium].

(A)



(B)

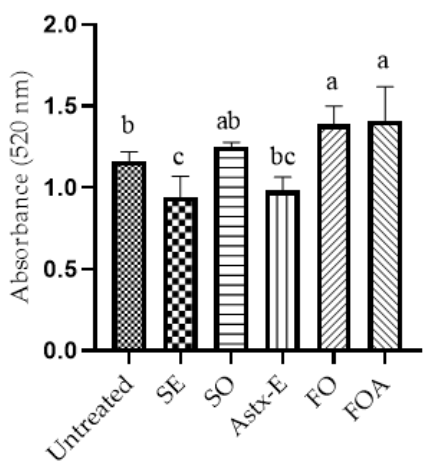


Figure 4.3. Shrimp extract decreased, while fish oil increased fat accumulation in 3T3-L1 mature adipocytes

Preadipocytes were differentiated to mature adipocytes in the presence or absence of various treatments for 8 days; Oil Red O staining and quantification was performed as explained in the methods section. (A) representative images of the cells stained with Oil Red O on day 8 as explained in the methods section (400 X magnification); (B) relative fat accumulation measured spectrophotometrically as explained in the methods

section. Values are expressed as mean ± SD, n = 3. Data were analyzed using one-way ANOVA and Tukey's post-hoc test; $p<0.05$ was considered significant. Superscripts (a, b, c) represent significant differences. Untreated: Untreated cells received cell culture media only, SE: shrimp extract, SO: shrimp oil, Astx-E: esterified astaxanthin, FO: fish oil, FOA: FO+Astx-E.

4.2.3 Shrimp oil decreased, while fish oil increased the mRNA expression of *Pparγ* and *Srebp1c* in mature 3T3-L1 adipocytes

The mRNA expression of *Pparγ* was significantly lower in 3T3-L1 mature adipocytes after treatment with SO (p=0.0006), SE (p=0.004), and Astx-E (p=0.0001), compared to untreated cells (Fig. 4.4A). However, the mRNA expression of *Pparγ* was significantly higher after treatment with FO (p=0.01) and FOA (p=0.01) (Fig. 4.4A). Both FO and FOA had higher mRNA expression of *Pparγ,* compared to SO, SE and Astx-E ($p<0.05$). There was no effect of respective doses of the vehicle controls (PC, DMSO, PC+DMSO) on the mRNA expression of *Pparγ*, compared to the untreated cells (Appendix III-A).

The mRNA expression of *Srebp1c* was significantly lower in 3T3-L1 mature adipocytes after treatment with SO (p=0.0004), SE ($p<0.0001$), and Astx-E (p=0.001), compared to untreated cells (Fig. 4.4B). Treatment with SO, SE and Astx-E also revealed significantly lower ($p<0.0001$) mRNA expression of *Srebp1c*, compared to FO. However**,** the mRNA expression of *Srebp1c* was significantly higher after treatment with FO (p=0.01), compared to untreated cells (Fig. 4.4B). There was no statistical difference between the cells treated with FOA and untreated normal control (Fig. 4.4B); however, FOA revealed lower ($p<0.05$) mRNA expression of *Srebp1c,* compared to FO (Fig. 4.4B). There was no effect of respective doses of the vehicle controls (PC, DMSO, PC+DMSO) on the mRNA expression of *Srebp1c*, compared to the untreated cells (Appendix III-B).

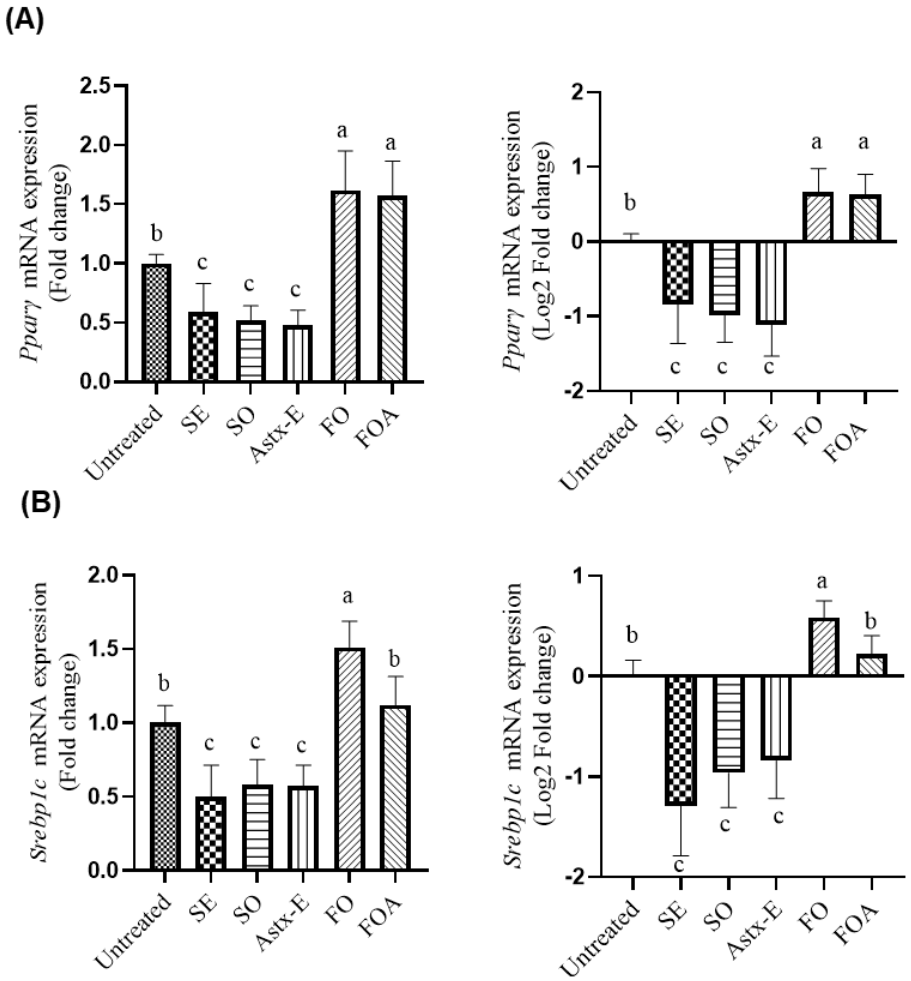


Figure 4.4. Shrimp oil decreased, while fish oil increased the mRNA expression of Pparγ and Srebp1c in 3T3-L1 mature adipocytes

The cells were differentiated to mature adipocytes, for 8 days, as explained in the methods section. Total RNA was extracted, and the mRNA expression analysis of (A) Peroxisome proliferator-activated receptor (Pparγ), (B) Sterol regulatory element-binding protein (Srebp1c) was measured as explained in the methods section and normalized to RPLP0 as the reference gene. The mRNA expression data were expressed in both fold change and Log2 fold change. Data were analyzed using one-way ANOVA and Tukey's post-hoc test. $p<0.05$ was considered significant. Superscripts (a, b, c) represent significant differences, n=3. Untreated: Untreated cells received cell culture media only, SE: shrimp extract (0.25 mg/mL culture medium), SO: shrimp oil (0.25 mg/mL culture medium), Astx-E: esterified astaxanthin (15.9 ng/mL culture medium), FO: fish oil (0.25 mg/mL culture medium), FOA: FO+Astx-E [(0.25 mg + 15.9 ng)/mL culture medium].

4.2.4 Shrimp oil decreased, while fish oil increased the mRNA expression of lipogenic genes in mature 3T3-L1 adipocytes

The mRNA expression of *Dgat2* was significantly lower after treatment with SO (p=0.01) and SE (p<0.0001), compared to untreated cells (Fig. 4.5A). *Dgat2* mRNA expression was also significantly lower after treatment with SO and SE (p<0.0001), compared to FO. There was no effect of Astx-E treatment on the expression of *Dgat2*, compared to untreated cells. Interestingly, FO treatment showed significantly higher (p<0.05) mRNA expression of *Dgat2*, compared to untreated cells (Fig. 4.5A). There was no statistical difference between the cells treated with FOA and untreated cells (Fig. 4.5A); however, treatment with FOA had lower (p<0.05) mRNA expression of *Dgat2*, compared to FO (Fig. 4.5A). There was no effect of respective doses of the vehicle controls (PC, DMSO, PC+DMSO) on the mRNA expression of *Dgat2*, compared to untreated cells (Appendix III-C).

Vehicle controls (PC, DMSO, PC+DMSO) had no effect on *Fasn* mRNA expression, compared to untreated cells (Appendix III-D). SO, SE and Astx-E had no effect on the mRNA expression of *Fasn*, compared to untreated cells (Fig. 4.5B). However, FO (p=0.01) and FOA (p=0.007) showed significantly higher mRNA expression of *Fasn*, compared to untreated cells (Fig. 4.5B).

SO, SE and Astx-E had no significant effect on the mRNA expression of *Scd1*, compared to untreated cells (Fig. 4.5C). Interestingly, treatment with FO showed significantly higher (p=0.0009) mRNA expression of *Scd1*, compared to control cells. There was no statistical difference between the cells treated with FOA and untreated cells (Fig. 4.5C); however, *Scd1* mRNA expression was lower in the FOA (p=0.003) treated cells, compared to FO (Fig. 4.5C). There was no effect of respective doses of the vehicle controls (PC, DMSO, PC+DMSO) on the mRNA expression of *Scd1*, compared to the untreated cells (Appendix III-E).

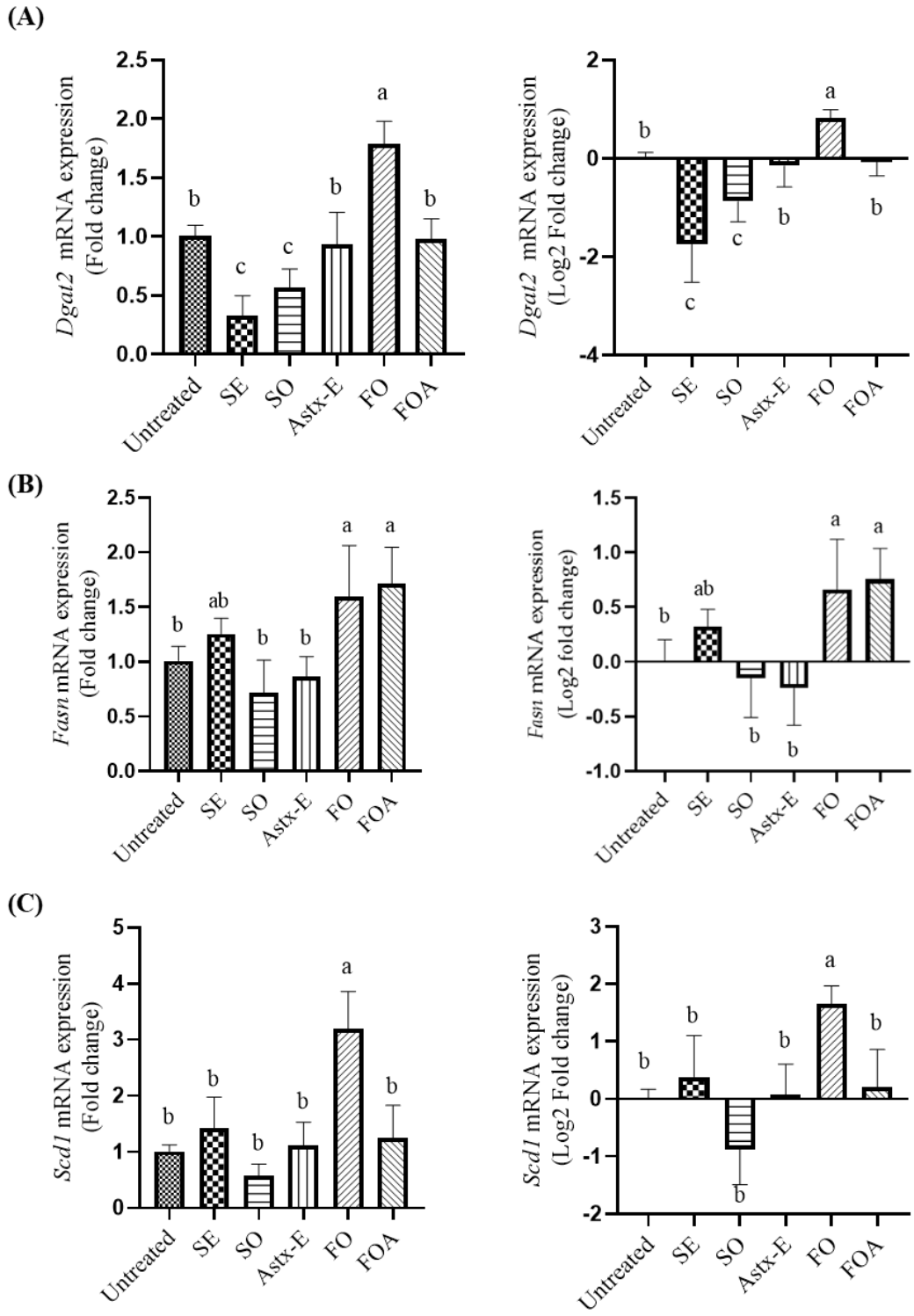


Figure 4.5. Shrimp oil decreased, while fish oil increased the mRNA expression of lipogenic genes in mature 3T3-L1 adipocytes.

The cells were differentiated to mature adipocytes, for 8 days, as explained in the methods section. Total RNA was extracted, and the mRNA expression analysis of (A) diacylglycerol O-acyltransferase 2 (Dgat2), (B) Fatty acid synthase (Fasn), (C) Stearoyl-CoA desaturase-1 (Scd1) gene was performed. Expression of target gene was normalized to RPLP0 as the reference gene. The mRNA expression data were expressed in both fold change and Log2 fold change. Data were analyzed using one-way ANOVA and Tukey's post-hoc test. $p<0.05$ was considered significant. Superscripts (a, b, c) represent significant differences, n=3. Untreated: Untreated cells received cell culture media only, SE: shrimp extract (0.25 mg/mL culture medium), SO: shrimp oil (0.25 mg/mL culture medium), Astx-E: esterified astaxanthin (15.9 ng/mL culture medium), FO: fish oil (0.25 mg/mL culture medium), FOA: FO+Astx-E [(0.25 mg + 15.9 ng)/mL culture medium].

4.2.5 Fish oil increased the mRNA expression of *Glut-4* in mature 3T3-L1 adipocytes

Treatment with SE, SO and Astx-E showed no significant effect on the mRNA expression of *Glut-4*, compared to untreated cells (Fig. 4.6). Interestingly, FO (p=0.0003) and FOA (p=0.01) showed significantly higher mRNA expression of *Glut-4*, compared to untreated cells, while SO and Astx-E revealed lower mRNA expression of *Glut-4* ($p<0.05$), compared to both FO and FOA (Fig. 4.6). There was no effect of respective doses of the vehicle controls (PC, DMSO, PC+DMSO) on the mRNA expression of *Glut-4*, compared to the untreated cells (Appendix III-F).

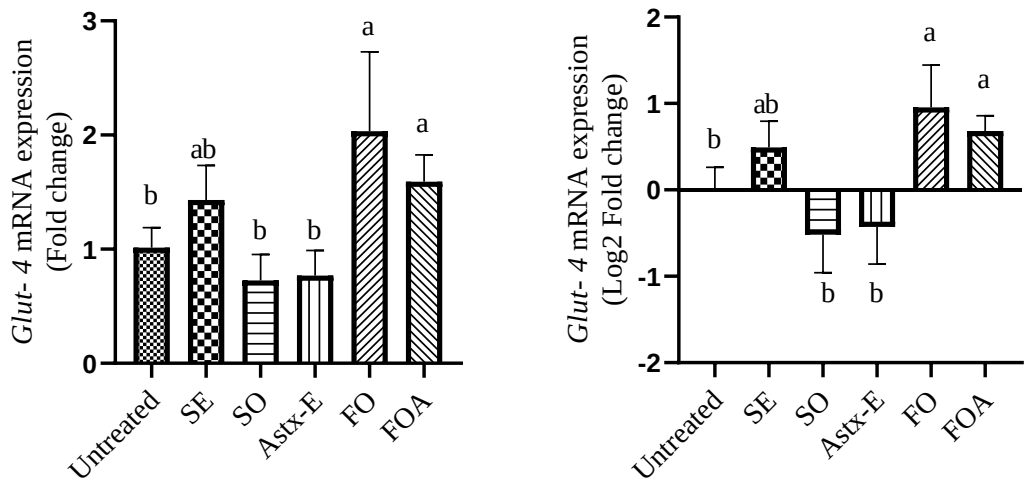


Figure 4.6. Fish oil increased the mRNA expression of Glut-4 in mature 3T3-L1 adipocytes

The cells were differentiated to mature adipocytes, for 8 days, as explained in the methods section. Total RNA was extracted, and gene expression analysis was performed. The mRNA expression of Glucose transporter type 4 (Glut-4) was normalized to RPLP0 as the reference gene. The mRNA expression data were expressed in both fold change and Log2 fold change. Data were analyzed using one-way ANOVA and Tukey's post-hoc test. $p<0.05$ was considered significant. Superscripts (a, b, c) represent significant differences, n=3. Untreated: Untreated cells received cell culture media only, SE: shrimp extract (0.25 mg/mL culture medium), SO: shrimp oil (0.25

mg/mL culture medium), Astx-E: esterified astaxanthin (15.9 ng/mL culture medium), FO: fish oil (0.25 mg/mL culture medium), FOA: FO+Astx-E [(0.25 mg + 15.9 ng)/mL culture medium].

4.3 The composition of oil extracted from shrimp processing by-product using fish oil extracted from waste fish as a solvent

4.3.1 The lipids, fatty acids, and total carotenoids content of oil extracts

Oil extracts (FOS, FS1, FS2 and FS3) were received from Dr. Hawboldt's laboratory. The lipid composition of the oil extracts is given in Appendix IV-A. FOS, FS1, FS2 and FS3 showed that oils are rich in TAG: FOS, FS1, FS2 and FS3 contained 98.48, 80.95, 80.10 and 97.26 (Wt. %) of TAG, respectively. Other lipid components included alcohols (17.59 Wt. %) in FS1, hydrocarbons (1.07 Wt. %) in FS2, free fatty acids (1.22 Wt. %) in FS3. However, FOS, FS2 and FS3 contained no alcohols; hydrocarbons class of lipids was not found in FS1 and FS3 and free fatty acids were not detected in FOS, FS1 and FS2. Furthermore, FS2 contained 2.95 (Wt. %) of phospholipids, whereas this class of lipids was not found in FOS, FS1 and FS3. Moreover, FS1, FS2 and FS3 contained 1.06, 5.0, 1.52 (Wt. %) of sterols, respectively. FOS, FS1 and FS2 contained 1.49, 0.4 and 10.88 (Wt. %) of acetone mobile polar lipids, respectively. Overall, the lipids content of FOS, FS1, FS2 and FS3 was not consistent.

Fatty acid composition revealed that FOS, FS1, FS2 and FS3 are rich in MUFAs, followed by PUFA and SFA (Appendix IV-B). MUFA comprised of C16:1n7, C18:1n9, C18:1n7 and C20:1n9. Amongst MUFA, C18:1n9 was the major fatty acid (FOS; 41.25 %, FS1, 43.77 %; FS2, 41.85 %; FS3,44.22 %). SFA content of the oil extracts comprised of C14:0, C16:0 and C18:0, where FOS, FS1, FS2 and FS3 contained 15.07, 15.94, 15.38 and 16.00 (%) of C16:0, respectively, and the C18:0 content was 3.89, 4.12, 3.91 and 4.05 (%), respectively. PUFA content of the oil extracts comprised of both n-6 PUFA and n-3 PUFA. The major n-6 PUFA was C18:2 (FOS, 16.88 %; FS1, 17.75 %; FS2, 16.87 %, and FS3 17.77 %). The amount of total n-6 PUFA in

FOS, FS1, FS2 and FS3 was 18.15, 18.94, 17.96 and 18.93, respectively. Amongst n-3 PUFA, C18:3n3 in FOS was 3.29 %, in FS1 was 3.45 %, in FS2 was 3.31 % and in FS3 was 3.43 %, while C20:5n3 in FOS, FS1, FS2 and FS3 was 4.34, 4.32, 3.93 and 4.13 (%), respectively. The amount of C22:5n3 in FOS, FS1, FS2 and FS3 was 1.85, 1.89, 1.72 and 1.85 (%), respectively, while C22:6n3 in FOS, FS1, FS2 and FS2 was 3.75, 3.90, 3.59 and 3.83 (%), respectively. The amount of total n-3 PUFA in FOS, FS1, FS2 and FS3 was 13.23, 13.56, 12.55, and 13.25, respectively.

Total carotenoids content in FS2 was 5.78 µg/g of oil, followed by FS3 (4.77 µg/g of oil), and FS1 (2.3 µg/g of oil) (Appendix IV-C). Further characterization of the carotenoids to measure the amount of astaxanthin was not performed due to a low amount of total extracted carotenoids. Overall, the composition of the extracts was similar to fish oil that was used as a solvent, and did not reflect extraction of shrimp oil. Moreover, in this method of extraction, there is a possible risk of impurities coming from shrimp processing by-product such as fine solid particles/residues that were observed in my extracts. It is likely that there is an added benefit of recovering astaxanthin associated proteins in these oil extracts, which needs to be tested in the future studies.

4.4 Effect of shrimp oil extracted from shrimp processing by-product using fish oil extracted from waste fish on fat accumulation and adipogenesis in 3T3-L1 adipocytes

4.4.1 Treatments with oil extracts maintained the cell metabolic activity of 3T3-L1 preadipocytes

The 3T3-L1 preadipocytes treated with various doses (respective doses used in the treatments) of the vehicle (PC) revealed no significant effect on the cell metabolic activity, compared to the untreated cells (Fig. 4.7A). There was no difference in the cell metabolic activity of 3T3-L1 preadipocytes treated with various doses of the individual treatments (FOS, FS1, FS2, FS3), compared to untreated cells (Fig. 4.7B).

4.4.2 FS1 and FS3 increased fat accumulation in mature 3T3-L1 adipocytes

The Oil Red O staining of mature 3T3-L1 adipocytes treated with FOS, FS1, FS2 and FS3 revealed higher fat accumulation, compared to untreated cells (Fig. 4.8A). Quantification of extracted Oil Red O confirmed significantly higher fat accumulation after treatment with FS1 (P=0.007) and FS3 (P=0.009), compared to untreated cells (Fig. 4.8B).

4.4.3 Effect of oil extracts on the mRNA expression of *Pparγ* and *Srebp1c* in mature 3T3-L1 adipocytes

Treatment with FOS and FS3 showed significantly (p<0.0001) higher mRNA expression of *Pparγ*, compared to untreated cells (Fig. 4.9A). However, FS1 and FS2 had no significant effect on the mRNA expression of *Pparγ*, compared to untreated cells (Fig. 4.9A). Both FOS and FS3 had significantly higher mRNA expression of *Pparγ*, compared to FS1 and FS2 (p<0.05). There was no effect of respective dose of vehicle control (PC) on the mRNA expression of *Pparγ*, compared to the untreated cells (Appendix V-A).

Treatments with FOS, FS1, FS2 and FS3 had no significant effect on the mRNA expression of *Srebp1c*, compared to untreated cells (Fig. 4.9B). There was no effect of respective dose of vehicle control (PC) on the mRNA expression of *Srebp1c*, compared to the untreated cells (Appendix V-B).

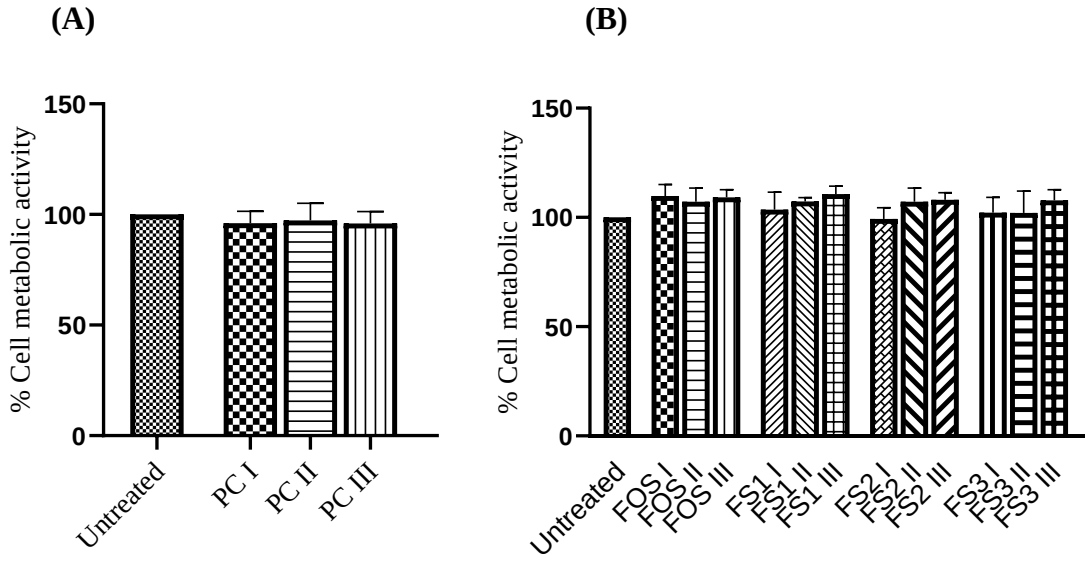
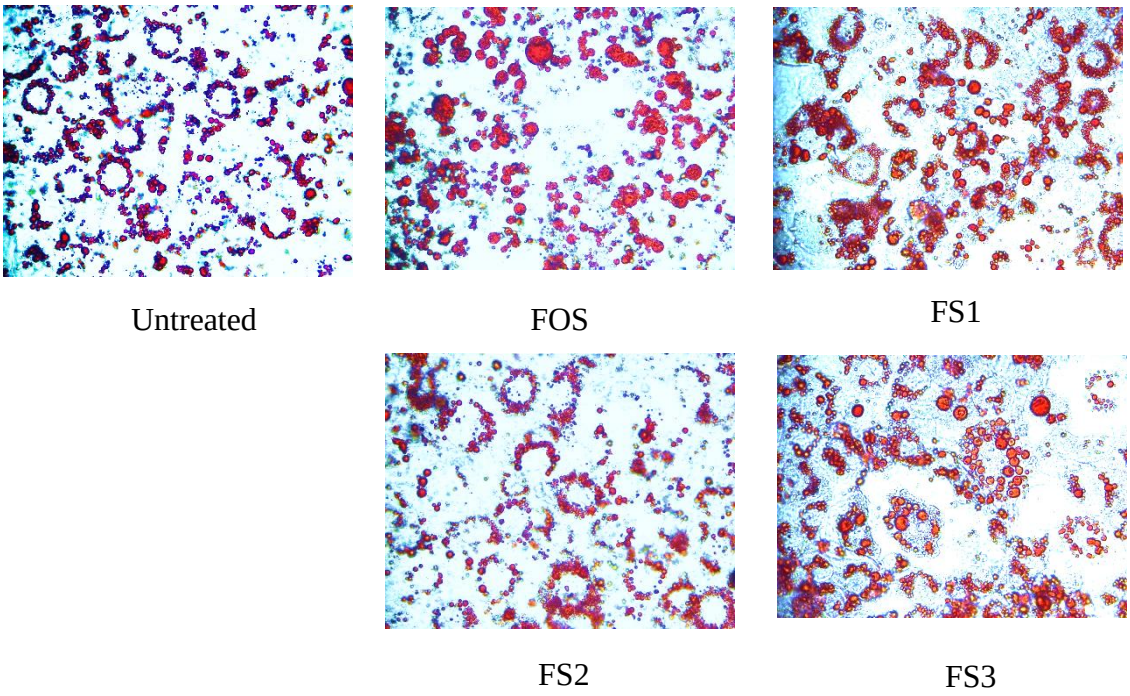


Figure 4.7. Cell metabolic activity of 3T3-L1 preadipocytes treated with various concentrations of treatments and vehicles

The cell metabolic activity of 3T3-L1 preadipocytes was measured using MTT assay after treatment with various concentrations of treatments, and the vehicles as explained in the methods section. (A) PC Vehicle; (B) FOS, FS1, FS2, FS3. Data were analyzed using one-way ANOVA to determine significance ($P<0.05$). Values are expressed as means ± SD, n=3. Untreated: Untreated cells received cell culture media only, PC: L-α-phosphatidylcholine (15 µg, 30 µg, 60 ug/mL culture medium), FOS: fish oil extracted from waste fish that was used as a solvent for shrimp oil extraction (0.125 mg, 0.25 mg, 0.5 mg/mL culture medium), FS1: 50ºC oil extract sample 1 (0.125 mg, 0.25 mg, 0.5 mg/mL culture medium), FS2: 60ºC oil extract sample 2 (0.125 mg, 0.25 mg, 0.5 mg/mL culture medium), FS3: 70ºC oil extract sample 3 (0.125 mg, 0.25 mg, 0.5 mg/mL culture medium).

(A)



(B)

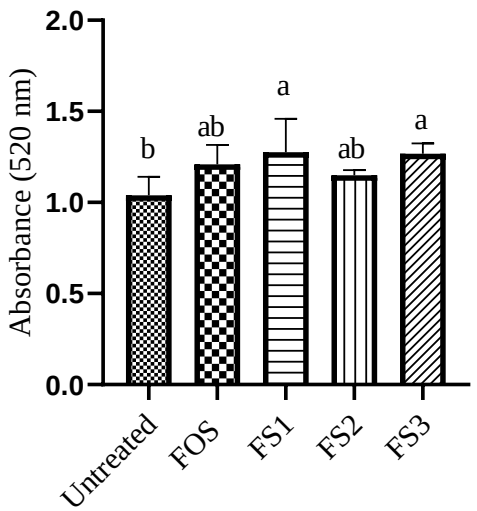


Figure 4.8. Effect of oil extracts on fat accumulation in mature 3T3-L1 adipocytes

Preadipocytes were differentiated to mature adipocytes in the presence or absence of treatments for 8 days, and Oil Red O staining and quantification was performed as explained in the methods section. B) Relative fat

accumulation measured spectrophotometrically as explained in the methods section, and A) representative images of the cells stained with Oil Red O on day 8 as explained in the methods section (400 X magnification). Values are expressed as mean ± SD, n=3. Data were analyzed using one-way ANOVA and Tukey's post-hoc test; p<0.05 was considered significant. Superscripts (a, b, c) represent significant differences. Untreated: Untreated cells received cell culture media only, FOS: fish oil extracted from waste fish that was used as a solvent for shrimp oil extraction (0.25 mg/mL culture medium), FS1: 50ºC oil extract sample 1 (0.25 mg/mL culture medium), FS2: 60ºC oil extract sample 2 (0.25 mg/mL culture medium), FS3: 70ºC oil extract sample 3 (0.25 mg/mL culture medium).

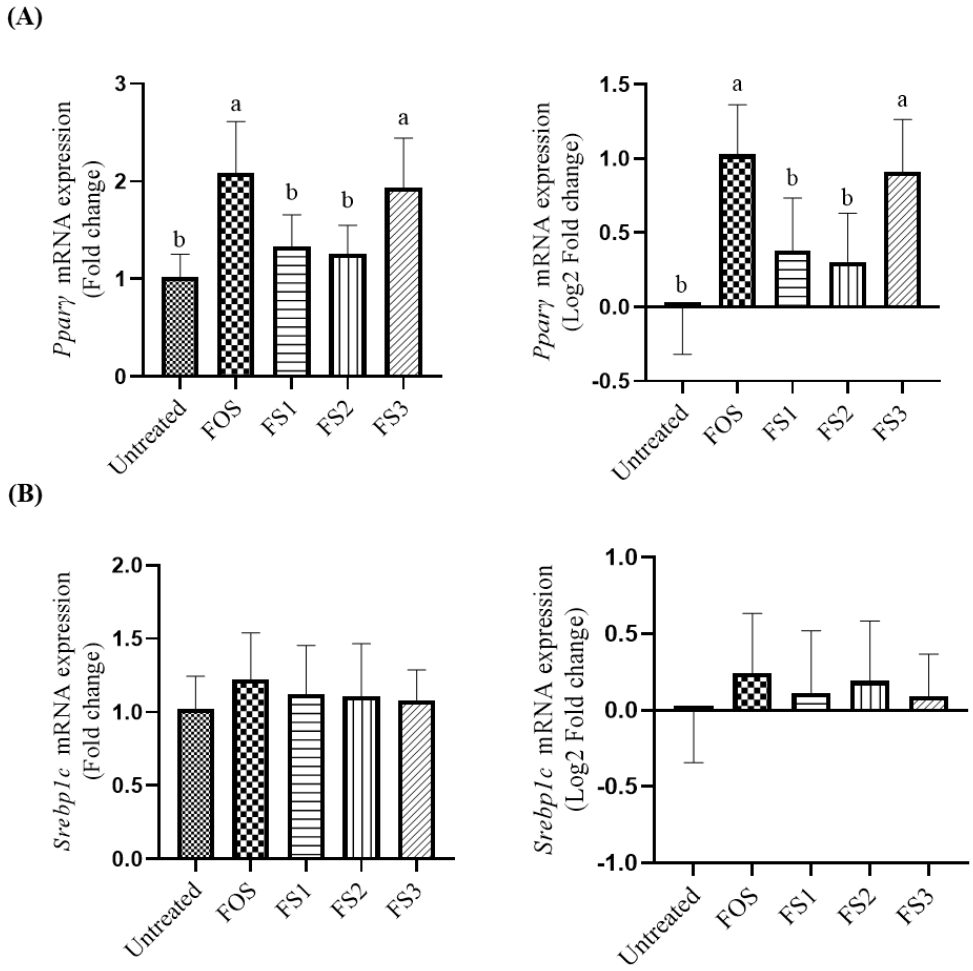


Figure 4.9. Effect of oil extracts on the mRNA expression of Pparγ and Srebp1c in mature 3T3-L1 adipocytes

The cells were differentiated to mature adipocytes, for 8 days, as explained in the methods section. Total RNA was extracted, and the mRNA expression analysis of (A) Peroxisome proliferator-activated receptor (Pparγ), (B) Sterol regulatory element-binding protein (Srebp1c) was measured as explained in the methods section and normalized to RPLP0 as the reference gene. The mRNA expression data were expressed in both fold change and Log2 fold change. Data were analyzed using one-way ANOVA and Tukey's post-hoc test. $p<0.05$ was considered significant. Superscripts (a, b, c) represent significant differences, n=3. Untreated: Untreated cells received cell culture media only, FOS: fish oil extracted from waste fish that was used as a solvent for shrimp oil extraction (0.25 mg/mL culture medium), FS1: 50ºC oil extract sample 1 (0.25 mg/mL culture medium), FS2: 60ºC oil extract sample 2 (0.25 mg/mL culture medium), FS3: 70ºC oil extract sample 3 (0.25 mg/mL culture medium).

4.4.4 Effect of oil extracts on the mRNA expression of lipogenic genes in mature 3T3-L1 adipocytes

Treatments with FOS, FS1, FS2 and FS3 had no significant effect on the mRNA expression of *Dgat2*, compared to untreated cells (Fig. 4.10A). There was no effect of respective dose of vehicle control (PC) on the mRNA expression of *Dgat2*, compared to the untreated cells (Appendix V-C).

The mRNA expression of *Fasn* was significantly higher after treatment with FOS (p=0.01), FS1 (p=0.0003) and FS3 (p=0.01), compared to untreated cells. However, treatment with FS2 had no significant effect on the mRNA expression of *Fasn*, compared to untreated cells (Fig. 4.10B). There was no statistical difference in the mRNA expression of *Fasn* between the cells treated with FS2 and FOS, FS1, FS3 (Fig. 4.10B). There was no effect of respective dose of vehicle control (PC) on the mRNA expression of *Fasn*, compared to the untreated cells (Appendix V-D).

The mRNA expression of *Scd1* was significantly higher after treatment with FOS ($p<0.0001$), FS1 (p=0.0003), FS2 ($p<0.0001$) and FS3 ($p<0.0001$), compared to untreated cells (Fig. 4.10C). There was no effect

of respective dose of vehicle control (PC) on the mRNA expression of *Scd1*, compared to the untreated cells (Appendix V-E).

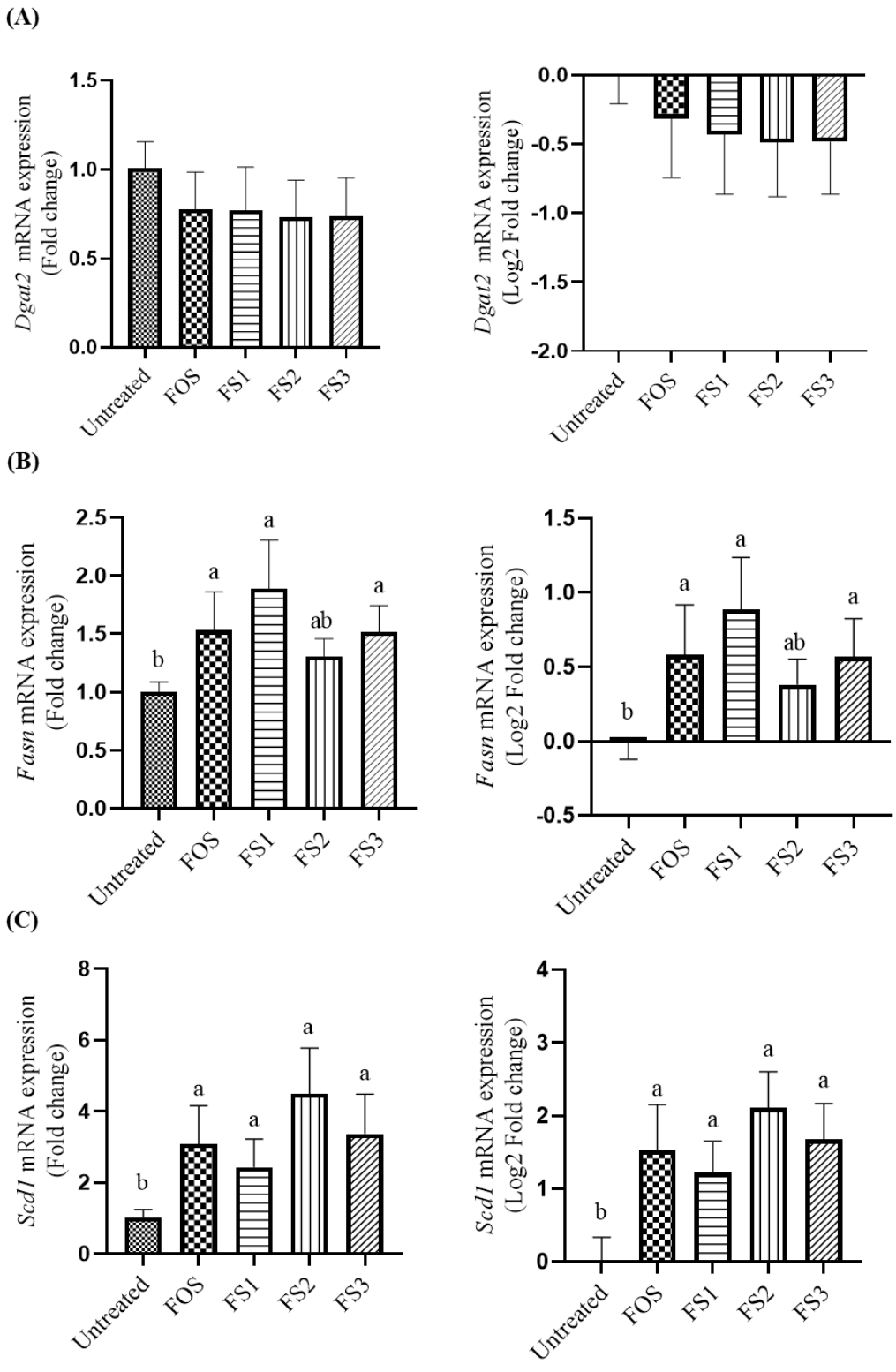


Figure 4.10. Effect of oil extracts on the mRNA expression of lipogenic genes in mature 3T3-L1 adipocytes

The cells were differentiated to mature adipocytes, for 8 days, as explained in the methods section. Total RNA was extracted, and the mRNA expression analysis of (A) diacylglycerol O-acyltransferase 2 (Dgat2), (B) Fatty acid synthase (Fasn), (C) Stearoyl-CoA desaturase-1 (Scd1) gene was performed. Expression of target gene was normalized to RPLP0 as the reference gene. The mRNA expression data were expressed in both fold change and Log2 fold change. Data were analyzed using one-way ANOVA and Tukey's post-hoc test. $p<0.05$ was considered significant. Superscripts (a, b, c) represent significant differences, n=3. Untreated: Untreated cells received cell culture media only, FOS: fish oil extracted from waste fish that was used as a solvent for shrimp oil extraction (0.25 mg/mL culture medium), FS1: 50ºC oil extract sample 1 (0.25 mg/mL culture medium), FS2: 60ºC oil extract sample 2 (0.25 mg/mL culture medium), FS3: 70ºC oil extract sample 3 (0.25 mg/mL culture medium).

4.4.5 Oil extracts increased the mRNA expression of *Glut-4* in mature 3T3-L1 adipocytes

The mRNA expression of *Glut-4* was significantly higher after treatment with FOS (p=0.001), FS1 (p=0.0001), FS2 (p<0.0001) and FS3 (p<0.0001), compared to untreated cells (Fig. 4.11). There was no effect of respective doses of PC on the mRNA expression of *Glut-4*, compared to the untreated cells (Appendix V-F).

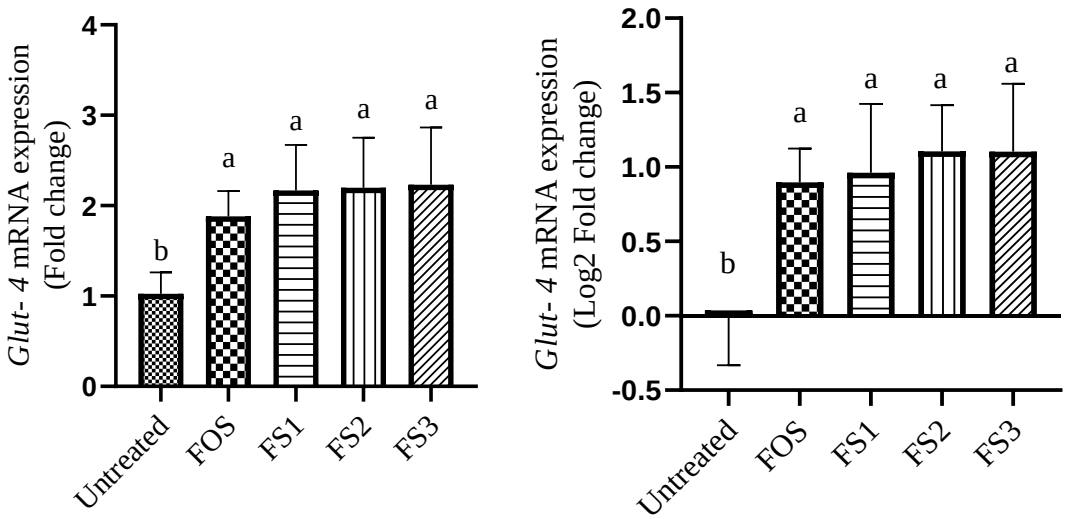


Figure 4.11. Oil extracts increased the mRNA expression of Glut-4 in mature 3T3-L1 adipocytes

The cells were differentiated to mature adipocytes, for 8 days, as explained in the methods section. Total RNA was extracted, and gene expression analysis was performed. The mRNA expression of Glucose transporter type 4 (Glut-4) was normalized to RPLP0 as the reference gene. The mRNA expression data were expressed in both fold change and Log2 fold change. Data were analyzed using one-way ANOVA and Tukey's post-hoc test. p<0.05 was considered significant. Superscripts (a, b, c) represent significant differences, n=3. Untreated: Untreated cells received cell culture media only, FOS: fish oil extracted from waste fish that was used as a solvent for shrimp oil extraction (0.25 mg/mL culture medium), FS1: 50ºC oil extract sample 1 (0.25 mg/mL culture

medium), FS2: 60ºC oil extract sample 2 (0.25 mg/mL culture medium), FS3: 70ºC oil extract sample 3 (0.25 mg/mL culture medium).

4.5 The composition of oils extracted from a mixture of shrimp and fish waste

Oils were extracted from a mixture of shrimp and fish waste using different ratios: 2:1 (fish waste + shrimp processing by-product) [2:1 (F+S)], 2.5:1 (fish waste + shrimp processing by-product) [2.5:1 (F+S)], 3:1 (fish waste + shrimp processing by-product) [3:1 (F+S)], and from only fish waste (salmon gut) as explained in the methods section. Total oil recovered from fish waste was 26.86 mL per 90 grams of waste; while it was 18.87 mL per 90 grams of waste mixture from 2:1 (F+S) (Appendix I-A). However, no oil was recovered from 2.5:1 (F+S) extraction mixture, while 3:1 (F+S) mixture gave 8.6 ml of oil per 90 grams of waste mixture (Appendix I-A). Lipids, fatty acids composition, and carotenoids content of the extracted oils was highly variable and is given in Appendix I. Lipid composition showed that the extracted oils are rich in TAG: fish waste oil, 2:1 (F+S) and 3:1 (F+S) contained 51.37, 56.80 and 55.39 (Wt. %) of TAG, respectively (Appendix I-B). Lipid composition revealed that the extracted oils had a significant amount of FFA fish waste oil, 2:1 (F+S) and 3:1 (F+S) contained 13.34, 12.69 and 12.44 (Wt. %) of free fatty acids, respectively (Appendix I-B). Other lipids components included hydrocarbons, sterols, acetone mobile polar lipids and phospholipids. Fish waste oil, 2:1 (F+S) and 3:1 (F+S) contained 2.69, 2.29 and 2.38 (Wt. %) of hydrocarbons, and 2.17, 2.76 and 3.04 (Wt. %) of sterols, respectively. Furthermore, 27.62, 24.45 and 24.89 (Wt. %) of acetone mobile polar lipids were found in fish waste oil, 2:1 (F+S) and 3:1 (F+S), respectively. The phospholipids content was found to be around 2.79, 2.36 and 1.83 (Wt. %) in fish waste oil, 2:1 (F+S) and 3:1 (F+S), respectively (Appendix I-B).

Fatty acid composition revealed that fish waste oil, 2:1 (F+S) and 3:1 (F+S) oils are rich in MUFAs, followed by PUFA and SFA (Appendix I-C). Overall, no difference was observed in the fatty acids composition between all extracted oils (Appendix I-C).

Total carotenoids content of 2:1 (F+S) was 30 µg/g of oil, and yield was 6.32 µg/g of shrimp processing by-product; 3:1 (F+S) contained 35.47 µg/g of oil, and yield was 3.39 µg/g of shrimp processing by-product (Appendix I-D).

Overall, discrepancy was observed in oil recovery and in carotenoid yield of respective extracted oils. Furthermore, there was no overall difference observed between lipids and fatty acids composition between fish waste oil, 2:1 (F+S), and 3:1 (F+S) extracted oils, and there was a high content of free fatty acids suggesting poor quality of the extracted oils. Hence, I did not proceed with further analysis or experiments using these samples.

4.6 Astx-E prevents oxidation of fish oil

Global Organization for EPA and DHA n-3 PUFA (GOED) considers the oils to be rancid when the PV is 10 mEq/kg or more (Omega-3 G. GOED Omega-3; Halvorsen and Blomhoff, 2011). The PV for both FO and FO+Astx-E samples at various time points are given in Fig. 4.12. The PV value for both FO and FO+Astx-E was 4.76 mEq/kg at 0 hour. The PV for FO was less than 10 mEq (9.52 mEq/kg) at 4 hours; however, the PV increased to 11.90, 14.28, 19.04 and 21.42 mEq at 8, 12, 24 and 48 hours, respectively. Addition of Astx-E at all concentrations (50, 75, 100 µg/g) maintained the PV of FO below 10 mEq, compared to FO without Astx-E. Astx-E at 50 µg/g FO showed a PV of 7.14 mEq at 8 hours of exposure to oxidation; however, the PV increased to 11.90, 11.90, and 16.66 mEq at 12, 24 and 48 hours, respectively. However, the addition of Astx-E at 50 µg/g maintained the PV significantly lower, compared to FO without Astx-E at 24 ($p<0.0001$) and at 48 ($p<0.0001$) hours. Addition of Astx-E at 75 µg/g FO showed a PV of 7.14 mEq at 8 hours of oxidation; however, the PV increased to 9.52, 11.90 and 16.66 mEq at 12, 24 and 48 hours, respectively. Interestingly, the PV after addition of Astx-E at 75 µg/g were significantly lower at 8 ($p=0.01$), 12 ($p=0.0003$), 24 ($p<0.0001$) and at 48 ($p<0.0001$) hours, compared to FO without Astx-E. Addition of Astx-E at 100 µg/g showed a PV of 7.00 mEq at 12 hours; however, the PV increased to 11.68 and 14.79 mEq at 24 and 48 hours, respectively. The PV after addition of

Astx-E at 100 μg/g were significantly lower at 8 ($p=0.006$), 12 ($p<0.0001$), 24 ($p<0.0001$) and at 48 ($p<0.0001$) hours, compared to FO without Astx-E.

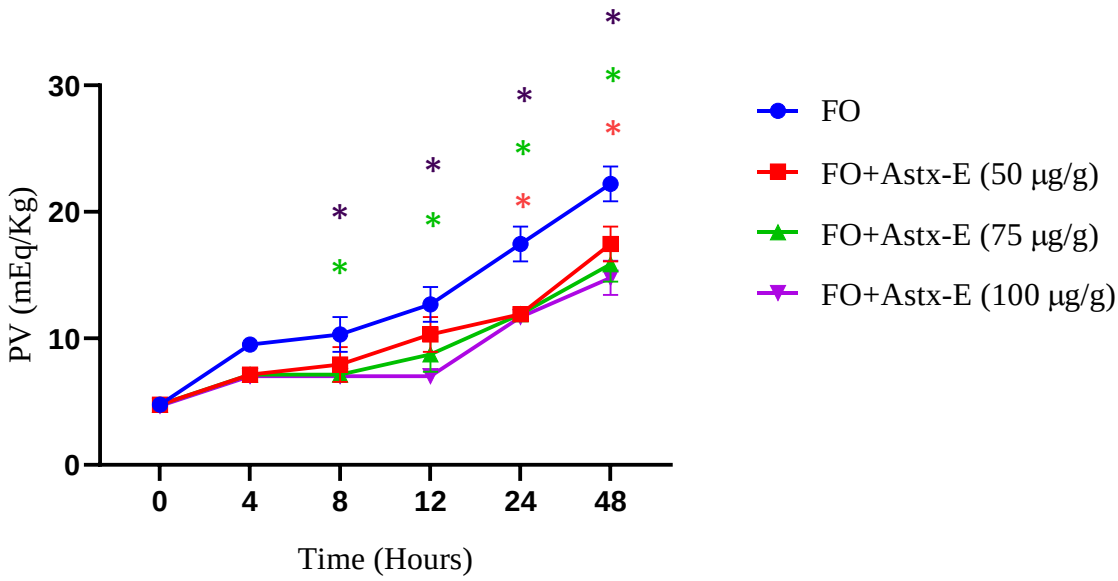


Figure 4.12. Astx-E prevents oxidation of fish oil

*Fish oil (FO), alone or in combination with Astx-E (50, 75, 100 µg/g) was exposed to oxidation as described in the methods section, and peroxide value (PV) was measured. Data were assessed using Two-way ANOVA followed by Tukey's multiple comparisons test, P<0.05 was considered significant; n=3. *Represent significant difference at a given time point between FO and FO+Astx-E (50 µg/g), *Represent significant difference at a given time point between FO and FO+Astx-E (75 µg/g), *Represent significant difference at a given time point between FO and FO+Astx-E (100 µg/g).*

CHAPTER FIVE

Discussion

5.1 Discussion

5.1.1 Soxhlet extraction method provided the best quality shrimp oil, compared to the other extraction methods

Our laboratory has previously shown that marine species, such as blue mussels and sea cucumber that are rich in n-3 PUFA and other nutrients, affect adipose tissue function to elicit anti-obesity effects (Vaidya et al., 2017). N-3 PUFA, such as EPA and DHA, play a crucial role in maintaining lipid metabolism (Buckley and Howe, 2009), and adipogenesis (Todorčević and Hodson, 2015). N-3 PUFA specifically target *Pparγ* gene expression to decrease lipogenesis, thereby inducing beneficial effects on adipose tissue metabolism and function (Todorčević and Hodson, 2015). Besides n-3 PUFA, free Astx found in marine species, also acts as an antagonist for PPARγ to inhibit lipid accumulation in 3T3-L1 adipocytes (Inoue et al., 2012). Shrimp oil is rich in both n-3 PUFA and Astx; however, there are no reports to date to show whether shrimp oil extracted from shrimp processing by-product affects the process of adipogenesis and lipogenesis in 3T3-L1 cells. In the current study, shrimp oil was extracted from shrimp processing by-product using various extraction methods; the effects of shrimp extracts and shrimp oil was then tested on fat accumulation, and the mRNA expression of genes involved in adipogenesis and lipogenesis in 3T3-L1 adipocytes. I also investigated the effects of a combination of fish oil plus Astx-E on fat accumulation, and the mRNA expression of genes involved in adipogenesis and lipogenesis, compared to fish oil alone. My findings demonstrated that the Soxhlet method of extraction using hexane: acetone (2:3) provided shrimp oil enriched in n-3 PUFA, phospholipids and Astx-E. My findings further demonstrated that treatment of 3T3-L1 cells with shrimp oil and shrimp extract decreased fat accumulation, which was due to a decrease in the mRNA expression of genes involved in adipogenesis and lipogenesis. However, fish oil, alone or in combination with Astx-E, showed an increase in fat accumulation, which was due to an increase in the mRNA expression of genes involved in adipogenesis and lipogenesis.

Shrimp oil from Soxhlet method was rich in phospholipids (64.20 Wt. %). Previously, it has been shown that phospholipid-bound n-3 PUFA such as EPA (C20:5n3) and DHA (C22:6n3) are better absorbed due to better bioavailability, and therefore more efficiently delivered (Maki et al., 2009; Schuchardt et al., 2011; Ramprasath et al., 2013, 2015). Shrimp oil from Soxhlet method was also rich in n-3 PUFA; the amount of total n-3 PUFA was 37.09 %, with 21 % EPA (C20:5n3) and 13.89 % DHA (C22:6n3). A high proportion of phospholipids in shrimp oil extracted from shrimp processing by-product, along with a high amount of n-3 PUFA may suggest that these fatty acids are associated with phospholipids. Free fatty acids formed a very small component of shrimp oil (0.33 Wt. %). Generally, high free fatty acids cause hydrolytic rancidity and affect the quality of oils; thus, the amount of free fatty acids is considered as a quality parameter for oils (Yu et al., 2012). The acceptable range of free fatty acids in commercially available krill oils is suggested to be up to 2 Wt. % (Burgess et al., 2020). Overall, my findings suggest that the SO extracted using Soxhlet method contained much lower amounts of free fatty acids confirming superior quality.

Lipid composition of oil extracts where waste fish oil was used for extractions (FOS, FS1, FS2 and FS3) showed that oils are rich in TAG. FS2 contained 2.95 (Wt. %) of phospholipids, whereas this class of lipids was not found in FS1 and FS3. The total n-3 PUFA content of these oil was: 13.23, 13.56, 12.55 and 13.25 (%) for FOS, FS1, FS2 and FS3, respectively. The amount of EPA in FOS, FS1, FS2 and FS3 was 4.34, 4.32, 3.93 and 4.13 (%), respectively and DHA in FOS, FS1, FS2 and FS3 was 3.75, 3.90, 3.59 and 3.83 (%), respectively. Fish oil is generally high in TAG, comprising of 80-90 % of total lipids (Kaitaranta and Ke, 1981; Zhoul et al., 1995). FS1, FS2 and FS3 were rich in TAG and not in phospholipids, whereas oil extracted using Soxhlet method was rich in phospholipids; it is thus acceptable to assume that this method of extraction failed to extract phospholipids from shrimp. Besides, the lipids content of these oil extracts was not consistent. Although I used these extracts for further experiments, this method of extracting shrimp oil from shrimp processing by-product was not considered ideal. The last method where fish waste and shrimp processing by-product were extracted together

showed no difference in fatty acids composition of fish waste oil, 2:1 (F+S), and 3:1 (F+S) extracted oils, and showed a high content of free fatty acids. Furthermore, the lipids, fatty acids and carotenoids composition data was inconsistent between repeats thus no further experiments were performed with these extracts.

My findings confirmed the presence of Astx in shrimp oil extracted using the Soxhlet method, along with other unknown carotenoids. Interestingly, my shrimp oil extract showed significantly higher amounts of Astx-E compared to free Astx; the Astx-E content was found to be almost 8 times higher than free Astx. It has been reported that Astx-E has higher antioxidant activity compared to free Astx (Régnier et al., 2015). Further health benefits have also been linked specifically to Astx-E. For example, Aoi et al. (Aoi et al., 2018) suggested that Astx-E promoted energy production and protected tissues from oxidative damage during exercise due to its favorable absorption properties, compared to free Astx. Thus, in view of these studies, it is likely that Astx-E, compared to free Astx has better health potential than free Astx. Moreover, considering the high amounts of Astx-E found in my shrimp extract compared to free Astx, in my subsequent experiments, I focused on Astx-E.

In regard to using waste fish oil to extract shrimp processing by-product, I found that FS2 had more total carotenoids, compared to FS1 and FS3 but the amount of carotenoids was low in these samples. Thus, I was not able to perform further characterization of total carotenoids to measure the amount of astaxanthin in respective samples. I also measured total carotenoids in the extracted oils where fish waste and shrimp processing by-product were extracted together. There was discrepancy in oil recovery, lipids, and fatty acids composition, and in carotenoid yield. This could be due to differences in the texture of fish waste and shrimp processing by-product, where shrimp processing by-product had harder shells, thus extractions did not work well. There was also a high content of free fatty acids in extracted oils; thus, the overall extractions were not satisfactory, and I did not proceed with further experiments using these samples.

Overall, our findings suggest that the Soxhlet extraction method yields good quality oil from shrimp processing by-product. It has been reported that the method of extraction impacts the distribution of lipids and yield of Astx in the final extract (Ahmadkelayeh and Hawboldt, 2020). While Soxhlet is a useful extraction method at the laboratory scale, scaling up is costly (economically and environmentally). Moreover, it has been stated that the use of edible oils as a "Green" solvent for extraction, and as a "Green" co-solvent in supercritical extraction can serve as an alternative to organic extractions (Ahmadkelayeh and Hawboldt, 2020). Preliminary results from Dr. Hawboldt's lab show that the "Greener" extraction methods have the potential for a higher quality product; however, these products need to be tested in cell culture and animal studies in the future.

5.1.2 Shrimp oil reduced fat accumulation by targeting the regulation of adipogenic and lipogenic genes

Our laboratory has previously reported that marine sources such as, blue mussels and sea cucumber extracts rich in n-3 PUFA decreased TAG levels in 3T3-L1 adipocytes (Vaidya and Cheema, 2014). Studies have also reported that EPA and DHA reduce fat accumulation in 3T3-L1 cells (Kim et al., 2006; Manickam et al., 2010). Moreover, Inoue et al. (Inoue et al., 2012) stated that free Astx reduced fat accumulation in 3T3-L1 adipocytes. Shrimp oil from my extractions was rich in both n-3 PUFA and Astx-E; thus, I investigated the effects of shrimp oil, shrimp extract, Astx-E, and fish oil on fat accumulation in 3T3-L1 cells. Oil Red O staining of mature 3T3-L1 adipocytes treated with shrimp oil, shrimp extract and Astx-E revealed a decrease in fat accumulation, compared to untreated cells. Oil Red O quantification confirmed that fat accumulation was lower in adipocytes treated with shrimp extract. On the other hand, Oil Red O quantification of mature 3T3-L1 adipocytes treated with fish oil and fish oil plus Astx-E revealed an increase in fat accumulation, compared to untreated cells. Interestingly, cells treated with FOS, FS1, FS2 and FS3 also revealed an increase in fat accumulation in mature 3T3-L1, compared to untreated cells. These effects were similar to the effects of fish oil observed in the above study, suggesting that the extracted oils using this method were mainly from fish, and that very little was extracted from shrimp.

Fat accumulation in the adipose follows a sequential expression of genes involved in TAG synthesis and storage (Nagai et al., 2018). The crucial steps towards differentiation of preadipocytes into mature lipid laden adipocytes involves regulatory genes such as *Pparγ*, and activation of the genes and enzymes necessary for fatty acid synthesis (Rosen et al., 2000). *Pparγ* is an important regulator of adipogenesis (Rosen et al., 2002); EPA and DHA, and their metabolites are natural ligands for *Pparγ* (Magee et al., 2012), and have been shown to act as antagonists. Since, shrimp oil is rich in both n-3 PUFA and Astx-E, I compared the effects of shrimp oil, shrimp extract, Astx-E, and fish oil on adipogenesis and lipogenesis in 3T3-L1 adipocytes. Shrimp oil, shrimp extract and Astx-E decreased the mRNA expression of *Pparγ* in 3T3-L1 mature adipocytes, compared to untreated cells, suggesting downregulation of adipogenesis. Inoue et al. (Inoue et al., 2012) has previously reported that free Astx inhibits adipogenesis by functioning as an antagonist for *Pparγ* in 3T3-L1 adipocytes. My findings demonstrate that Astx-E also inhibits adipogenesis by reducing the mRNA expression of *Pparγ.* Interestingly, fish oil and fish oil plus Asx-E treatments revealed an increase in the mRNA expression of *Pparγ* in 3T3-L1 mature adipocytes, compared to untreated cells. Li et al. (Li et al., 2017) has reported that EPA and DHA downregulate the mRNA expression of *Pparγ* in 3T3-L1 adipocytes. Other studies have also stated that EPA and DHA reduced the mRNA expression of *Pparγ* (Lorente-Cebrián et al., 2006; Manickam et al., 2010), while I found an increase. It is important to note that I used fish oil to treat 3T3-L1 cells, and not pure n-3 PUFA, such as EPA or DHA. Although fish oil is rich in EPA and DHA, it also contains other fatty acids and antioxidants that may exert a different effect on adipogenesis. That said, EPA and DHA rich-fish oil supplementation in animal studies have shown to reduce mRNA expression of *Pparγ* (Amaral et al., 2014). Unfortunately, there are no studies reported in the literature to date where an effect of fish oil or shrimp oil emulsion has been studied on adipogenesis in 3T3-L1 adipocytes. Previously, our laboratory has shown that arachidonic acid, an n-6 PUFA, has a dominant effect on the regulation of lipogenic genes when given along with EPA and DHA in 3T3-L1 adipocytes (Vaidya and Cheema, 2015). It is thus possible that the effects observed with fish oil on adipogenesis

in my study are due to a combination of different fatty acids and other components in fish oil. Future studies will focus on animal studies to compare the effects of fish oil and shrimp oil on adipose tissue function.

Interestingly, oil extracts FOS and FS3 also revealed an increase in the mRNA expression of *Pparγ*; while FS1 and FS2 had no significant effect on the mRNA expression of *Pparγ*, compared to untreated cells. FOS and FS3 also had significantly higher mRNA expression of *Pparγ*, compared to FS1 and FS2. Previously it has been studied that MUFAs, such as oleic acid upregulates adipogenesis (Regassa and Kim, 2013), and stearic acid, a SFA also increases fat accumulation and stimulate adipogenesis (Yanting et al., 2018). Since these oil extracts had a significant amount of MUFA and SFA (MUFA; FOS, 49.64, FS1, 47.37; FS2, 50.18; and FS3, 47.69 (%), SFA; FOS, 18.97, FS1, 20.14, FS2, 19.31 and FS3, 20.14 (%), which may have been associated with increased lipogenesis and adipogenesis. Overall, my findings suggest that shrimp oil from the Soxhlet method decreased the mRNA expression of *Pparγ,* whereas oil extracts using fish oil to extract shrimp oil increased *Pparγ* gene expression that were similar to the effects observed by fish oil in the Soxhlet extraction study.

The regulation of lipogenesis in adipocytes involves another important transcription factor called *Srebp1c* (Eberlé et al., 2004). *Srebp1c* belongs to the helix-loop-helix leucine zipper family, and promotes adipocyte differentiation by regulating the expression of genes linked to fatty acid synthesis (Kim and Spiegelman, 1996), and is an important marker of lipogenesis (Sekiya et al., 2007). Shrimp oil, shrimp extract and Astx-E showed a decrease in the mRNA expression of *Srebp1c* in 3T3-L1 mature adipocytes, compared to untreated cells, suggesting downregulation of lipogenesis. N-3 PUFA have been shown to reduce the mRNA expression of *Srebp1c* (Dentin et al., 2005; Kaur et al., 2011). Interestingly**,** fish oil showed an increase in the mRNA expression of *Srebp1c*. Increased lipogenesis could be due to an increase in the mRNA expression of *Srebp1c*. Fish oil plus Astx-E treated cells revealed a decrease in the mRNA expression of *Srebp1c,* compared to fish oil, suggesting Astx-E likely has a dominant effect. Previously, it has been reported that Astx inhibited Akt activity and thus reduced *Srebp1c* phosphorylation, which delayed nuclear translocation of *Srebp1c* and subsequent

lipogenesis (Jia et al., 2015), which may explain the overall effects of shrimp oil on *Srebp1c*. We did not measure phosphorylation or nuclear translocation of *Srebp1c* with various treatments. It is possible that the treatments affect post-transcriptional and post-translations modifications of *Srebp1c*, thereby affecting lipogenesis, which will be explored in the future. Furthermore, FOS, FS1, FS2 and FS3 had no significant effect on the mRNA expression of *Srebp1c*, compared to untreated cells. Overall, findings suggest that shrimp oil from the Soxhlet study decreased the mRNA expression of *Srebp1c* compared to untreated cells, whereas other oil extracts (FOS, FS1, FS2 and FS3) showed no significant effect. Previously, studies have shown that n-3 PUFA from fish oil decreased the mRNA expression of *Srebp1c* (Dentin et al., 2005; Kaur et al., 2011). Interestingly, we observed that fish oil increased the mRNA expression of *Srebp1c;*further studies are needed to understand the mechanisms involved in the up-regulation of *Srebp1c by fish oil in 3T3-L1 preadipocytes.*

Fasn is a key lipogenic gene that catalyzes the steps involved in the synthesis of palmitate (C16:0) from acetyl-CoA and malonyl-CoA (Wang et al., 2004). N-3 PUFA have been shown to downregulate the mRNA expression of *Fasn* in 3T3-L1 cells (Li et al., 2017). Shrimp oil, shrimp extract and Astx-E showed no significant effect on the mRNA expression of *Fasn*, compared to untreated cells. On the other hand, treatment with fish oil and fish oil plus Astx-E revealed an increase in the mRNA expression of *Fasn* compared to untreated cells, suggesting upregulation of lipogenesis. Interestingly, FOS, FS1 and FS3 also revealed an increase in the mRNA expression of *Fasn*, compared to untreated cells. However, FS2 had no significant effect; this could be attributed to the higher content of total carotenoids in FS2 compared to FS1 and FS3. This observation was consistent with *Pparγ* mRNA expression, where FS2 had no significant effect on the mRNA expression of *Pparγ* compared to FOS and FS3.

Synthesis of C16:0 by *Fasn* provides substrates for the synthesis of MUFA, specifically oleic acid (C18:1) from stearic acid (C18:0), a reaction catalyzed by *Scd1* (Paton and Ntambi, 2009). SCD1 is the rate-limiting enzyme for the synthesis of MUFA from SFA; and MUFA are important for the synthesis of TAG

(Ntambi and Miyazaki, 2004). N-3 PUFA have been shown to reduce the mRNA expression of *Scd1* in 3T3-L1 adipocytes (Manickam et al., 2010; Li et al., 2017). Treatment with shrimp oil, shrimp extract and Astx-E revealed no significant effect on the mRNA expression of *Scd1* compared to untreated cells. Interestingly**,** fish oil showed an increase in the mRNA expression of *Scd1* indicating upregulation of lipogenesis. However, fish oil plus Astx-E had no effect on *Scd1* gene expression, compared to untreated cells, which could be attributed to the dominant effect of Astx-E. Furthermore, fish oil plus Astx-E revealed a decrease in the mRNA expression of *Scd1,* compared to fish oil alone, supporting the dominating effects of Astx-E. Kawada et al. (Kawada et al., 2000) stated that the effects of carotenoids on inhibiting adipogenesis are mainly obtained by interfering with nuclear receptors such as retinoic acid receptor (RAR), retinoic X receptor (RXR) or PPAR. FOS, FS1, FS2 and FS3 increased the mRNA expression of *Scd1* compared to untreated cells, indicating an increase in the lipogenesis. Overall, my findings suggest that shrimp oil from the Soxhlet study showed no significant effect on the mRNA expression of *Fasn* and *Scd1* compared to untreated cells, whereas oil extracts from waste fish oil and shrimp processing by-product revealed an increase. These effects were similar to the effects observed by fish oil during the experiment conducted with Soxhlet method, further supporting that the effects of fish oil on adipogenesis and lipogenesis are different from those observed for shrimp oil.

I also studied the mRNA expression of *Dgat2* that plays vital role in TAG synthesis and storage (Chitraju et al., 2019). *Dgat2* catalyzes the final step of mammalian TAG synthesis (Harris et al., 2011); thus, it is an important lipogenic gene responsible for fat accumulation in adipocytes. Our laboratory has previously reported that blue mussel and sea cucumber extracts rich in n-3 PUFA decreased TAG levels in 3T3-L1 adipocytes (Vaidya and Cheema, 2014). Other studies have also reported that EPA and DHA reduce TAG accumulation in 3T3-L1 cells (Kim et al., 2006b; Manickam et al., 2010). Moreover, Inoue et al. (Inoue et al., 2012) stated that free Astx reduced TAG accumulation in 3T3-L1 adipocytes. Shrimp oil and shrimp extract showed a decrease in the mRNA expression of *Dgat2,* compared to untreated cells, suggesting downregulation of TAG synthesis

and storage. On the other hand, fish oil revealed an increase in the mRNA expression of *Dgat2*. Increase in lipogenesis could be due to an increase in the mRNA expression of *Dgat2*. Remarkably, fish oil plus Astx-E revealed a decrease in the mRNA expression of *Dgat2*, compared to fish oil, again suggesting a dominant effect of Astx-E. Suppression of *Dgat2* is protective against excessive fat accumulation, obesity, and improved insulin resistance (Cheol et al., 2007). Overall, my findings suggest that shrimp oil from the Soxhlet study decreased the mRNA expression of *Dgat2*, which corresponds with fat accumulation data from Oil Red O staining. My findings suggest that Astx-E may regulate *Dgat2* mRNA via *Srebp1c*. Since shrimp oil and shrimp extract had no significant effects on lipogenic genes, it is possible that the decrease in fat accumulation may be due to an increase in β-oxidation (Ukropec et al., 2003; Yang et al., 2011). Recently, it has been suggested that the effect of astaxanthin on fat accumulation and lipogenic genes is dose dependent (Tsai et al., 2020). These authors found that a dose of 5 μg/mL of astaxanthin decreased fat accumulation, however, there was no effect on the mRNA expression of lipogenic genes. Increasing the astaxanthin dose to 25 μg/mL decreased fat accumulation, along with a decrease in lipogenic genes. It is possible that shrimp oil and fish oil have a dose dependent effect on fat accumulation and adipogenic and lipogenic gene expression, which will be explored in the future. Future studies will also measure protein expression levels of the studied genes.

It is important to note that fat accumulation in adipocytes is an essential process, and its main role is to store energy in the form of lipids (Saponaro et al., 2015). However, excess fat accumulation predisposes cells towards insulin resistance (Grundy, 2015). Adipogenesis is associated with insulin sensitivity via insulin-mediated glucose uptake (Barr, 2018). Increased uptake of glucose via GLUT-4 is positively linked with insulin sensitivity in adipose tissue (Govers, 2014). Previously, Peyron-Caso et al. (Peyron-Caso et al., 2002) reported that fish oil improves insulin sensitivity by regulating glucose transport as a result of increasing the GLUT-4 protein and mRNA levels in adipocytes of Sucrose-fed rats. In the present study, I measured the mRNA expression of *Glut-4*. I did not measure the sequestered and active versions of the *Glut-4*. Treatment of shrimp

oil, shrimp extract and Astx-E showed no significant effect on the mRNA expression of *Glut-4* compared to untreated cells. Interestingly, fish oil and fish oil plus Astx-E revealed an increase in the mRNA expression of *Glut-4*, which may be consistent with an increase in fat accumulation. I am speculating that the increase in the mRNA expression of *Glut-4* will relate to insulin sensitivity, and may be responsible for an increase in fat accumulation. Similarly, it was also interesting to see the treatments with FOS, FS1, FS2 and FS3 significantly increased the mRNA expression of *Glut-4*, in mature 3T3-L1 adipocytes, compared to untreated cells. Our findings indicate that the effects of these oil extracts on fat accumulation and adipogenesis are mainly due to fish oil that was used as a solvent to extract lipids and carotenoids from shrimp processing by-product. Further investigations were planned to study the effects of fish oil towards improving insulin sensitivity in 3T3-L1 cells by measuring glucose uptake, which could not be undertaken due to Covid-19 lockdown. My findings are however promising in that they indicate fish oil and shrimp oil regulate adipogenesis and lipogenesis via independent pathways. It is likely that the effects of fish oil in 3T3-L1 adipocytes are associated with improving insulin sensitivity, whereas the effects of shrimp extract and shrimp oil are associated with the inhibition of adipogenesis and lipogenesis, or increased β-oxidation. Moreover, the effects of shrimp oil and shrimp extract were not always consistent, even though we used the same amount of shrimp oil and shrimp extract in our treatments. Shrimp extract contained a lower amount of oil compared to shrimp oil, while shrimp extract likely contained both water-soluble and insoluble components due to the combination of polar and nonpolar solvent used for extraction. Further investigations are needed to understand the molecular mechanisms by which fish oil, shrimp oil, and shrimp extract regulate adipocyte function. Overall, my findings demonstrate that the shrimp processing by-product is a valuable source for extracting bioactives, and for developing nutraceuticals with potential anti-obesity properties. Effective solutions to the problems of modern life are often found at the local level. Innovations which advocate approaches towards a circular economy are essential as we seek to move towards a more sustainable future. This study intends to lay the groundwork for one such innovation. Such

scientific approaches that encourage the concept of circular economy are especially pertinent to advance towards more sustainable ways of production and processing to develop novel nutraceuticals.

5.1.3 Astx-E prevented oxidation of fish oil

Interestingly, the majority of the tested fish oil supplements in the market have been suggested to be oxidized (Jackowski et al., 2015). Addition of antioxidants to fish oil supplements have been shown to reduce the oxidation of oil (Zuta et al., 2007). The stabilizing effects and antioxidant activity of carotenoids extracted from *H. pluvialis* cells have been shown in the edible oils such as coconut, rice bran, groundnut, mustard, gingelly, olive, palm, and sunflower (Rao et al., 2007). These authors suggested that the stabilizing and antioxidant effects are due to Astx, since Astx represented a major proportion of the total carotenoids (Rao et al., 2007). I investigated the effects of Astx-E on oxidation of fish oil. Being highly potent antioxidant (Hussein et al., 2006), Astx when combined with oil rich in n-3 PUFA, may prevent oxidation of oil. The initial oxidative products of fats and oils are peroxides; thus, the peroxide value of the oils measures the state of oxidation, and predicts the oxidative rancidity in fats and oils (Barden et al., 2011; Jackowski et al., 2015). Erkan et. al (Erkan et al., 2009) stated the kinetics of lipid oxidation where purified sunflower oil exposed to the oxidation in a temperature-controlled microwave oven as a function of time and temperature at 40, 60 and 80 ºC for 2-10 hours, and further reported the antioxidative effects of added natural antioxidants. It has been reported that storing the oils for 24 hours in a forced-air oven at 60°C is equivalent to 1 month of storage at ambient temperatures (Tian and Dasgupta, 1999; Li and Wang, 2018). I incubated fish oil, with and without Astx-E at 37 ºC temperature up to 48 hours and measured the peroxide values. The Global Organization for n-3 PUFA considers the oils to be rancid when the peroxide value is 10 mEq/kg or higher (Omega-3 G. GOED Omega-3; Halvorsen and Blomhoff, 2011). My findings showed that addition of Astx-E (50, 75, 100 µg/g) to fish oil maintained peroxide values below 10 mEq, compared to fish oil alone. The peroxide value for fish oil alone was below 10 mEq for 4 hours of exposure to oxidation. Whereas Astx-E at 50 µg/g fish oil showed peroxide values below 10 mEq for 8 hours.

Astx-E at 75 μg/g and 100 μg/g fish oil showed peroxide values below 10 mEq for 12 hours. Addition of Astx-E at 50 μg/g fish oil showed significantly lower peroxide values at 24 and 48 hours, compared to fish oil alone; while Astx-E at 75 and 100 μg/g fish oil showed significantly lower peroxide values at 8, 12, 24 and 48 hours, compared to fish oil alone. On the other hand, the peroxide value for fish oil was less than 10 mEq (9.52 mEq/kg) only for 4 hours when exposed to oxidation; however, it increased significantly thereafter. These findings demonstrate that Astx-E prevents oxidation of fish oil and can be utilized as a strategy to increase the shelf-life of fish oil by preventing oxidation.

5.2 Limitations and future direction

In this study, I focused on free and esterified astaxanthin found in shrimp extract. However, it will also be interesting to study protein-associated astaxanthin from shrimp extract in the future. I focused mainly on fat accumulation in 3T3-L1 adipocytes using Oil Red O staining. Further experiments such as lipid extraction and TAG analysis from 3T3-L1 adipocytes upon treatments could not be conducted due to the limited amount of shrimp oil to repeat experiments in larger cell culture dishes to extract lipids. Also, in current study, I only measured the mRNA expression of the adipogenic and lipogenic genes. These genes may also be regulated at the post-transcriptional levels (Strable and Ntambi, 2010; Xu et al., 2013; Brunmeir and Xu, 2018; Song et al., 2018). Thus, it would be important to look at the protein expression of the genes. We observed that cells treated with shrimp oil, shrimp extract and Astx-E reduced fat accumulation. Thus, it will be important to investigate decrease in fat accumulation via β-oxidation of fatty acids in 3T3-L1 adipocytes. It is also important to look at the effects of shrimp oil, shrimp extract and Astx-E on lipolysis in 3T3-L1 adipocytes. I measured mRNA expression of *Glut-4,* which is not the sequestered and active versions of the *Glut-4.* Interestingly, we observed an increase in the mRNA expression of *Glut-4,* which may be consistent with an increase in fat accumulation upon treatment with fish oil and fish oil plus Astx-E. However, our understanding of the role of fish oil in improving insulin sensitivity and thereby increasing adipogenesis in 3T3-L1 cells is still very limited. Further

investigations were planned to study the potential effects of fish oil towards improving the insulin sensitivity in 3T3-L1 cells. However, analysis such as glucose uptake assay could not be conducted due to Covid-19 lockdown. Lastly, I observed the oxidation preventive effect of only Astx-E when combined with fish oil. It would be interesting to investigate the effects of a combination of fish oil and free Astx on oxidation of fish oil using PV assay to establish which Astx form is better at preventing oxidation.

5.3 Conclusion

Overall, my findings revealed that shrimp oil extracted from shrimp processing by-product using Soxhlet method showed a decrease in the mRNA expression of adipogenic and lipogenic genes, with no significant change in *Glut-4* compared to the untreated cells. I have proposed the mechanism of action of shrimp oil on adipogenesis and lipogenesis in figure 5.1. It seems likely that the effects observed due to the shrimp oil on adipogenesis are not just because of n-3 PUFA, but due to shrimp oil as a combination of all fatty acids. Furthermore, Astx along with other unknown carotenoids could also be additional contributing factors. Interestingly, the oil extracts recovered from shrimp processing by-product using waste fish oil as a solvent showed similar effects on adipogenesis and lipogenesis compared to the effects of the fish oil observed in the Soxhlet study. The proposed mechanism of action of oil extracts on adipogenesis and lipogenesis is in figure 5.2. Treatment with oil extracts showed an increase in fat accumulation in adipocytes, which was consistent with an increase in the mRNA expression of adipogenic and lipogenic genes, and *Glut-4* compared to the untreated cells. This is analogous to the effect of fish oil observed in the Soxhlet study. Therefore, it seems that fish oil and shrimp oil regulate adipogenesis via independent pathways. Findings from this study may have significant implications towards potential anti-adipogenic properties of shrimp oil extracted from shrimp processing by-product and may find extensive applications in the aquaculture, nutraceutical, and pharmaceutical industries.

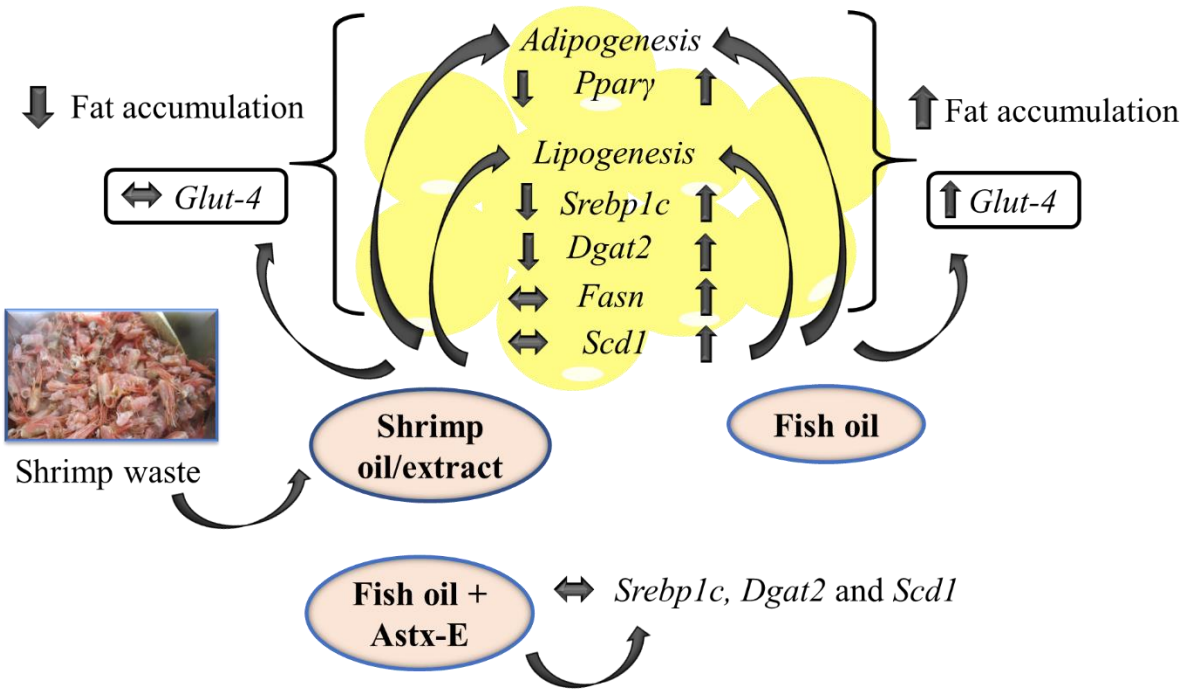


Figure 5.1. Schematic representation of the effect of shrimp oil, shrimp extract from shrimp processing by-product, and fish oil on the mRNA expression of genes involved in adipogenesis and lipogenesis in 3T3-L1 adipocytes

Astx-E, Esterified astaxanthin; Pparγ, Peroxisome proliferator-activated receptor-gamma; Srebp1c, Sterol regulatory element-binding protein 1; Dgat2, Diacylglycerol O-acyltransferase 2; Fasn, Fatty acid synthase; Scd1, Stearoyl-CoA desaturase; Glut-4, Glucose transporter type 4. Up arrow indicates increase in the mRNA expression of respective gene; down arrow indicates decrease in the mRNA expression of respective gene; straight arrow indicates no change in the mRNA expression of respective gene compared to untreated cells.

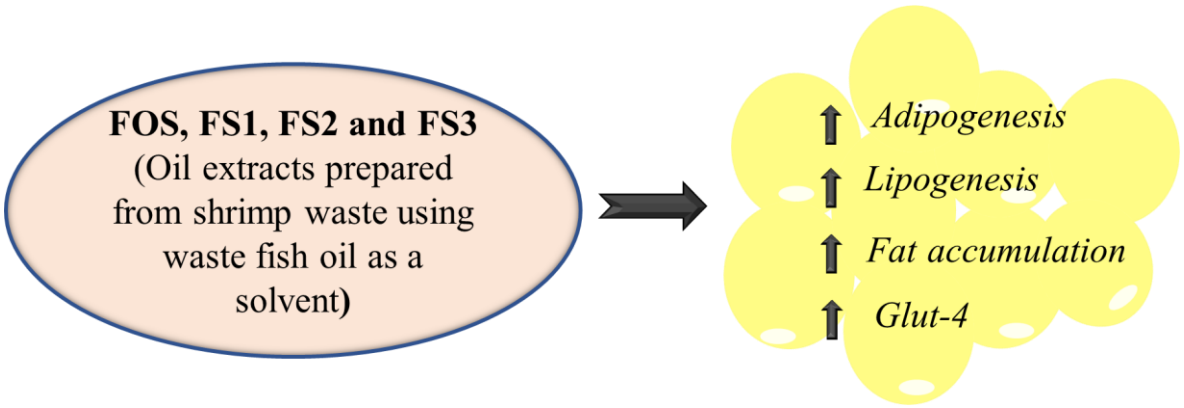


Figure 5.2. Schematic representation of the effect of oil extracts on the mRNA expression of genes involved in adipogenesis and lipogenesis in 3T3-L1 adipocytes

FOS; fish waste oil that was used as a solvent for extracting shrimp processing by-product , FS1; 50^{o}C oil extract sample 1, FS2; 60^{o}C oil extract sample 2, FS3; 70^{o}C oil extract sample 3, Glut-4; Glucose transporter type-4. Up arrow indicates increase in the mRNA expression of genes involved in adipogenesis and lipogenesis.

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APPENDICES

The data presented in appendix I and II includes the composition of oil extracted from shrimp plus fish waste mixture, and the effect of fish oil (0.25,0.5,0.75 and 1 mg/mL) on differentiation in 3T3-L1 cells, respectively. The data from appendix III and V shows that vehicle controls had no significant effect on the mRNA expression of adipogenic and lipogenic genes. The data from appendix IV includes the composition of oil extracted from shrimp processing by-product using waste fish oil as a solvent. The data in appendix VI includes the optimization of Astx standards and preliminary analysis of shrimp extract using mass spectrometry (MS). Dr. Robert Brown (Biochemistry, Memorial University) spent his valuable time performing optimization of Astx standards on MS, and also performed preliminary run of shrimp extract. These data are important to provide further insights into respective analyses.

Appendix I

The composition of oil extracted from a mixture of shrimp plus fish waste

A) Oil recovery

Fish + shrimp processing by-product (Total=90 grams)	**Oil recovered (ml)**
2:1 (60+30 g)	18.67
2.5:1 (64.3+25.7 g)	N/A
3:1 (67.5+22.5 g)	8.6
Fish waste (Salmon Gut) Total=90 grams	26.86

B) Lipid composition of shrimp plus fish waste oils

Lipid Composition (Wt. %)	Fish waste oil	2:1 (F+S)	3:1 (F+S)
Hydrocarbons	2.69	2.29	2.38
Triacylglycerols	51.37	56.80	55.39
Free Fatty Acids	13.34	12.69	12.44
Sterols	2.17	2.76	3.04
Acetone Mobile Polar Lipids	27.62	24.45	24.89
Phospholipids	2.79	2.36	1.83

C) Fatty acids composition of shrimp plus fish waste oils

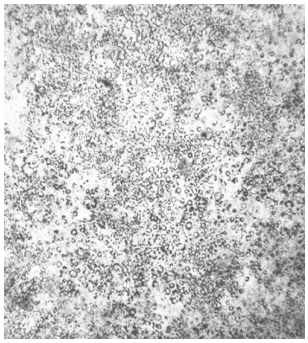
Fatty acids (% nmol)	Fish waste oil	2:1 (F+S)	3:1 (F+S)
C14:0	2.73	2.97	2.81
C16:0	12.58	12.79	12.64
C16:1n7	6.32	6.54	6.12
C18:0	3.18	3.10	3.16
C18:1n9	40.65	39.74	40.82
C18:1n7	3.61	3.57	3.62
C18:2n6	17.13	16.87	16.76
C18:3n6	0.21	0.22	0.19
C18:3n3	3.75	3.69	3.89
C20:1n9	0.95	0.84	0.81
C20:4n6	0.50	0.58	0.57
C20:5n3	3.53	3.71	3.54
C22:4n6	0.00	0.14	0.13
C22:5n3	1.66	1.63	1.44
C22:6n3	3.21	3.61	3.50
∑ SFA	18.49	18.86	18.60
∑ MUFA	51.53	50.69	51.37
∑ PUFA	29.98	30.45	30.03

D) Total carotenoids content in shrimp plus fish waste oils

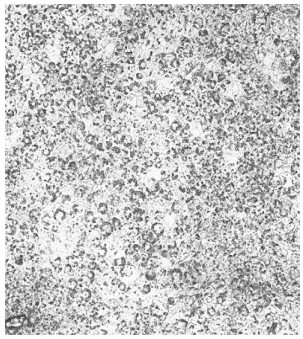
Sample	Total waste used (g)	Total oil recovery (mL)	Concentration of total carotenoids (µg/g of oil)	Yield of total carotenoids, µg/g of shrimp processing by-product
2:1 (F+S)	90	18.67	30.48	6.32
3:1 (F+S)	90	8.6	35.47	3.39

Appendix II

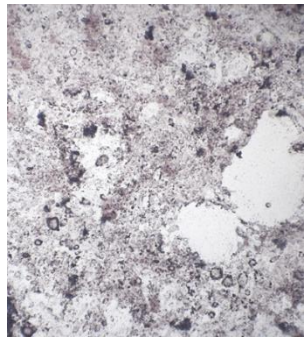
The effect of fish oil (0.25, 0.5, 0.75, 1 mg/mL culture medium) on adipogenesis in 3T3-L1 adipocytes



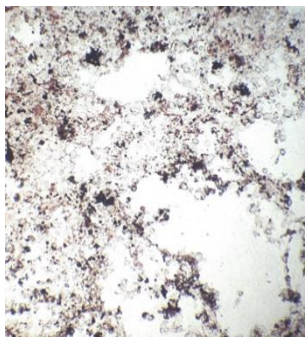
Untreated



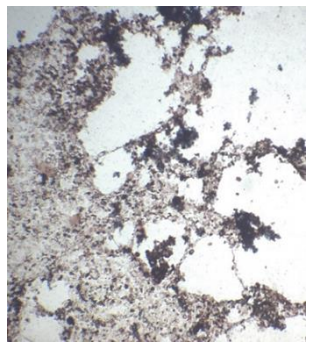
FO emulsion (0.25 mg)



FO emulsion (0.5 mg)



FO emulsion (0.75 mg)

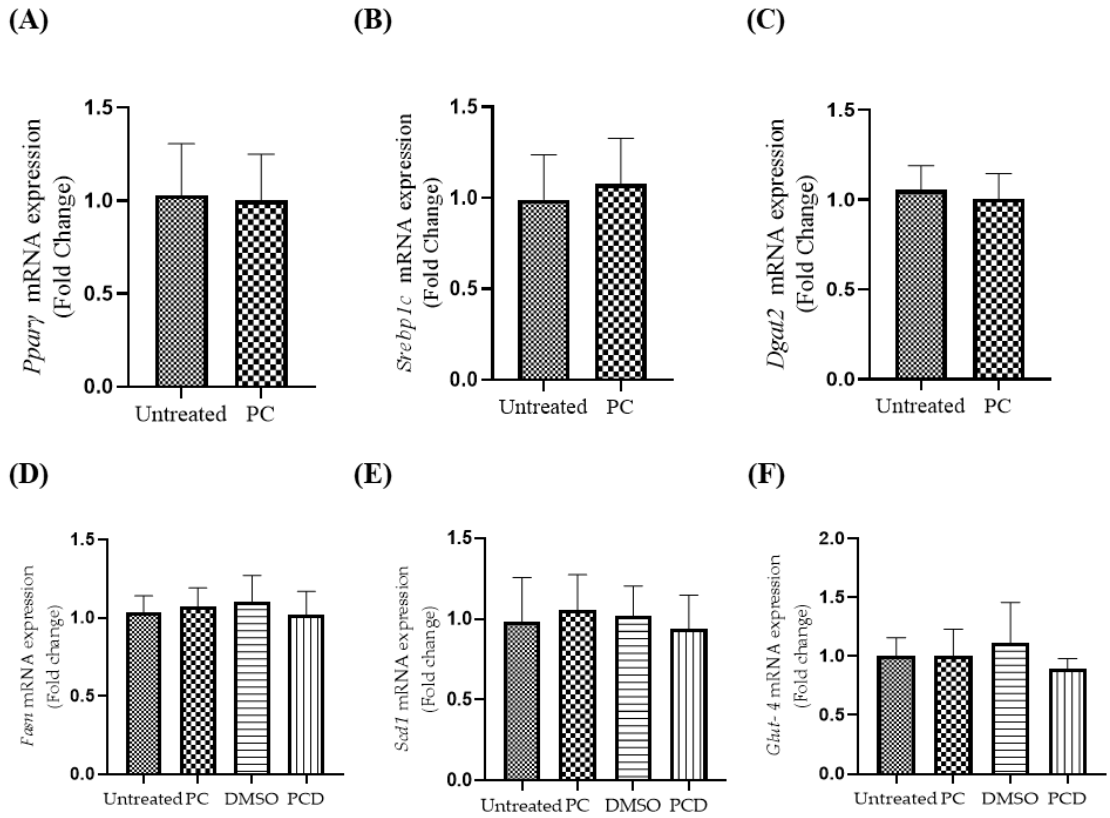


FO emulsion (1 mg)

Preadipocytes were differentiated to mature adipocytes in the presence or absence of different concentrations of fish oil emulsions for 8 days, as explained in the methods section. On day 8, the cells were viewed using a Leica DMIL LED Microscope at 40x magnification, and Infinity Camera Analyze Software (version 6.5.5) was used for capturing the images. Untreated=untreated cells with cell culture media only, Fish oil (FO) emulsion= 0.25, 0.5, 0.75 and 1 mg/mL of culture medium.

Appendix III

The vehicle controls (PC, DMSO, PC+DMSO) had no significant effect on the mRNA expression of *Pparγ, Srebp1c, Dgat2, Fasn, Scd1* and *Glut-4,* compared to the untreated cells.



The cells were differentiated to mature adipocytes, for 8 days, as explained in the methods section. Total RNA was extracted, and the mRNA expression analysis of (A) Peroxisome proliferator-activated receptor (Pparγ), (B) sterol regulatory element-binding protein (Srebp1c), (C) diacylglycerol O-acyltransferase 2 (Dgat2), (D) Fatty acid synthase (Fasn), (E) Stearoyl-CoA desaturase-1 (Scd1), (F) Glucose transporter type 4 (Glut-4) genes was performed. Expression of target genes was normalized to RPLP0 as the reference gene. The mRNA expression data were expressed in fold change. Vehicles: PC, DMSO, PCD. Data were analyzed using one-way ANOVA and Tukey's post-hoc test. $p<0.05$ was considered significant. n=3. Untreated=untreated cells with cell culture media only, PC=L-α-phosphatidylcholine (30 ug/mL culture medium), DMSO=dimethyl sulfoxide (0.06 %), PCD=PC+DMSO [(30 μg + 0.06 %)/mL culture medium]

Appendix IV

The composition of oil extracted from shrimp processing by-product using waste fish oil as a solvent

A) Lipid composition of the oil extracts

Lipid composition (Wt. %)	FOS	FS1	FS2	FS3
Hydrocarbons	0.03	0.00	1.07	0.00
Triacylglycerols	98.48	80.95	80.10	97.26
Free Fatty Acids	0.00	0.00	0.00	1.22
Alcohols	0.00	17.59	0.00	0.00
Sterols	0.00	1.06	5.00	1.52
Diacylglycerols	0.00	0.00	0.00	0.00
Acetone Mobile Polar Lipids	1.49	0.40	10.88	0.00
Phospholipids	0.00	0.00	2.95	0.00

Data are expressed as the percentage weight of the total extracted lipids. FOS=fish waste oil that was used as a solvent for extraction, FS1=50ºC oil extract sample 1, FS2=60ºC oil extract sample 2, FS3=70ºC oil extract sample 3; details are given in the methods section.

B) Fatty acids composition of the oil extracts

Fatty acids (% nmol)	FOS	FS1	FS2	FS3
C14:0	0.01	0.08	0.02	0.08
C16:0	15.07	15.94	15.38	16.00
C16:1n7	7.36	2.54	7.33	2.55
C18:0	3.89	4.12	3.91	4.05
C18:1n9	41.25	43.77	41.85	44.22
C18:1n7	0.17	0.15	0.15	0.00
C18:2n6	16.88	17.75	16.87	17.77
C18:3n6	0.42	0.35	0.37	0.35
C18:3n3	3.29	3.45	3.31	3.43
C20:1n9	0.85	0.91	0.84	0.92
C20:4n6	0.69	0.67	0.59	0.63
C20:5n3	4.34	4.32	3.93	4.13
C22:4n6	0.16	0.18	0.13	0.18
C22:5n3	1.85	1.89	1.72	1.85
C22:6n3	3.75	3.90	3.59	3.83
∑ SFA	18.97	20.14	19.31	20.14
∑ MUFA	49.64	47.37	50.18	47.69
∑ n-3 PUFA	13.23	13.56	12.55	13.25
∑ n-6 PUFA	18.15	18.94	17.96	18.93

Data are expressed as the percentage nmol of the total extracted fatty acids. ΣSFA = sum of saturated fatty acids, ΣMUFA= sum of monounsaturated fatty acids, ΣPUFA = sum of polyunsaturated fatty acids, Σn-3 PUFA = sum of omega-3 PUFA, Σn-6 PUFA = sum of omega-6 PUFA. FOS=fish waste oil that was used as a solvent for extraction, FS1=50ºC oil extract sample 1, FS2=60ºC oil extract sample 2, FS3=70ºC oil extract sample 3.

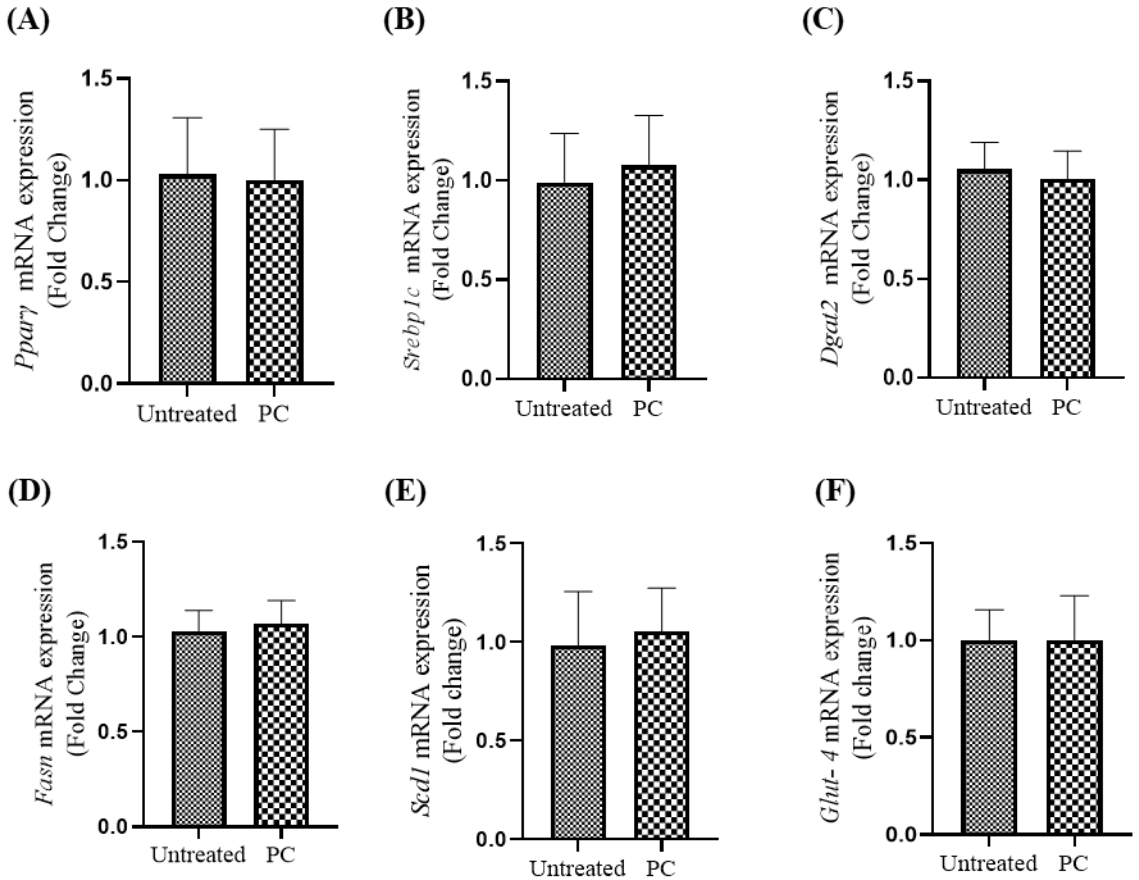
(C) Total carotenoids content in the oil extracts

Oil extracts	Carotenoids concentration, µg/g of oil
FS1	2.3
FS2	5.78
FS3	4.77

Total carotenoid content was measured following the method of Meyers and Bligh. FOS was used as a blank and the absorbance was recorded at 505 nm using a spectrophotometer as explained in the methods section. Data are expressed as µg/g of oil. FS1=50ºC oil extract sample 1, FS2=60ºC oil extract sample 2, FS3=70ºC oil extract sample 3.

Appendix V

The vehicle control (PC) had no significant effect on the mRNA expression of *Pparγ, Srebp1c, Dgat2, Fasn, Scd1* and *Glut-4,* compared to the untreated cells.



The cells were differentiated to mature adipocytes, for 8 days, as explained in the methods section. Total RNA was extracted, and the mRNA expression analysis of (A) Peroxisome proliferator-activated receptor (Pparγ), (B) sterol regulatory element-binding protein (Srebp1c), (C) diacylglycerol O-acyltransferase 2 (Dgat2), (D) Fatty acid synthase (Fasn), (E) Stearoyl-CoA desaturase-1 (Scd1), (F) Glucose transporter type 4 (Glut-4) genes was performed. Expression of target genes was normalized to RPLP0 as the reference gene. The mRNA expression data were expressed in fold change. Data were analyzed using one-way ANOVA and Tukey's post-hoc test. $p<0.05$ was considered significant. n=3. Untreated=Untreated cells with cell culture media only, PC=L-α-phosphatidylcholine (30 ug/mL culture medium).

Appendix VI

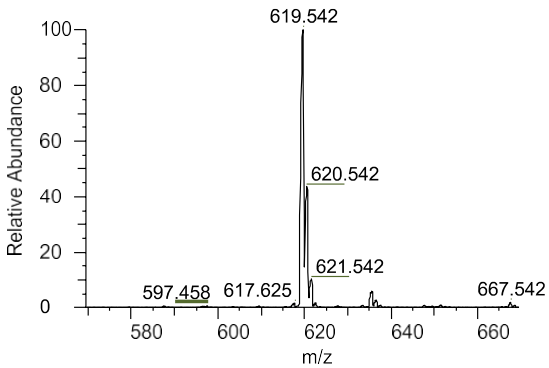
Primary analysis of astaxanthin from shrimp extract using Mass spectrometry

(A) Compound optimization of free Astx standard

PASSED - Compound Optimization Result Report- Free Astx

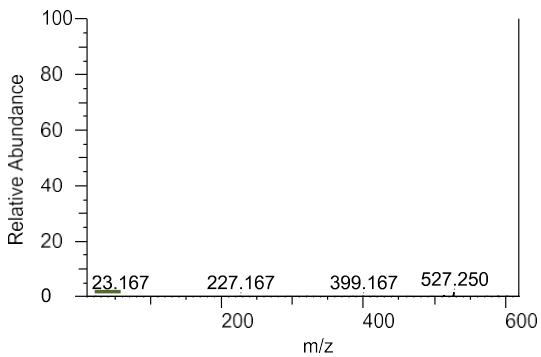
Precursor Ion Spectrum

Averaged Spectrum - Q3MS [569.375-669.375] Max Intensity: 7.59E+006



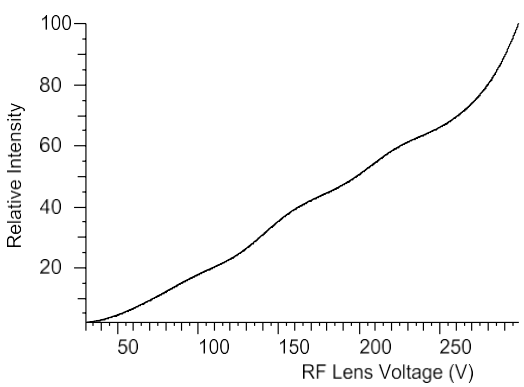
Product Spectrum

Averaged Spectrum - Full MS2 Max Intensity: 1.21E+006



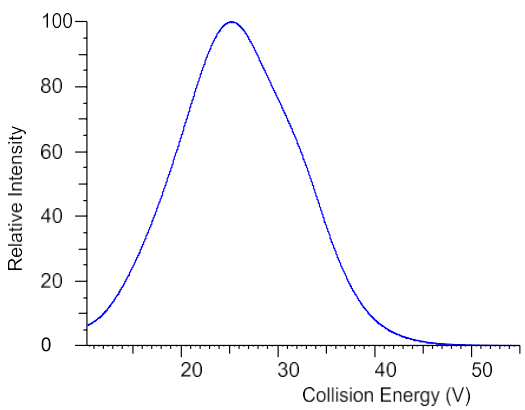
Optimizing RF Lens of Ion 619.500 in Q1MS (+)

Optimal Value: 299 V; Optimal Intensity: 1.31E+008



Breakdown Curve of Ion 619.375 m/z at 1 mTorr (+)

Max Intensity: 4.03E+004

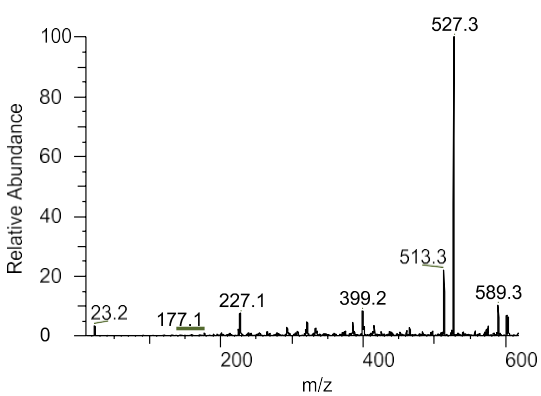


Product ions Col. Energy

527.25 m/z 25 V

Product Scan of Ion 619.375 m/z (+)

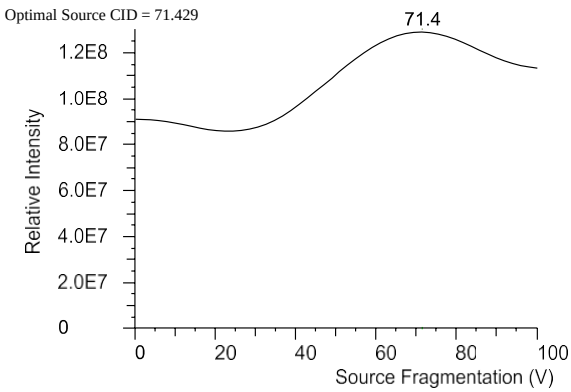
Max Intensity: 3.93E+004



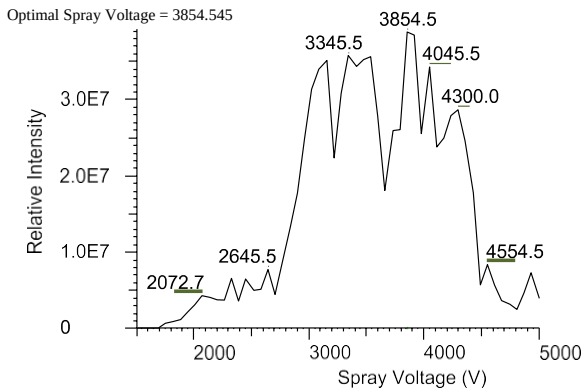
Compound optimization result in raw file:
C:\Thermo\Instruments\TNG\TSQQuantis\3.0\data\Optimization\ASTX_2019090932438.raw

RT (min)	Message
0.00	Initial source device setting are as follows: SPRAY_V: 3663.6; CAP_T: 325.0; VAP_T: 30.0; SHEATH_P: 3.2; AUX_P: 13.0; SWEEP_P: 0.4.
0.30	Sweep Gas has optimal tuning of 0.00 for precursor ion at m/z= 619.5 in positive polarity.
0.62	Spray Voltage has optimal tuning of 3854.55 for precursor ion at m/z= 619.5 in positive polarity.
1.42	Sheath Gas has optimal tuning of 2.42 for precursor ion at m/z= 619.5 in positive polarity.
1.80	Auxiliary Gas has optimal tuning of 8.54 for precursor ion at m/z= 619.5 in positive polarity.
1.95	Precursor with m/z=619.500 (positive polarity) has optimal SRIG RF setting 298.635 V.
2.07	Source CID has optimal tuning of 71.43 for precursor ion at m/z= 619.5 in positive polarity.
2.22	Optimal Q1 isolation mass for precursor with m/z=619.500 (positive polarity) is 619.375. Maximum intensity=130841370.003. Peak width=0.7.
2.70	Transition from precursor m/z=619.375 to product with m/z=527.250 is optimized with collision energy set to 25.240 V. Intensity=40348.243.
2.85	Optimal Q3 isolation mass for product with m/z=527.250 is 527.232. Maximum intensity=529744.257.
2.85	Compound optimization completed.

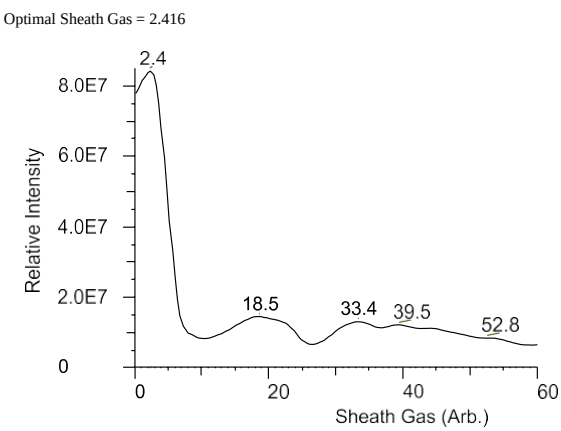
Optimizing Source Fragmentation of Ion 619.500 m/z (+)



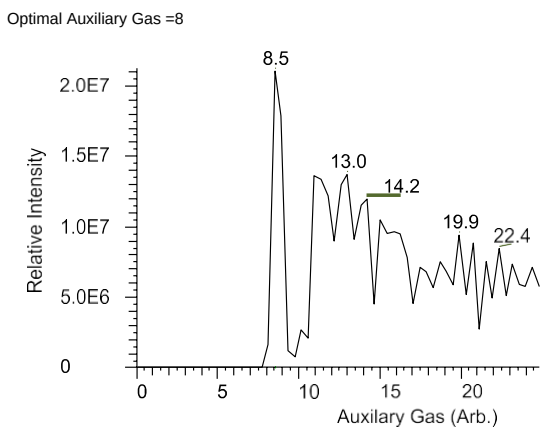
Optimizing Spray Voltage for Ion 619.500 in Q1MS (+)



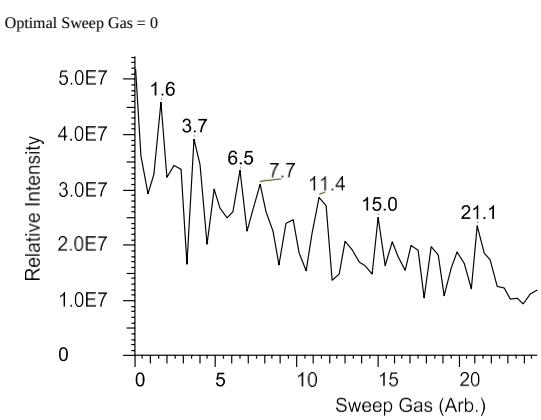
Optimizing Sheath Gas for Ion 619.500 in Q1MS (+)



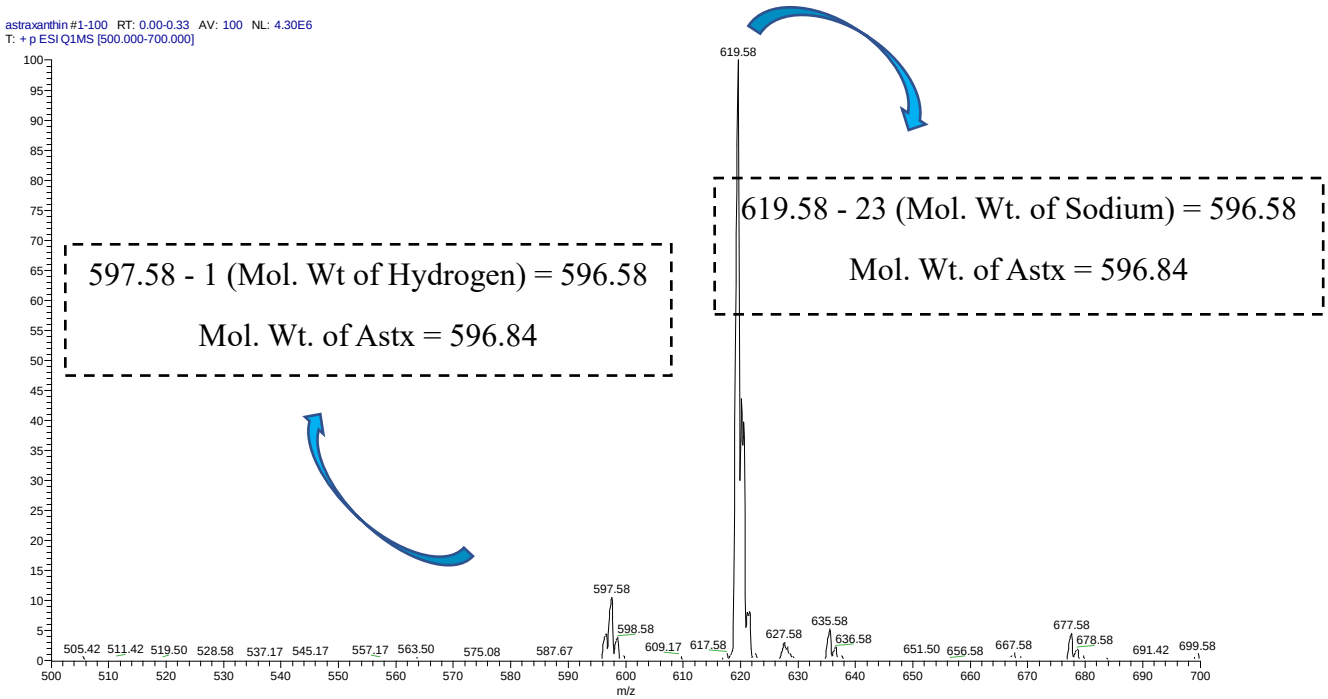
Optimizing Auxiliary Gas for Ion 619.500 in Q1MS (+)



Optimizing Sweep Gas for Ion 619.500 in Q1MS (+)



A. Free Astx standard optimization

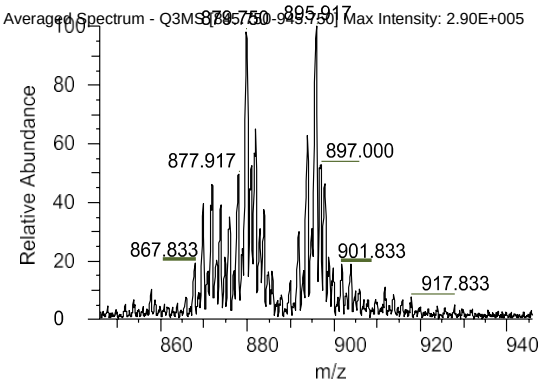


Compound optimization of free Astx standard was performed; the m/z 619.58 of free Astx standard represents [M+Na (23)] $^{+}$*, whereas the m/z 597.58 peak represents [M+H (1)]* $^{+}$*. Furthermore, the product scan of ion m/z 619.58 showed fragment ions m/z 527.3, m/z 589.3, m/z 513.3 and m/z 3.99.2.*

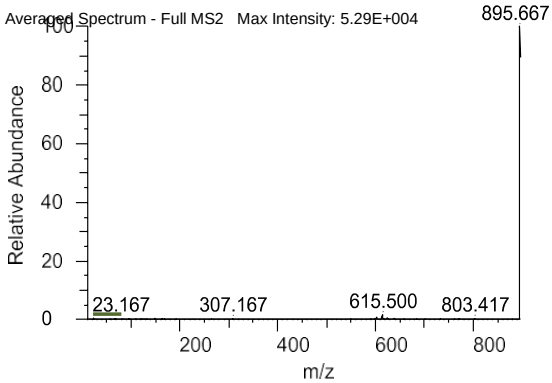
(B) Compound optimization of esterified Astx (Astx-E) standard

PASSED - Compound Optimization Result Report- Esterified Astx

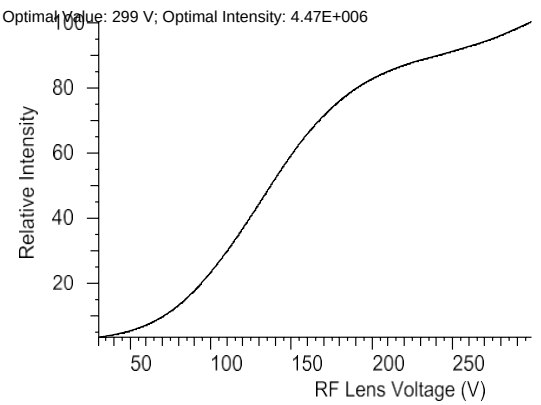
Precursor Ion Spectrum



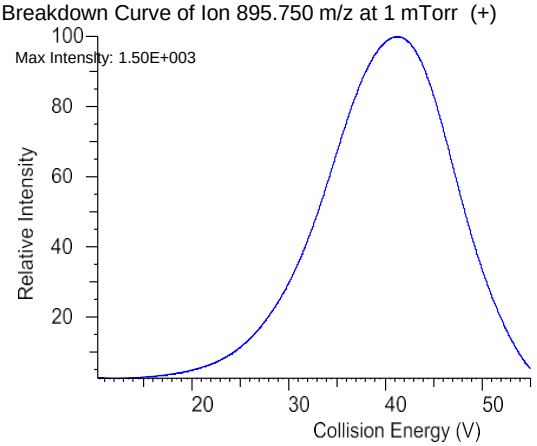
Product Spectrum



Optimizing RF Lens of Ion 895.750 in Q1MS (+)

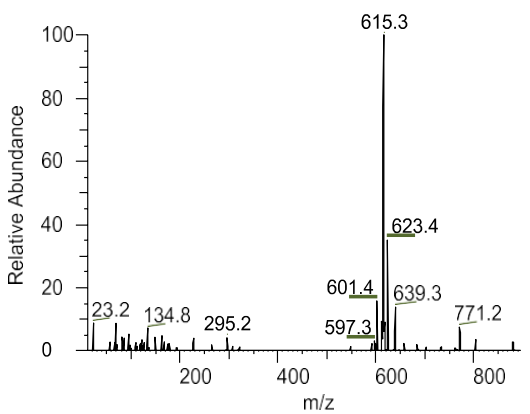


Breakdown Curve of Ion 895.750 m/z at 1 mTorr (+)



Product ions	Col. Energy
615.333 m/z	41 V

Product Scan of Ion 895.750 m/z (+)

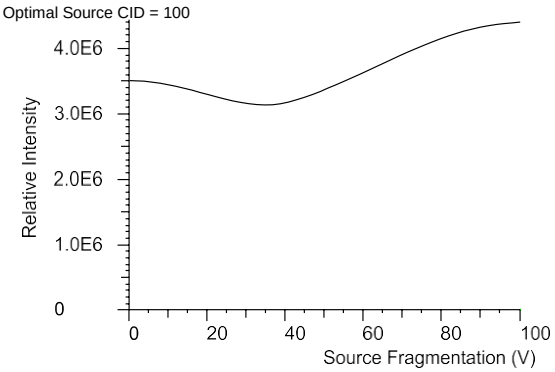


Compound optimization result in raw file: C:\Thermo\Instruments\TNG\TSQQuantis\3.0\data\Optimization\1_2019092011231.raw

RT (min)	Message
0.00	Initial source device setting are as follows: SPRAY_V: 4554.5; CAP_T: 325.0; VAP_T: 30.0; SHEATH_P: 1.2; AUX_P: 4.5; SWEEP_P: 2.8.
0.31	Sweep Gas has optimal tuning of 2.85 for precursor ion at m/z= 895.8 in positive polarity.
0.63	Spray Voltage has optimal tuning of 3409.09 for precursor ion at m/z= 895.8 in positive polarity.
1.41	Sheath Gas has optimal tuning of 0.00 for precursor ion at m/z= 895.8 in positive polarity.
1.78	Auxiliary Gas has optimal tuning of 4.47 for precursor ion at m/z= 895.8 in positive polarity.
1.93	Precursor with m/z=895.750 (positive polarity) has optimal SRIG RF setting 298.635 V.
2.05	Source CID has optimal tuning of 100.00 for precursor ion at m/z= 895.8 in positive polarity.
2.21	Optimal Q1 isolation mass for precursor with m/z=895.750 (positive polarity) is 895.750. Maximum intensity=4469947.599. Peak width=0.7.
2.80	Transition from precursor m/z=895.750 to product with m/z=615.333 is optimized with collision energy set to 41.276 V. Intensity=1504.932.
2.96	Optimal Q3 isolation mass for product with m/z=615.333 is 615.458. Maximum intensity=21436.921.
2.96	Compound optimization completed.

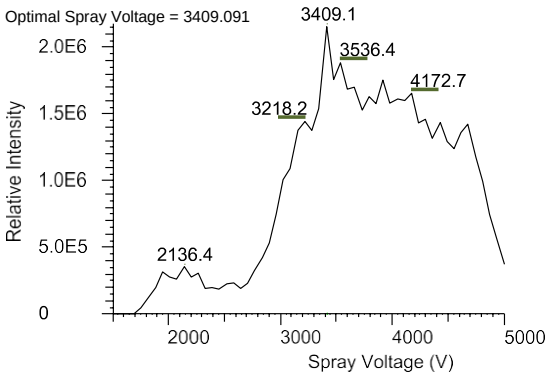
Optimizing Source Fragmentation of Ion 895.750 m/z (+)

Optimal Source CID = 100



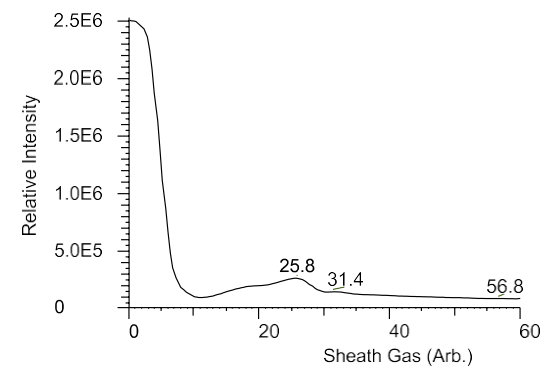
Optimizing Spray Voltage for Ion 895.750 in Q1MS (+)

Optimal Spray Voltage = 3409.091



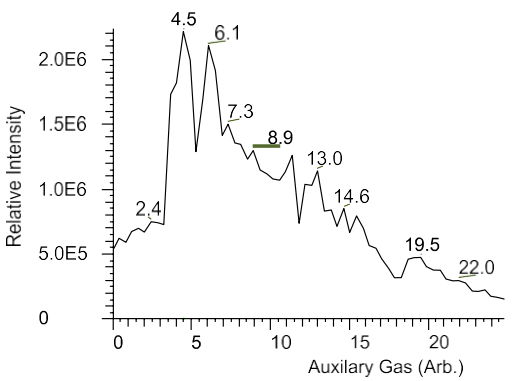
Optimizing Sheath Gas for Ion 895.750 in Q1MS (+)

Optimal Sheath Gas = 0



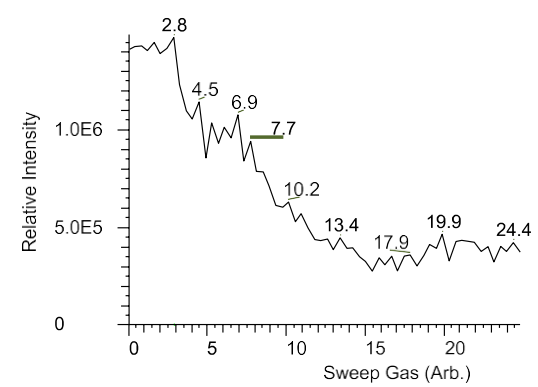
Optimizing Auxiliary Gas for Ion 895.750 in Q1MS (+)

Optimal Auxiliary Gas = 4.47

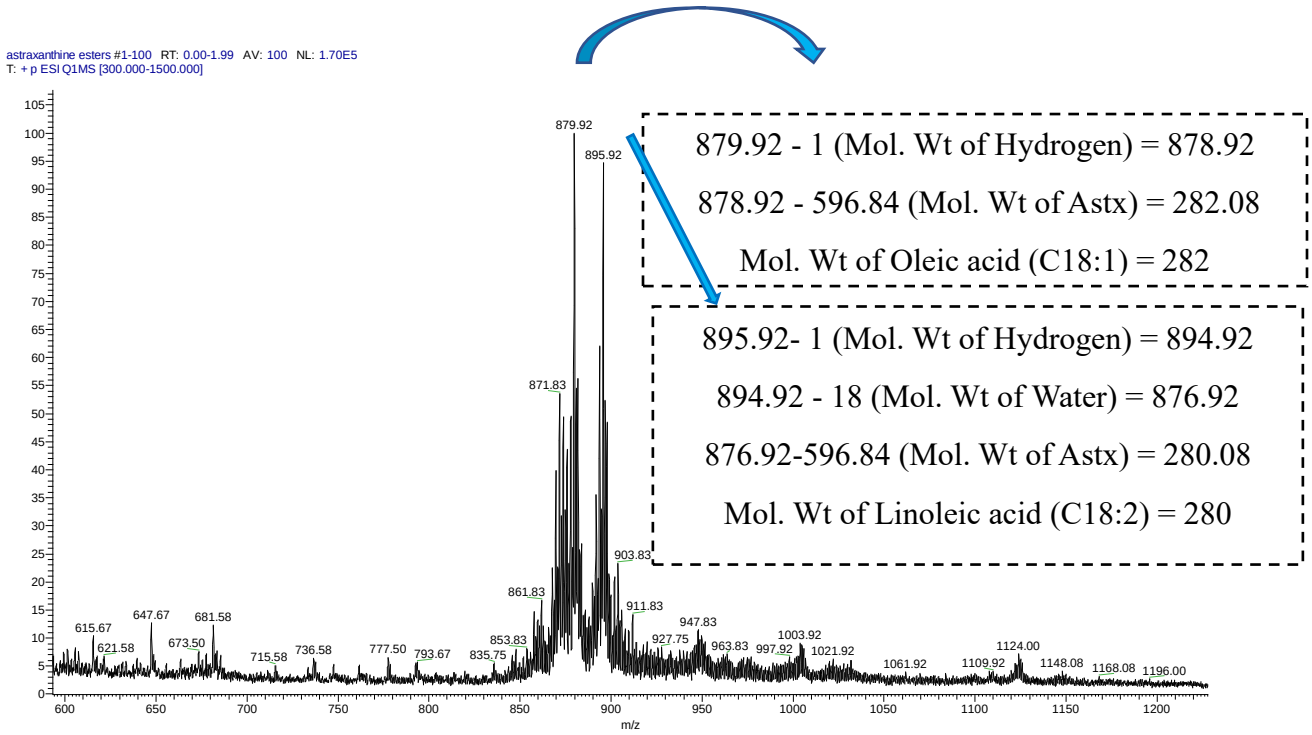


Optimizing Sweep Gas for Ion 895.750 in Q1MS (+)

Optimal Sweep Gas = 2.846

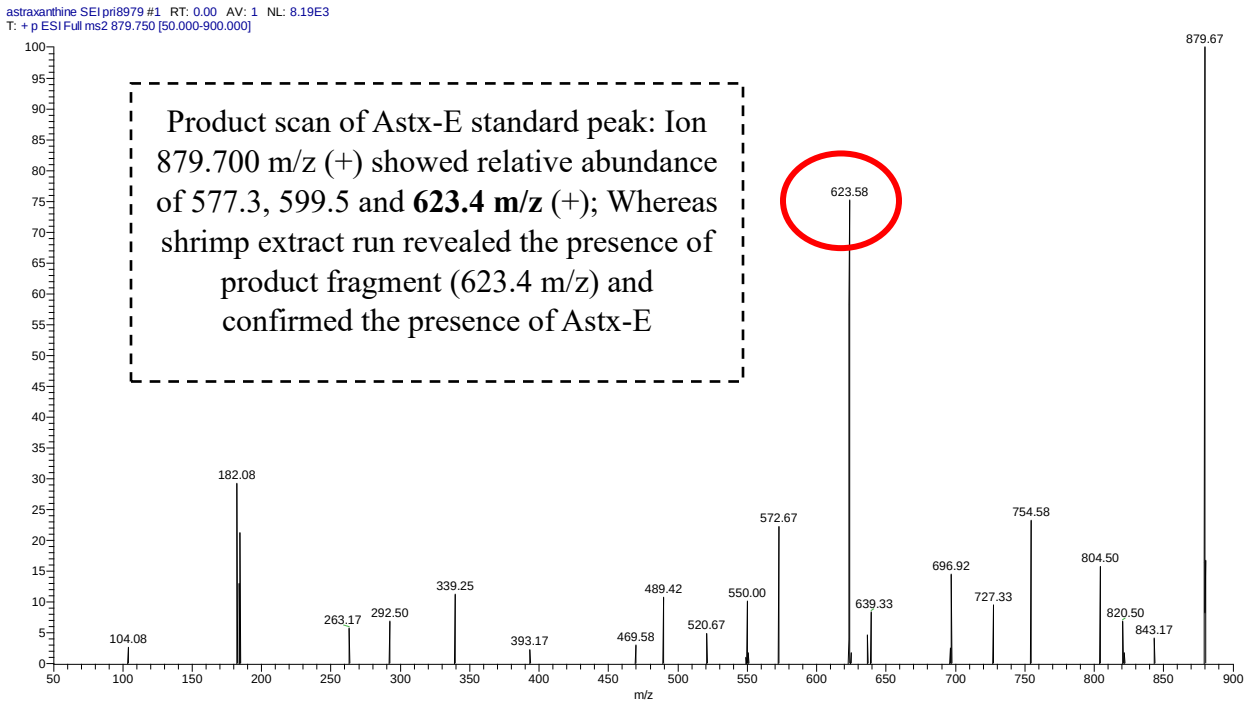


B. Esterified Astx (Astx-E) standard optimization confirmed the presence of C18:1 fatty acid



Compound optimization of Astx-E standard revealed the presence of C18:1/C18:2 fatty acid. The m/z 879.92 of Astx-E standard appears to be [MH+C18:1(282)] $^{+}$*, whereas the m/z 895.92 appears to be [MH+C18:2(280) +* H_2O*]* $^{+}$*. The product scan of ion m/z 895.91 represents fragment ions m/z 615.3 and m/z 623.4.*

(C) Preliminary analysis revealed the presence of the fragment of Astx-E [879.92 peak: Product scan of Ion 879.700 m/z (+)] in shrimp extract



Preliminary analysis of shrimp extract using MS showed the presence of Astx-E product fragment along with other unknown carotenoids peaks in shrimp extract. The fragment ions m/z 577.3, m/z 599.5 and m/z 623.4 are produced in shrimp extract, which is obtained after the product scan of ion m/z 879.700 of Astx-E standard (Appendix VI-B), and as mentioned above the ion m/z 623.4 appears to be produced from the Astx-E major peak 895.91 (Appendix VI-B).